Interactions Between Gut Microbiota and the Central Nervous System, with Emphasis on Quorum Sensing Between Commensal Lactic Acid Bacteria and Human Cells

by

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Declaration

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Prof. Leon M.T. Dicks

Summary

The human gut hosts close to 4 trillion microorganisms, which is nearly equivalent to the estimated 3.0×10^{13} human cells in a 70 kg body. Although the composition of gut microbiota changes with age, variation in diet, medication, hormone levels, stress and other environmental factors, a core group of autochthonous bacteria, between 400 and 500 species, are always present. More than 90% of the gut microbiome is represented by Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes, with the latter in the majority. Fusobacteria and Verrucomicrobia make up the remaining 10% of the gut microbiome. The human gut microbiome supersedes the number of cells in our bodies ten-fold. Since lactic acid bacteria (LAB) are the predominant gut microbiota, it is safe to conclude that changes in this group will affect the entire microbiome, ultimately leading to adjustments in the behaviour of intestinal epithelial cells (IECs). Changes in the immune system and quorum sensing (QS) signals instigated by an altering gut environment trigger a cascade of hormonal and neurological reactions. Activation of Toll-like receptors, for instance, induce strong immune and inflammatory reactions, but at the same time stimulate the secretion of hormones such as 5-hydroxytryptamine (5-HT, or serotonin), glucagon-like peptide-1 (GLP-1), peptide tyrosine tyrosine (PYY), glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CCK), ghrelin, leptin, pancreatic polypeptide (PP), oxyntomodulin and neurotensin. Serotonin act as neurotransmitter but also regulates diverse functions such as platelet aggregation, bone development, immune response, cardiac function and gut homeostasis, and control enteric motor and secretory reflex. Gut bacteria also synthesize, or regulate, the production of serotonin and other neurotransmitters such as glutamine (Glu), gamma-amino butyric acid (GABA), dopamine (DA), norepinephrine and histamine. These molecules communicate with the central nervous system (CNS) via afferent fibers in the Vagus nerve (VN), autonomic sympathetic and parasympathetic nervous systems, but also the hypothalamic-pituitary-adrenal axis (HPA). Intermediate compounds such as short-chain fatty acids (SCFAs), tryptophan and secondary bile acids produced by gut bacteria also communicate with the CNS. Signals received from the brain are sent back to entero-epithelial cells (EECs) via the HPA and efferent VN fibers to complete the circle of communication referred to as the gut-brain axis (GBA). The modulation, development, and renewal of neurons in the enteric nervous system (ENS) are controlled by gut microbiota, especially those with the ability to produce and metabolize hormones. Minor activation of the ENS and VN results in drastic changes in the production of neurotransmitters, which also affects digestion, intestinal permeability, gastric motility, and

immune regulation. GABA, in addition to other metabolites, play an important role in anti-inflammatory responses and help alleviate psychiatric symptoms stemming from inflammation. Treatment of schizophrenic and bipolar patients with probiotics alleviated symptoms associated with irritable bowel disease (IBD), and autistic children benefitted from probiotic treatment. Obsessive compulsive disorder (OCD)-like behavior could also be controlled by treatment with LAB.

Inter- and intra-species signalling systems have been well studied, but far less is known about interkingdom quorum sensing (QS), especially between gut bacteria and intestinal epithelial cells (IECs). Although the auto-inducer 3 (AI-3)/epinephrine (Epi)/norepinephrine (NE) QS signalling system described for pathogenic *Escherichia coli, Salmonella typhimurium* and *Ctirobacter rodentium* are widely used by Gram-negative pathogenic bacteria, not all species have receptors that recognize these signals. Instead, they have developed "broad-range" "solo" LuxR-type receptors such as SdiA (a LuxR homolog) and QscR to improve their communication abilities. Despite our knowledge on QS, the effect of these signalling molecules on the CNS is ill-researched. Several QS peptides (QSPs) have the ability to diffuse through the intestinal mucosa and enter the circulatory system, from where they may penetrate the blood-brain barrier (BBB). It may be that LAB communicate with the CNS using small linear or cyclized oligopeptides (QS peptides, QSPs) of 5 to 17 amino acids long, as reported for other Gram-positive bacteria. In our own research we have shown that bacteriocins can indeed transverse epithelial (Caco-2) and endothelial (HUVECs) monolayers without changing the integrity of the membranes and with no toxic effect. Once in the blood stream, bacteriocins may cross the BBB, similar to that reported for the heptapeptide PapRIV produced by *Bacillus*.

Our understanding of exactly how gut microorganisms control cognitive behavior, mood, and neuropsychiatric disorders remains limited. However, the more we discover about the gut microbiome, QS, neurotransmitters and the GBA, the greater the chance of developing novel therapeutics, probiotics and psychobiotics to treat gastro-intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), but also improve cognitive functions and prevent or treat mental disorders. This calls for in-depth deciphering of the complex, everchanging network between cells and neurons. Research on the quenching of QS signals need to be prioritised. We need to understand how quorum quenching (QQ) therapy will affect beneficial gut microbiota. Biomarkers need to be developed to identify differences in the gut microbiome of individuals suffering from psychological disorders. Interactions between drugs used in treatment and gut microbiota need to be studied in greater

depth. We need to understand the effect psychiatric medication may have on the composition of the gut microbiome. Are intestinal microbiota able to metabolise these drugs? Studies should include multi-omics of gut and oral microbiota to have a better understanding of the mutual interplay between phyla. Will it be possible to develop probiotics to treat dysbiosis and neuropsychiatric abnormalities?

Opsomming

Die ingewande van die mens huisves bykans 4 triljoen mikroörganisms, min of meer gelykstaande aan die beraamde 3.0×10^{13} selle teenwoordig in ‘n persoon met ‘n liggaamsmassa van 70 kg. Alhoewel die samestelling van ingewandsmikrobes varieer met ouderdom en verandering van dieët, medikasie, hormoonvlakke, angs en ander omgewingsfaktore, is ‘n kerngroep endemiese bakterieë van 400 tot 500 spesies altyd teenwoordig. Meer as 90% van die ingewandsmikrobioom bestaan uit Proteobacteria, Actinobacteria, Bacteroidetes en Firmicutes, met laasgenoemde in die meerderheid. Die oorblywende 10% ban die ingewandsmikrobioom bestaan uit Fusobacteria en Verrucomicrobia. Die ingewandsmikrobioom van die mens oorskry die aantal selle in ons liggame tienvoudig.. In die lig daarvan dat melksuurbakterieë (MSB) die ingewandsmikrobioom oorheers, kan afgelei word dat verandering in hierdie groep die mikrobioom in geheel sal beïnvloed en uiteindelik die gedrag van intestinale epiteelselle (IESe) sal verander. Verandering van die immuunsisteem en produksie van kworum-aanvoelbare (QS) seine tweeggebring deur ‘n veranderde ingewands-omgewing gee aanleiding tot ‘n opeenvolging van hormoon- en neurologiese reaksies. Aktivering van Toll-tipe reseptore sal byvoorbeeld ‘n sterk immuun- en inflammatoriese reaksie tot gevolg hê, maar terselfteryd ook die uitskeiding van hormone soos 5-hidroksietriptamien (5-HT of serotonien), glukagon-tipe peptied-1 (GLP-1), peptied tirosien-tirosien (PYY), glukose-afhanklike insulienotropiese peptied (GIP), cholesistokinien (CCK), ghrelin (kortisol), leptien, pankreatien polipeptied (PP), oksientomodulien en neurotensien stimuleer. Serotonien tree op as neurongeleier, maar reguleer ook verskeie ander funksies soos die aggregasie van bloedplaatjies, beenontwikkeling, immuunreaksie, hartfunksie, bevordering van ingewandshomeostase, asook die beheer van enteriese beweging en sekretoriese refleksie. Ingewandsbakterieë sintetiseer of reguleer die produksie van serotonien en ander neuro-geleiers soos glutamien (Glu), gamma-amino butiriensuur (GABA), dopamien (DA), norepinefrien en histamien. Hierdie molekules kommunikeer met die sentrale sunuweestelsel (CNS) via opwaarts-gerigte vesels in die Vagus senuwee (VN), outonome simpatiese- en parasimpatiese senuweesisteme, maar ook die hipotalamiese-pituitêre-adrenale as (HPA). Intermediëre verbindings soos kortketting vetsure (SCFAs), triptofaan en sekondêre galsoute geproduseer deur ingewandsbakterieë, kommunikeer ook met die CNS. Seine vanaf die brein word deur middel van die HPA en afwaarts-gerigte vesels in die VN na entero-epiteel selle (EECs) gelei en voltooi sodoende die siklus van kommunikasie, waarna verwys word as die ingewandsbrein as (GBA). Die verandering, ontwikkeling en herstel van die enteriese neuronsisteem (ENS) word deur ingewandsmikrobe beheer, veral dié wat die vermoë het om hormone te produseer

en te metaboliseer. Kleinskaalse aktivering van die ENS en VN bring drastiese veranderinge mee in die produksie van neurongeleiers, wat op hul beurt die vertering van voedsel, intestinale deurlaatbaarheid, mobiliteit van die ingewands- en immuunregulering beïnvloed. GABA, asook ander metaboliete, speel ‘n belangrike rol in anti-inflammatoriese reaksies en help met die verligting van psigiatriese simptome wat deur inflammasie veroorsaak word. Behandeling van skisofreniese en bipolêre pasiente met probiotika het simptome geassosieer met prikkelbare dermsindroom (IBS) verlig en outistiese kinders het ook deur die behandeling baat gevind. Obsessiewe kompulsiewe wangedrag (OCD) is ook met behandeling van MSB beheer.

Inter- en intra-spesie seinsisteme is reeds goed bestudeer, maar min is bekend oor inter-koninkryk kworum sein (QS) sisteme, veral tussen bakterieë en intestinale epiteelselle (IECs). Alhoewel die outo-induseerder 3 (AI-3)/epinefrien (Epi)/norepinefrien (NE) QS sisteem beskryf vir patogene *Escherichia coli, Salmonella typhimurium* en *Ctirobacter rodentium* deur verskeie Gram-negatiewe ingewandsbakterieë gebruik word, het nie al die patogene die reseptore om hierdie seine te herken nie. In die plek daarvan het hulle “breë-spektrum” “solo” LuxR-tipe reseptore soos SdiA (‘n LuxR homoloog) en QscR ontwikkel om hule kommunikasie vaardighede te verbeter. Ten spyte van ons kennis oor QS is die effek van hierdie sein molekules op die CNS nog nie goed bestudeer nie. Verskeie QS peptiede (QSPs) het die vermoë om deur die ingewandsmukosa te diffundeer en die sirkulêre sisteem binne te dring vanwaar die bloed-brein versperring (BBB) gepenetreer word. Dit mag wees dat MSB deur middel van klein liniêre peptiede of sikliese oligopeptiede (QS peptiede, OSP) van 5 tot 17 aminosure lank met die CNS kommunikeer, soos die geval blyk te wees met ander Gram-positiewe bakterieë. Ons eie navorsing het getoon dat bakteriosiene wel deur enkellaag Caco-2 epiteelselle en HUVEC endoteelselle kan migreer sonder om die intergriteit van die membrane te verander of toksies te wees. Sodra dit in die bloedstroom is, kan bakteriosiene deur die BBB beweeg, soortgelyk aan die heptapeptied PapRIV wat deur *Bacillus* geproduseer word.

Ons verstaan van presies hoe ingewandsmikrobe kognitiewe gedrag, gemoed en neuropsigiatriese wanbalanse beheer, is beperk. Nietemin, hoe meer ontdekkings ons oor die ingewandsmikrobioom, QS, neuro-geleiers en die GBA maak, hoe groter is die kans om nuwe terapeutiese middels, probiotika en psigobiotika te ontwikkel om gastrointestinale wanbalanse soos inflammatoriese dermsiekte (IBD) en IBS te behandel, maar ook kognitiewe funksies te verbeter en geestelike versteurings te voorkom. Dit verg intense ontrafeling van die komplekse, alomveranderde netwerk tussen selle en neurone. Navorsing in onderdrukking van QS seine

moet prioriteit geniet. Ons moet verstaan hoe kworum-onderdrukkings (QQ)-terapie voordelige ingewandsmikrobe beïnvloed. Biomerkers moet ontwikkel word om verskille in die ingewandsmikrobioom van individue wat aan psigiatriese wanbalanse lei te identifiseer. Die interaksie tussen medikasie en ingewandsmikrobe moet beter bestudeer word. Ons moet verstaan wat die effek van psigiatriese medikasie is op die samestelling van die ingewandsmikrobioom. Is ingewandsmikrobe daartoe instaat om hierdie middels te mataboliseer? ‘n Multi-omiese benadering behoort gevolg te word ten einde die interaksies tussen orale en ingewandsmikrobe beter te verstaan. Is dit moontlik om probiotika te ontwikkel vir die behandeling van disbiose en neuropsigiatriese abnormaliteite?

Biographical Sketch

I was born in Port Elizabeth, Eastern Cape, on the 15th of January 1961, as first child of Trevor Milner Theodore Dicks and Anna Elizabeth (née du Plessis). I attended Newton Park Primary School in Port Elizabeth and matriculated in 1978 from De Vos Malan High School in King William's Town, Eastern Cape. The next year I enrolled as BSc student at Free State University, Bloemfontein, and graduated in 1981. After completing BSc Hons in 1982 we moved as newlyweds to Stellenbosch, Western Cape. I enrolled as MSc student in Microbiology at Stellenbosch University (SU), graduated (*cum laude*) in 1985 and obtained the PhD degree in 1989. During my PhD studies (1986), I was appointed as Senior Technician in the Department of Microbiology (SU) and later (1988) as Lecturer. This took me to Senior Lecturer in 1991, Associate Professor in 1996, Full Professor in 2001 and Distinguished Professor in 2014 - the position I am presently holding at SU. I am married to Sarina (née Scholtz) and we have three children, Anelda, Elisna and Leon. Elisna is married to Chris Joubert, and we have a granddaughter, Ayva. We have been fortunate to raise our children in Stellenbosch, where we have spent most of our lives. My research and lecturing career has been very productive, filled with many innovations and the launching of a very successful probiotic. My hobbies include product development, real estate, painting, woodworking, and writing.

Preface

The reason for the study is explained in the Introduction (first chapter). Although this dissertation is primarily about interactions amongst gut microbiota and lactic acid bacteria (LAB), and their influence on the human body, it is only appropriate to include a chapter on the current taxonomic status of the species (Chapter 2, Lactic Acid Bacteria on a Taxonomic Highway) and look back with nostalgia to earlier days when I was actively involved in the classification and reclassification of LAB. Research on LAB is nothing but a gene-toggling, plug-and-play, exercise if we do not understand their nutritional requirements. This is reviewed in Chapter 3, Metabolism of Lactic Acid Bacteria Summarised. LAB have been part of my life from, I guess, before birth. It thus makes sense to look back at where it all started and walk the journey from cradle to casket (Chapter 4, The walks of Life – From a Microbial Perspective). This is an introduction to Chapter 5, Probiotic Properties and Health Benefits of Lactic Acid Bacteria and their Bacteriocins. Interest in the anti-carcinogenic properties of LAB is gaining momentum and this is reviewed in Chapter 6, Do Bacteria Provide an Alternative to Cancer Treatment and What Role Does Lactic Acid Bacteria Play?. Communication amongst intestinal LAB and commensal species, and their interactions with human cells is discussed in Chapter 7, Quorum Sensing between Gut Microbiota and Lactic Acid Bacteria, and its Effect on the Host. This is followed up with a review on bacterial communication with the central nervous system (Chapter 8, Lactic Acid Bacteria, Neurotransmitters, and Neuropsychiatric Disorders). Chapter 8 is divided into two parts, each published in separate review papers: (i) Gut Bacteria and Neuropsychiatric Disorders and (ii) Gut Bacteria and Neurotransmitters. The latter addressed the role commensal gut microbiota, including LAB, play in the gut-brain-axis (GBA). Chapter 9 is a general discussion on interactions amongst gut microbiota and their effect on the human body, including the central nervous system (CNS). Quorum sensing (QS) amongst gut microbiota, their effect on the CNS and human health, including mental health, is a vast research field that captures the interest of many scientists. As with all research, some areas require more in-depth investigations, as pointed out in Chapter 9.

Species names used for the genus *Lactobacillus* are as listed in the Approved Lists of 1980. This is done for cross-referencing purposes and is not meant to disagree with the reclassification of the genus proposed by Zheng *et al*. (2020). Most recent changes in the taxonomy of LAB, especially *Lactobacillus*, as suggested by Zheng *et al*. (2020), are summarized in Chapter 2.

The format and style of headings, in-text references and reference lists vary, depending on the Journal in which chapters were published. Chapters 6, 7 and 8 have been written exclusively for this dissertation and were published in 2021 and 2022.

Reference

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., *et al*., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae.* Int. J. Syst. Evol. Microbiol. 70: 2782-2858.

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my wonderful wife, Sarina, our children, Anelda, Elisna and Leon, son-in-law (Chris), granddaughter (Ayva) and my mother,
our Heavenly Father. He allowed me discover things already known to Him, and He made it a joyful experience.

This dissertation is dedicated to my wife Sarina

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Chapter 1

General Introduction

Where do we find Lactic Acid Bacteria (LAB)?

How well are LAB presented in the human gastro-intestinal tract (GIT)?

Rationale behind the study

General Introduction

Lactic acid bacteria (LAB) are omnipresent and have been isolated from fresh and fermented plant material, meat, dairy products, nutrient-rich soil, water, flowers, and the gastrointestinal tract (GIT) of humans, animals, reptiles, and insects. Cells are Gram-positive, rod-shaped, or coccoid, single, or arranged in chains, pairs or tetrads. Although catalase negative, some strains exhibit pseudocatalase activity when grown on media rich in heme, such as blood agar (Singh *et al.*, 2007). All species are non-motile, except for members of the genus *Vagococcus* (Collins *et al*., 1989), *Lactobacillus ghanensis* (Nielsen *et al*., 2007), *Lactobacillus nagelii* (Edwards *et al*., 2000), *Lactobacillus satsumensis* (Endo and Okada, 2005), *Lactobacillus vini* (Rodas *et al.*, 2006), *Lactobacillus agilis* (Weiss *et al*., 1981), *Lactobacillus mali* (Kaneuchi *et al*., 1988), *Lactobacillus capillatus* (Chao *et al*., 2008) and *Lactobacillus ruminis* (Sharpe *et al*., 1973). All species produce lactic acid under microaerophilic conditions (Carr *et al*., 2002). Carbohydrates are fermented via the Embden Meyerhof Parnas (EMP) pathway to lactic acid and the phosphoketolase pathway to lactic acid, CO_2 and ethanol, depending on the presence of aldolase or phosphoketolase (reviewed by Endo and Dicks, 2014). Although lactic acid is the main product produced from the fermentation of sugars, several secondary compounds are formed from the degradation of proteins and lipids (Endo and Dicks, 2014). These include various alcohols, aldehydes, acetic acid, esters, and sulphur-containing compounds.

The human gut hosts close to 4 trillion microorganisms, which is more-or-less equivalent to the estimated 3.0×10^{13} human cells in a 70 kg body (Sender *et al*., 2016). Although the composition of gut microbiota changes with age, variation in diet, medication, hormone levels, stress and other environmental factors, a core group of autochthonous bacteria, ranging between 400 and 500 species, are always present (Sender *et al*., 2016; Shreiner *et al*., 2015; Tiihonen *et al*., 2010). Recent findings did indeed put a different perspective on the human gut microbiome. The general view that we host 500 to 1 000 species may soon change, as the number of unique genotypes (sub-species) may be orders of magnitude greater than this. More than 90% of the gut microbiome is represented by Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Bilen *et al*., 2018; Hugon *et al*., 2015; Li *et al*., 2014). The Firmicutes phylum is composed of more than 200 different genera such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus*. *Clostridium* represents 95% of the Firmicutes phyla. Bacteroidetes consists of predominant genera such as *Bacteroides* and *Prevotella*. The Actinobacteria phylum is proportionally less abundant and is mainly represented by the *Bifidobacterium* genus (Arumugam *et al*., 2011). Fusobacteria and Verrucomicrobia make up the remaining 10% of

the gut microbiome (Eckburg *et al*., 2005). As expected from a stable environment such as the GIT, drastic changes in the core gene pool need to be avoided. Strain dominance is achieved by neutral evolution, as illustrated with studies on an intestinal strain of *Escherichia coli* (Ghalayini *et al*., 2018). Genome sequences of 24 isolates of *E. coli* ED1a, studied over a year, showed a mutation rate of only 6.9×10^{-7} per base (Ghalayini *et al*., 2018). This was, however, complemented by a reduction in population size (Ghalayini *et al*., 2018), suggesting that external stress factors have a profound influence on the survival of bacteria in the GIT. Roodgar *et al*. (2022) have shown that strains exposed to stress, such as antibiotics, develop resistance rapidly and changes the overall composition of the gut microbiome. Although the outcome of the reports by Ghalayini *et al*. (2018) and Roodgar *et al*. (2022) were different, both studies illustrated that the frequency at which strains adapt to an ever-changing and stressful environment, such as the GIT, is unpredictable. Strains within the same species isolated from different individuals have at least one variation in every hundred base pairs (Costea *et al*., 2017; Garud *et al*., 2019; Schloissnig *et al*., 2013; Truong *et al*., 2017). Species that cannot adapt or compete are replaced by novices that are able to regulate their own gene expressions, or alter their genetic composition (Maurice *et al*., 2013; Schloissnig *et al*., 2013). Once adapted to the GIT, strains are not easily replaced or expelled (Costea *et al*., 2017). *Bacteroides fragilis* adapted so well to humans that the species is represented by a single strain (Verster *et al*., 2017). *Helicobacter pylori, Mycobacterium tuberculosis* (Comas *et al*., 2013; Falush *et al*., 2003; Linz *et al*., 2007), *Eubacterium rectale* (Karcher *et al*., 2020) and *Prevotella copri* are host-specific, i.e., strains are associated with individuals from specific geographical regions (Tett *et al*., 2019). Most species in the GIT are represented by strains with unique phenotypic and genotypic characteristics (Browne *et al*., 2016; Garud and Pollard, 2020; Yassour *et al*., 2018). Beneficial and non-beneficial (pathogenic) microbiota are in constant competition for survival. For a review on the health benefits of lactic acid bacteria, the reader is referred to Dicks and Botes (2010), and Dicks and Endo (2022).

Diet has a profound influence over the composition of the gut microbiome. A diet rich in fat and sugars supports the growth of Bacteroidetes, whereas a high-fibre diet shifts the balance towards Firmicutes (reviewed by Dicks and Endo, 2022; Wu *et al.*, 2011). A comprehensive study by Odamaki *et al*. (2016), based on 16S rRNA gene sequences of gut microbiota in Japanese subjects, provided an insight on the changing gut microbiota during human life. The authors identified four predominant phyla, i.e., Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, with profound changes in relative abundance of the latter two phyla, especially in the elderly. Of interest was the high cell numbers of *Enterobacteriaceae* recorded amongst

infants and the elderly. The reason for this may be that gut epithelial cells in infants are considered too immature and, in the elderly, too weakened to resist bacterial infections (Odamaki *et al.*, 2016). It could also be that *Enterobacteriaceae* represses the growth of bifidobacteria, as concluded by Wu *et al.* (2015) and Pérez *et al.* (2014). The lack in *Bifidobacterium* spp. may also be ascribed to a specific lifestyle, as observed in a study conducted on hunters of the Hadza tribe of Tanzania (Schnorr *et al*., 2014). Cellular interactions between *Enterobacteriaceae* and bifidobacteria and changing concentrations of acetate may influence the growth of commensal species. Acetate and lactate stimulate the growth of butyrate-producing bacteria, at least *in vitro* (Falony *et al*., 2006; Mahowald *et al*., 2009). This may explain the abundance of *Lachnospiraceae*; *Coprococcus*, *Roseburia* and *Faecalibacterium* (Odamaki *et al*., 2016). Butyrate is converted to lipids serving as energy, protects gut epithelial cells against cancer, helps maintain the integrity of the gut wall (den Besten *et al.*, 2013; Goncalvesa and Martel, 2016; Noble *et al*., 2017) and down-regulates pro-inflammatory responses (Bermudez-Brito *et al*., 2013; Groeger *et al*., 2013; Sagar *et al*., 2014).

Dicks and Endo (2022) were the first to point out that fructose-loving (fructophilic) lactic acid bacteria (FLAB) may be present in the human GIT and argued that species have not been detected due to the use of non-specific growth media and non-optimal growth conditions. In the past some strains have been incorrectly identified as members of the genus *Lactobacillus* (Endo and Dicks, 2014; Endo and Okada, 2008). On the other hand, FLAB are poor carbohydrate fermenters (glucose is only fermented in the presence of an electron acceptor such as oxygen) and may thus be under-presented in the GIT. Diet could play a major role. With a preference for fructose, FLAB may be present in higher cell numbers if a diet rich in plant material is followed, or less digestible polymeric forms of fructose (fructans) are consumed. Our understanding of the role FLAB may play in the human GIT is limited and warrants further research. For more information on the metabolism of FLAB the reader is referred to Dicks and Endo (2022), Endo and Dicks (2014), Endo *et al*. (2009), Endo *et al*. (2015), and Endo *et al*. (2018).

Treatment of gastric disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) with probiotic LAB led to in-depth studies on immune stimulation. The ability of LAB to stimulate our immune system and fight microbial infections may hold answers to more intriguing questions such as control over tumour growth and cancer. The untapped ability of gut microbiota and their influence on human health is supported by extensive human gut microbiome studies with data collected from more than a 1 000 participants (Gilbert *et al*., 2018). A review on the role bacteria, specifically LAB, play in the treatment of cancer was

published by Dicks and Vermeulen (2022), included as Chapter 6. The more we discover about the gut microbiome, gut-brain axis (GBA), hypothalamic pituitary adrenal axis (HPA), cognitive behavior and neuropsychiatric disorders, such as autism, depression, and schizophrenia (reviewed by Dicks *et al*., 2021 and included as Chapter 8), the more questions arise concerning the influence gut bacteria have on the production of prominent neurotransmitters, such as γ-aminobutyric acid (GABA), dopamine (DA), norepinephrine (NE, also called noradrenaline), serotonin (5-HT), and histamine.

What role does inter- and intra-bacterial communication play in all of this? To what extend does quorum sensing (QS) molecules produced by LAB influence our health? Answers to these questions are addressed in the review by Dicks (2022), included as Chapter 7. More in-depth research may help us to develop novel therapeutics, probiotics and psychobiotics to treat gastro-intestinal disorders, improve cognitive functions, and prevent or treat mental disorders and serious diseases such as cancer.

The effect of gut microbiota on human health should not be underestimated. A thousand bacterial species, each with an average of 2 000 genes relate to 2 000 000 genes, superseding the 20 000 human genes a hundred-fold. This correlates with the actual size of microbial gene catalogues obtained by MetaHIT (Qin *et al*., 2010.) and the Human Microbiome Project (Integrative HMP Research Network Consortium, 2014). This raises the question whether the human genome has any effect on the gut microbiome, especially since the gut microbial community is regulated by changing environmental factors. It is also evident that changes in immune responses shapes the gut microbiome (Karczewski *et al*., 2014).

This dissertation is a compilation of health benefits offered by LAB and summarises findings I have published over 35 years. The first few years of my career were devoted to the taxonomy of LAB, during which 28 research papers and 19 book chapters were published. The book chapters were on *Leuconostoc* in The Genera of Lactic Acid Bacteria (Dellaglio *et al*., 1995), *Pediococcus* and *Tetragenococcus* in The Prokaryotes (Holzapfel *et al*., 2006), and six chapters in the second edition of Bergey's Manual of Systematic Bacteriology, covering the genera *Pediococcus* (Holzapfel *et al*., 2009), *Melissococcus* (Dicks *et al*., 2009), *Tetragenococcus* (Dicks *et al*., 2009), *Leuconostoc* (Holzapfel *et al*., 2009), *Oenococcus* (Dicks and Holzapfel, 2009), and *Weissella* (Björkroth *et al*., 2009). These were followed by more book chapters on *Oenococcus* (Endo and Dicks, 2014), *Fructobacillus* (Endo and Dicks, 2014), *Cattelliococcus, Melissococcus* and *Pillibacter* (Dicks *et al*., 2014), the family *Leuconostocaceae* (Endo *et al*., 2014), *Leuconostoc* (Björkroth *et al*., 2014), *Weissella* (Björkroth *et al*., 2014), *Pediococcus* (Franz *et al*., 2014), and a comparison of *Pediococcus, Paralactobacillus* and *Sharpea* (Dicks *et al*., 2015).

Since 1998 my research focussed mostly on bacteriocins and probiotic properties of LAB. To date, I have contributed to 234 scientific papers (mostly research articles) and eight book chapters covering bacteriocins and probiotics.

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Chapter 2

Lactic Acid Bacteria on a Taxonomic Highway

By 2020 as many as 261 *Lactobacillus* species have been described

The genetic diversity within the genus is far more complex than reported for other bacterial genera, and even bacterial families

Lactobacillacea (*Lactobacillus* and *Pediococcus* species) have now been separated into 26 lineages based on average nucleotide identity (ANI), average amino acid identity (AAI), core-gene average amino acid identity (cAAI), core genome phylogeny, and the sequence of signature genes

Lactobacillus is separated into 25 genera, according to pairwise AAI and conserved proteins (POCP)

The description of *Lactobacillus* and *Paralactobacillus* is emended but the description of *Pediococcus* is left unchanged

The family *Leuconostocaceae* Schleifer 2010 may be considered a later synonym of the family *Lactobacillaceae* Winslow *et al.* 1917

Lactic Acid Bacteria on a Taxonomic Highway

Methods used to identify and classify LAB changed drastically over the past 40 years. Before 1980 the description of new species was based on the origin of the sample (clinical, environmental, or food), carbohydrate fermentation profiles, optimal growth temperature, metabolites produced, amino acid sequence of the peptidoglycan interpeptide bridge, G+C content and DNA homology (Schleifer and Stackebrandt, 1983). Later, total soluble cellular protein profiles were introduced (Dicks 1995; Dicks and Van Vuuren, 1987, 1990; Dicks *et al*., 1990, 1993, 1995a, 1995b, 1995c, 1996, 2000; Dellaglio *et al*., 1991; Fantuzzi *et al*., 1992; Klein *et al*., 1996; Silvester and Dicks, 2003; Torriani *et al*., 1996). Schleifer and Stackebrandt (1983) introduced 16S rRNA sequencing as one of the most suitable and widely used bacterial identification methods. Phylogenetic trees generated by 16SrRNA sequences were combined with sequences from household genes and were presented as concatenated phylogenetic trees. Although these are considered conserved genes and the data adequate to determine the phylogenetic position of a strain, sequence data do not always point out phylogenetic relationships between clades, as pointed out by Salvetti *et al*. (2012) and Zheng *et al*. (2015). These concerns were supported by several large-scale phylogenetic analyses performed on genome sequences from *Lactobacillus* and *Pediococcus* (Duar *et al*., 2017; Sun *et al*., 2015; Zheng *et al*., 2015). As genome sequences became more available, notably so during the past 15 years, full genome comparisons became the gold standard for description of new bacterial species (Chun *et al*., 2018; Konstantinidis and Tiedje, 2005). An average nucleotide identity (ANI) value between 94 and 96% is considered the benchmark in taxonomy (Kim *et al*., 2014; Jain *et al*., 2018; Richter and Rosselló-Móra, 2009).

The plethora of genome sequences generated through taxonomic studies is a rich source of identifying genes with known traits that may be incorporated in phenotypic screenings. The "unknown" genes are largely ignored and should be analysed in-depth. Current genetic methods should be adapted to focus on multiple signals, including genetic information stemming from an evolutionary (strain evolving and adaptational) background, and focus on gene duplications, gene losses and parallel amino acid changes. We need to utilize existing genome sequences and not only focus on the "known unknown", but also the "unknown unknown" loci. These endeavours need to be complemented by more in-depth analyses of transcriptomes, proteomes, metabolomes and immunomes, and where possible, supported by model systems. More efficient extraction of information from genomic sequences and genetic signals can lead to many interesting, and hopefully testable, hypotheses, and help us understand

quorum sensing on a different level. Phenotypic and genotypic characteristics of gut microbiota may be used to define biomarkers to detect certain diseases, as also supported by microbiome analyses and the Microbiome Wide Association Studies (MWAS). A holistic approach is important, since genes and genomes do not function on their own. The fundamental principles of taxonomy and phenotypic traits should, however, never be abandoned.

Members of the Subcommittee on the Taxonomy of *Bifidobacterium*, *Lactobacillus* and Related Organisms of the International Committee on the Systematics of Prokaryotes (ICSP) published minimal standards for the description of new taxa within the genera *Bifidobacterium* and *Lactobacillus* (Mattarelli *et al*., 2014). The authors stated that "the description of novel species should be based on phenotypic, genotypic and ecological characteristics to ensure a rich polyphasic characterization". This meant species descriptions should be based on DNA G+C content, DNA-DNA hybridization, 16S rRNA gene sequencing and the sequencing of at least two housekeeping genes (e.g. hsp60 and recA). At the time of publication (Mattarelli *et al*., 2014), the order Lactobacillales, to which all LAB belong, included the families *Aerococcaceae* (genera *Aerococcus, Abiotrophia* and *Facklamia* and the "minor genera" *Dolosicoccus, Eremococcus, Globicatella* and *Ignavigranum*), *Carnobacteriaceae* (*Carnobacterium* and the "minor genera" *Alkalibacterium, Allofustis, Alloiococcus, Atopobacter, Atopococcus, Atopostipes, Desemzia, Dolosigranulum, Granulicatella, Isobaculum, Lacticigenium, Marinilactibacillus, Pisciglobus* and *Trichococcus*), *Enterococcaceae* (*Enterococcus, Tetragenococcus* and *Vagococcus*, plus the "minor genera" *Bavariicoccus, Catellicoccus, Melissococcus* and *Pilibacter*), *Lactobacillaceae* (*Lactobacillus* and *Pediococcus*), *Leuconostocaceae* (*Leuconostoc, Fructobacillus, Oenococcus* and *Weissella*) and *Streptococcaceae* (*Lactococcus, Lactovum* and *Streptococcus*). Based on 16S rRNA sequences, LAB were divided into three lineages, i.e., the *Leuconostoc* group, the *Lactobacillus casei*/*Pediococcus* group and the *Lactobacillus delbrueckii* group (Mattarelli *et al*., 2014). The genera *Carnobacterium, Enterococcus, Vagococcus, Aerococcus* and *Tetragenococcus* were considered more closely related to each other than to any other LAB (Mattarelli *et al*., 2014). *Streptococcus* and *Lactococcus* spp. formed a separate branch (Schleifer and Ludwig, 1995). Based on complete genome sequences, the *Streptococcus-Lactococcus* branch formed the basal part of the *Lactobacillales* tree, with the *Pediococcus* group close to the *Leuconostoc* group within the *Lactobacillus* clade (Mattarelli *et al*., 2014). Based on these findings, the genus *Lactobacillus* does not include all descendants of a common ancestor and may be considered paraphyletic to the *Pediococcus–Leuconostoc* group. *Lactobacillus casei* is positioned at the base of the *L. delbrueckii* group (Makarova *et al*., 2006; Makarova and Koonin, 2007), which

contradicts previous suggestions that lactobacilli should be grouped with pediococci (Holzapfel *et al*., 2001). Zhang *et al*. (2011) confirmed that *Leuconostocaceae, Enterococcaceae* and *Streptococcaceae* are monophyletic based on an analysis of 232 genes from 28 LAB genomes. The authors placed *Enterococcaceae* and *Streptococcaceae* in one group, separate from *Lactobacillaceae* and *Leuconostocaceae.*

The genus *Fructobacillus* warrants special mentioning. As part of the *Leuconostocaceae,* grouped with *Leuconostoc, Oenococcus* and *Weissella*, all species are heterofermentative, i.e., produce CO_2 from the fermentation of D-glucose. Species are, however, defined as either obligate or facultative heterofermentative. Obligate HE-F FLAB prefer fructose but will ferment glucose if external electron acceptors such as pyruvate and fructose are present (Endo *et al*., 2009). The facultative HE-F species, *F. florum*, produce lactate, ethanol, and acetate at a ratio of 1:0.8:0.2 (Endo *et al*., 2010) and CO_2 from glucose. Arabinose, ribose, and xylose, which are usually metabolized by HE-F LAB, are not fermented by FLAB. *Fructobacillus* spp. have lost most of the genes involved in carbohydrate metabolism (Endo *et al.*, 2015). This is evident from the approximately 5% carbohydrate and transport genes reported for *Fructobacillus* spp, compared to almost double the number of genes in *Leuconostoc* spp. (Endo *et al.*, 2015).

By 2020 as many as 261 *Lactobacillus* species have been described. The genetic diversity within the genus is far more complex than reported for other bacterial genera, and even bacterial families. Phylogenetic groups within *Lactobacillus* are composed of species with phylogenetic and physiological differences similar to diversity patterns observed for other bacterial genera (Zheng *et al*., 2015). With such variation in diversity, the taxonomic status of some species is questionable. The addition of newly described species to an already heterogeneous collection of species complicated matters even further. To clarify the taxonomic status of species within the genus *Lactobacillus*, Zheng *et al*. (2020) separated the *Lactobacillacea* (*Lactobacillus* and *Pediococcus* species) into 26 lineages. The authors followed a polyphasic approach and based their reclassification of the family on metabolic and ecological criteria, average nucleotide identity (ANI), average amino acid identity (AAI), core-gene average amino acid identity (cAAI), core genome phylogeny and the sequence of signature genes. Twenty-three clades were proposed, each resembling a new genus. The description of *Lactobacillus* and *Paralactobacillus* is emended but the description of *Pediococcus* is left unchanged. The authors also suggested that the family *Leuconostocaceae* Schleifer 2010 be considered a later synonym of the family *Lactobacillaceae* Winslow *et al.* 1917.

Lactobacillus is separated into 25 genera, according to pairwise average amino acid identity (AAI) and conserved proteins (POCP). The authors argued that classification based on AAI is the more powerful of the two metrics, as it is based on actual protein sequences and rules out putative gene expressions. The cut-off value determined for AAI on genus level is 68% (Konstantinidis and Tiedje, 2005). 16S rRNA gene sequence homology values greater than 94.5% was used to assign species to one of the 25 genera within *Lactobacillaceae.* Zheng *et al*. (2020) proposed an amendment to the description of the family *Lactobacillaceae* to include all genera that were previously included in the families *Lactobacillaceae* and *Leuconostocaceae*. This was based on two datasets and bio-informatic approaches that grouped *Leuconostocaceae* as a monophyletic cluster within *Lactobacillaceae* (Zheng *et al*., 2020). Heterofermentative lactobacilli are more closely related to *Leuconostoc* and *Weissella* than they are to the *L. Delbrueckii* group. Based on the reclassifications proposed by Zheng *et al*. (2020), all genera previously included in the families *Lactobacillaceae* Winslow *et al*. 1917 (Skerman *et al*., 1980) and *Leuconostocaceae* Schleifer 2010, i.e., *Convivina*, *Fructobacillus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Paralactobacillus*, *Pediococcus* and *Weissella* will be included in the family *Lactobacillaceae*. Species within *Lactobacillaceae* are described as Gram-positive, non-spore-forming and facultative or strict anaerobic. Cells are coccoid or rod-shaped, and may form chains, pairs, or tetrads (genus *Pediococcus*). Lactate is the main end-product from the fermentation of glucose, but acetate, ethanol, CO_2, formate, and succinate may also be produced. Species are fastidious, with complex requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters, and fermentable carbohydrates. *Lactobacillaceae* is the only family in *Lactobacillales* containing homofermentative and heterofermentative species. The type genus is *Lactobacillus* Beijerinck 1901 212. Species within the genus *Lactobacillus* are described as homofermentative, usually unable to ferment pentoses, and thermophilic. Pentose-phosphate and pyruvate-formate lyase pathways are absent. The genus *Lactobacillus* includes all organisms that were previously assigned to the *L. delbrueckii* group. The nomenclature of species in the emended genus *Lactobacillus* remains unchanged. The type species for the genus *Lactobacillus* is *L. delbrueckii*. For the latest classification of the genus *Lactobacillus* the reader is referred to Zheng *et al*. (2020). A broad outline of the reclassification of the genus *Lactobacillus* is listed in Table 1. Species of industrial importance, and their new names, are listed in Table 2. A simplified core genome phylogenetic tree of *Lactobacillaceae*, redrawn from Zheng *et al*. (2020), is shown in Fig. 1.

Table 1. Reclassification of the genus *Lactobacillus* into 25 newly proposed genera

Species grouping before 2020[a]	New genus name according to (Zheng *et al*., 2020)
***L. buchneri* group**	***Lentilactobacillus***
***L. collinoides* group**	***Secundilactobacillus***
***L. brevis* group**	***Levilactobacillus***
***L. fructivorans* group**	***Fructilactobacillus***
***L. kunkeei* group**	***Apilactobacillus***
***L. reuteri* group**	***Limosilactobacillus***
***L. vaccinostercus* group**	***Paucilactobacillus***
***L. rossiae* group**	***Furfurilactobacillus***
L. plantarum group	*Lactiplantibacillus*
L. salivarius group[b]	*Ligilactobacillus* and *Liquorilactobacillus*
L. algidus group	*Dellaglioa*
L. coryniformis group	*Loigolactobacillus*
L. selangorensis group	*Paralactobacillus*
L. sakei group	*Latilactobacillus*
L. casei group	*Lacticaseibacillus*
L. composti group	*Agrilactobacillus*
L. perolens group	*Schleiferilactobacillus*
L. dextrinicus/*concavus* group	*Lapidilactobacillus*
L. alimentarius group	*Companilactobacillus*
L. mellifer/*mellis* group	*Bombilactobacillus*
L. delbrueckii group[c]	*Lactobacillus*
L. amylophilus group	*Amylolactobacillus*
L. floricola group	*Holzapfelia*
L. jinshani group (name not validly published)	***Acetilactobacillus***

[a]Groups represent several species, e.g., the *L. buchneri* group includes *L. curieae*, *L. diolivorans, L. farraginis, L. hilgardii, L. kefir, L. kisonensis, L. otakiensis, L. parabuchneri, L. parafarraginis, L. parakefiri, L. raoultii, L. rapi, L. senioris* and *L. sunkii.*

[b]*L. salivarius* is a heterogeneous group and is included in two new genera.

[c]*Lactobacillus delbrueckii* is the type species of the genus *Lactobacillus.* This species share many phenotypic and genotypic characteristics with *L. leichmannii*, *L. lactis* and *L. bulgaricus. L. lactis* and *L. leichmannii* are classified as *L. delbrueckii* subsp. *Lactis,* and *L. bulgaricus* as *L. delbrueckii* subsp. *Bulgaricus.*

Heterofermentative groups and genera are printed in bold.

Table 2. Reclassification of prominent starter cultures and probiotics

Previous species name	New species name	Abbreviation

Starter cultures		
Lactobacillus acidophilus	*Lactobacillus acidophilus*	*L. acidophilus*
Lactobacillus casei	*Lacticaseibacillus casei*	*L. casei*
Lactobacillus curvatus	*Latilactobacillus curvatus*	*L. curvatus*
Lactobacillus delbrueckii	*Lactobacillus delbrueckii*	*L. delbrueckii*
Lactobacillus fermentum	*Limosilactobacillus fermentum*	*L. fermentum*
Lactobacillus helveticus	*Lactobacillus helveticus*	*L. helveticus*
Lactobacillus plantarum	*Lactiplantibacillus plantarum*	*L. plantarum*
Lactobacillus sakei	*Latilactobacillus sakei*	*L. sakei*
Probiotics:		
Lactobacillus plantarum	*Lactiplantibacillus plantarum*	*L. plantarum*
Lactobacillus brevis	*Levilactobacillus brevis*	*L. brevis*
Lactobacillus casei	*Lacticaseibacillus casei*	*L. casei*
Lactobacillus fermentum	*Limosilactobacillus fermentum*	*L. fermentum*
Lactobacillus paracasei	*Lacticaseibacillus paracasei*	*L. paracasei*
Lactobacillus plantarum	*Lactiplantibacillus plantarum*	*L. plantarum*
Lactobacillus reuteri	*Limosilactobacillus reuteri*	*L. reuteri*
Lactobacillus rhamnosus	*Lacticaseibacillus rhamnosum*	*L. rhamnosus*

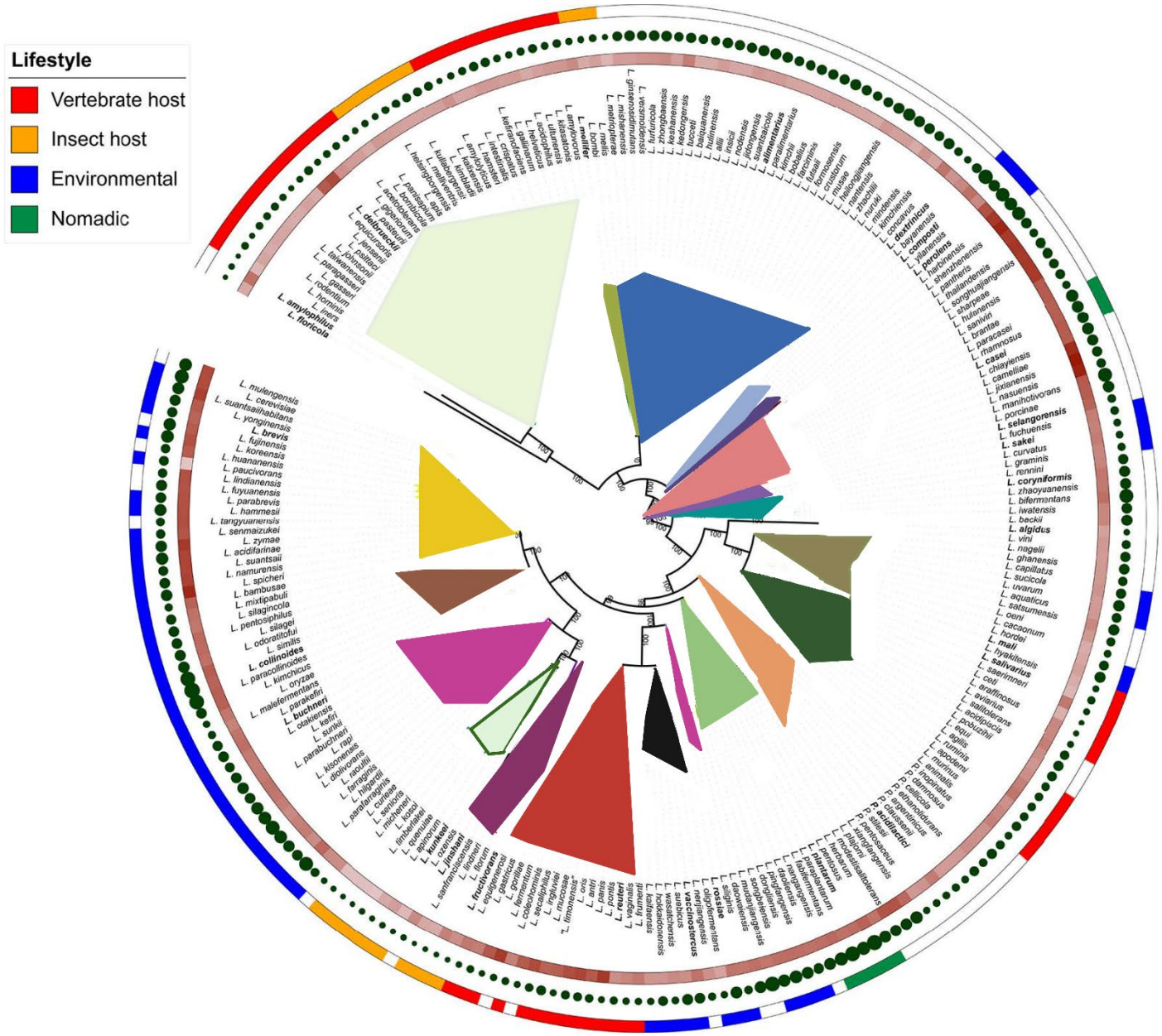

Figure 1. A simplified core genome phylogenetic tree of *Lactobacillaceae*, redrawn from Zheng *et al*. (2020). Phylogenomic relatedness was determined based on concatenated alignment of protein sequences for 114 single-copy core genes. The genome sequences of 244 *Lactobacillus* and *Pediococcus* spp. available on the NCBI database at time of publication was used. Midpoint rooting was used. Bootstrap support values were calculated from 500 replicates. Only values above 90% were taken into consideration. Members of the same phylogenetic group are included in the same triangle. Type strains are in bold. The outer circle denote genomic features and the natural habitat of the species. The inner circle depicts the GC content of each genome sequence. Higher GC contents are shown in darker red. The circle in the centre represent genome sizes, with larger genomes depicted as larger circles. This image is best viewed at 1.5x magnification.

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Chapter 3

Metabolism of Lactic Acid Bacteria Summarised

Species are classified as homofermentative if no CO_2 is produced from the fermentation of glucose; obligately homofermentative if only LA is produced from glucose and facultatively heterofermentative if LA is produced together with CO_2 and acetate

Separation of lactic acid bacteria (LAB) into physiological groups is practical, *but the species cannot read*

LAB are now grouped as either homofermentative (hexoses metabolised via the Embden–Meyerhoff pathway) or heterofermentative (hexoses fermented via the phosphoketolase pathway)

Species within the genus *Lactobacillus* conforms to this grouping, *but other LAB are even more dyslectic*, and the rule applies on family level

The ability to ferment pentoses is even more confusing, as this varies on species and strain level

Fructobacillus, of which some were *Lactobacillus* and others *Leuconostoc,* belongs to *Leuconostocaceae* and group with *Leuconostoc, Oenococcus* and *Weissella*

Ethanol production is less than 5%, due to a deletion of the bifunctional alcohol/acetaldehyde dehydrogenase (*adhE*)

Fructobacillus spp. have lost most of the genes involved in carbohydrate metabolism

Metabolism of Lactic Acid Bacteria Summarised

The metabolism of lactic acid bacteria (LAB) is discussed by Endo & Dicks (2014). All LAB ferment glucose either via the Embden-Meyerhof-Parnas (glycolytic) or phosphoketolase (6-phosphogluconate) pathway. The glycolytic pathway yields two moles lactic acid (LA) and two moles ATP per mol glucose fermented. Fructose-1,6-diphosphatase is the key enzyme in the pathway and pyruvate and acetyl-phosphate the key intermediates. Since LA is the only end-product formed, the fermentation is referred to as homolactic. Depending on growth conditions, some homofermentative species may revert to using additional metabolic pathways and produce a greater variety of end products. Despite this, species are classified as homofermentative if no CO_2 is produced from the fermentation of glucose and is further defined as obligately homofermentative if only LA is produced from glucose and facultatively heterofermentative if LA is produced together with CO_2 and acetate. The latter is only possible with the expression of genes encoding phosphoketolase that allows the fermentation of pentoses such as arabinose, ribose and xylose (obligately homofermentative LAB do not ferment pentoses). The ratio at which these end products are produced is influenced by the presence of electron acceptors and initial growth pH. Pentose metabolism is variable at not consistent amongst strains of the same species (Zheng *et al*., 2015). Pyruvate is converted to LA by lactate dehydrogenase (LDH) or formate by formate lyase. Sugars are generally transported across cell membranes by permeases, phosphorylated and converted to ribulose-5-phosphate or xylulose-5-phosphate by epimerase or isomerase. This results in the formation of 1 mol LA and 1 mol acetic acid per mol pentose, without the formation of CO_2. The phosphoketolase pathway is used by *Enterococcus, Lactococcus, Lactovum, Paralactobacillus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and group II *Lactobacillus* spp., as well as obligately heterofermentative LAB such as *Leuconostocaceae* (*Leuconostoc* and *Oenococcus*), *Weissella* and group III *Lactobacillus* spp. Members of *Carnobacteriaceae* (*Alkalibacterium, Carnobacterium, Marinilactibacillus* and *Trichococcus*) metabolises glucose to pyruvate using the glycolytic pathway, but converts additional pyruvate to formic acid, acetic acid, and ethanol, with or without CO_2 (De Bruyn *et al*., 1988; Ishikawa *et al*., 2009). From the latter group, *Carnobacterium* is the only genus that produces CO_2 (De Bruyn *et al*., 1988; Ishikawa *et al*., 2009; Liu *et al*., 2002). Within the genus *Lactobacillus*, homofermentative and heterofermentative species are separated into two distinct phylogenetic clades (Sun *et al*., 2015; Zheng *et al*., 2015).

Lactobacillus vini ferments pentoses via the pentose fermentation pathway. This pathway differs from the phospoketolase pathway by the presence of transketolase and transaldolase. In

this case 1.6-1.7 moles LA, and very little acetic acid or ethanol, is produced from arabinose and ribose (Barre, 1978; Rodas *et al.*, 2006). This is close to the theoretical value of 1.67 moles LA per mol pentose and is referred to as homolactic pentose fermentation. Genetically engineered strains of *L. vini*, with the ability to produce xylose isomerase and xylulose kinase, converted xylose to LA (Picataggio *et al*., 1998). Genetically engineered *Lactobacillus plantarum* NCIMB 8826 (strain WCFS1) metabolized xylose and arabinose to D-lactic acid (Okano *et al*., 2009a,b).

Obligately heterofermentative species ferment glucose via the phosphoketolase pathway. Heterolactic fermentation (HE-F) results in a net gain of one mol ATP per mol glucose consumed. Although *Bifidobacterium* is not regarded as true LAB, they metabolize glucose via the "bifidus pathway" (Scardovi, 1986) and produce 1.5 moles acetic acid and one mol LA per mol glucose. Phosphoketolase splits fructose-6-phosphate (F6P) to erythrose-4-phosphate (E4P) and AcP. The phosphoketolase of *Lactobacillus* spp. do not convert F6P to E4P (De Vries & Stouthamer, 1967).

Although the separation of LAB into physiological groups (obligately homofermentative, facultatively heterofermentative and obligately heterofermentative) is practical, this does not apply when species are compared using a phylogenetic and genomic approach. According to the latest reclassification of LAB (Zheng *et al*., 2020), LAB are grouped as either homofermentative (hexoses metabolised via the Embden–Meyerhoff pathway) or heterofermentative (hexoses fermented via the phosphoketolase pathway). This said, grouping based on hexose fermentation remains problematic and at times confusing. Species within the genus *Lactobacillus* conforms to this grouping, but in other LAB the rule applies on family level (Salvetti *et al*., 2012; Zheng *et al*., 2015). The ability to ferment pentoses is even more confusing, as this varies on species and strain level (Zheng *et al*., 2015).

LA is produced either in the D(-) or L(+) configuration and is considered one of the key characteristics to classify LAB into subgroups. Some species produce both configurations, whereas exceptions such as *Lactobacillus sakei* changes the ratio of LA isomers depending on the presence or absence of sodium acetate. Mainly L(+)-LA is produced in the presence of acetate, whereas almost equimolar amounts of the two isomers are produced in the absence of acetate (Iino *et al*., 2001).

The genus *Fructobacillus* warrants special mentioning. As part of the *Leuconostocaceae,* grouped with *Leuconostoc, Oenococcus* and *Weissella*, all species are heterofermentative, i.e., produce CO_2 from the fermentation of D-glucose. Species are, however, defined as either obligate or facultative heterofermentative. Obligate HE-F FLAB prefer fructose but will ferment glucose if external electron acceptors such as pyruvate and fructose are present (Endo *et al*., 2009). End products from the fermentation of fructose are lactate, acetate, CO_2, ethanol and mannitol. Almost equal molar ratios of lactate and acetate are produced (Fig. 1). Ethanol production is less than 5%, due to a deletion of the bifunctional alcohol/acetaldehyde dehydrogenase (*adhE*) gene (Endo *et al*., 2018). The lack of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) leads to an imbalance of NAD/NADH, and thus, the requirement of electron acceptors when glucose is metabolised. The facultative HE-F species, *F. florum*, produce lactate, ethanol, and acetate at a ratio of 1:0.8:0.2 (Endo *et al*., 2010) and CO_2 from glucose. Arabinose, ribose, and xylose, which are usually metabolized by HE-F LAB, are not fermented by FLAB.

Fructobacillus spp. have lost most of the genes involved in carbohydrate metabolism (Endo *et al.*, 2015). This is evident from the approximately 5% carbohydrate and transport genes reported for *Fructobacillus* spp, compared to almost double the number of genes in *Leuconostoc* spp. (Endo *et al.*, 2015). The phosphotransferase system (PTS) is not functional in *Fructobacillus*, as they have only one gene encoding enzymes in this pathway. *Leuconostoc* spp., on the other hand, have 13 PTS genes (Endo *et al.*, 2015). The number of genes encoding ATP-binding cassette (ABC) transporters are also less in *Fructobacillus* spp. (*ca.* 33 genes), compared to *Leuconostoc* spp. (50.6 genes; Endo *et al.*, 2015). Smaller genomes are characteristic of species in specialised habitats, e.g., *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners* and *Lactobacillus jensenii* isolated from the vaginal tract (Mendes-Soares *et al.*, 2014), *F. sanfranciscensis* from sourdough (Gobbetti & Corsetti, 1997) and *F. lindneri* from beer (Asano *et al*., 2009). It would be interesting to know which species of FLAB are best adapted to conditions in the human GIT and how changes in diet affect their survival. This is especially important as fructose is an integral part of our diet and present in fruits, honey and vegetables such as carrots, onions and sweet potatoes. The possible beneficial properties of FLAB is reviewed by Dicks & Endo (2022).

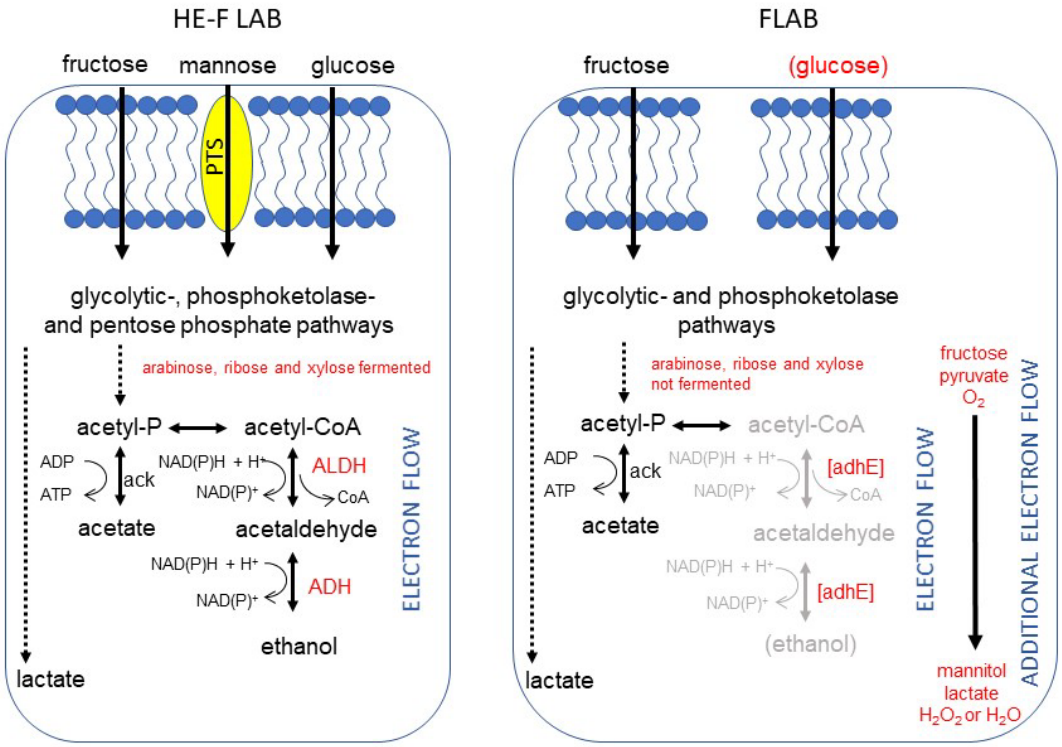

Fig. 1. Key differences between fructophilic lactic acid bacteria (FLAB) and heterofermentative lactic acid bacteria (HE-F LAB). For further reading, see Endo *et al.* (2018). PTS = phosphotransferase system; ack = acetate kinase; ALDH = acetaldehyde dehydrogenase; ADH = alcohol dehydrogenase; [adhE] = deletion in bifunctional alcohol/acetaldehyde dehydrogenase gene amongst obligate FLAB; (glucose) = poorly fermented in absence of fructose, pyruvate or O_2; (ethanol) = low concentrations produced, grey text denotes inactive or partially active pathways. From Dicks & Endo (2022).

Fructose, galactose and mannose are fermented by most LAB. Fructose and mannose enter the glycolytic or phosphoketolase pathway as G6P or F6P after isomerization or phosphorylation. Fructose does not only serve as substrate, but also as electron acceptor. If galactose is transported across the cell membrane via the phosphotransferase system (PTS), G6P is metabolized via the tagatose-6-phosphate pathway (Bissett & Anderson, 1973). If galactose is transported by galactose permease, it is metabolized by a combination of the Leloir and glycolytic pathways (Bissett & Anderson, 1974). LAB that prefers fructose over glucose, lacks the ability to ferment pentoses, and ferments glucose to almost equimolar amounts of lactic acid and acetic acid, with very little ethanol produced. Species with these characteristics, notably from the genus *Leuconostoc*, have been classified as fructophylic LAB (FLAB) and transferred to a newly formed genus *Fructobacillus* (Endo et al., 2009; Endo & Okada, 2008). The reason for not fermenting pentoses is most probably due to the absence of pentose kinase, epimerases

or isomerases. Of interest is that *Fructobacillus* spp. have significantly smaller genomes and less protein coding sequences compared to *Lactobacillus* spp. (Endo *et al*., 2015). Genes notably less prominent are those encoding the transport and metabolism of carbohydrates, including the phosphotransferase system (PTS). This explains why *Fructobacillus* spp. are less saccharoclastic than other LAB. *Fructobacillus* spp. have lost most of the genes involved in carbohydrate metabolism (Endo *et al.*, 2015). This is evident from the approximately 5% carbohydrate and transport genes reported for *Fructobacillus* spp, compared to almost double the number of genes in *Leuconostoc* spp. (Endo *et al.*, 2015). The phosphotransferase system (PTS) is not functional in *Fructobacillus*, as they have only one gene encoding enzymes in this pathway. *Leuconostoc* spp., on the other hand, have 13 PTS genes (Endo *et al.*, 2015). The number of genes encoding ATP-binding cassette (ABC) transporters are also less in *Fructobacillus* spp. (*ca.* 33 genes), compared to *Leuconostoc* spp. (50.6 genes; Endo *et al.*, 2015). Smaller genomes are characteristic of species in specialised habitats, e.g., *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners* and *Lactobacillus jensenii* isolated from the vaginal tract (Mendes-Soares *et al.*, 2014), *Fructobacillus sanfranciscensis* from sourdough (Gobbetti & Corsetti, 1997) and *Fructobacillus lindneri* from beer (Asano *et al*., 2009). Maeno *et al*. (2021) hypothesized that *Fructobacillus* spp. underwent specific reductive evolution when they adapted to D-fructose-rich niches. This may be true for LAB other than *Leuconostoc* spp, as seen from genomic characteristics reported for *Lactobacillus kunkeei* and *Lactyobacillus apinorum (*Maeno *et al.* 2016, 2017). Concluded from these findings, a drastic change in fermentable carbohydrates induces a certain level of gene reductions that gives rise to a phylogenetically distant group of bacteria (Fig. 2). Further studies revealed that *Fructobacillus* spp. lacks the bifunctional alcohol/acetaldehyde dehydrogenase gene (adhE) and respective enzyme activities (Endo *et al*., 2014; Endo *et al*., 2015). These specific characteristics are conserved within FLAB, but exceptions have been noted, such as *L. kunkeei* with a partially conserved *adhE* gene (Maeno *et al*., 2016). Absence of a functional *adhE* gene in *Fructobacillus* spp. may lead to a shortage of NAD^+ and the requirement of additional electron acceptors, in this case fructose. Maeno *et al*. (2019) cloned *adhE* from *Leuconostoc mesenteroides* NRIC 1541^T into a plasmid and transferred this to *Fructobacillus fructosus* NRIC 1058^T. Strain 1-11, transformed with the *adhE* gene, did not show any fructophilic characteristics, and the strain grew well on D-glucose without external electron acceptors. Accumulation of acetic acid, reported in the parental strain, was replaced with ethanol in the transformed strain. Furthermore, *in silico* analyses revealed that strain NRIC 1058^T lacked the sugar transporters/permeases and enzymes required to convert metabolic intermediates. This may be the reason why FLAB display such poor carbohydrate fermentation. Expression of

adhE in the recombinant strain is likely due to this leaky promoter activity that assists bacteria to produce acceptable levels of AdhE. Strain 1-11 that expressed the *adhE* gene did not show fructophilic characteristics, i.e., good growth on D-glucose in the absence of external electron acceptors and ethanol production from the metabolism of D-glucose. This is clear evidence that a lack in the *adhE* gene is the reason for fructophilic characteristics in *Fructobacillus* spp. The phosphoketolase pathway needs ethanol formation, supported by AdhE, to keep NAD/NADH in balance. FLAB, including *Fructobacillus* spp., *L. kunkeei* and *L. apinorum*, are the only heterofermentative LAB that partially or completely lack *adhE* (Maeno *et al*., 2016). Thus, FLAB require external electron acceptors such as pyruvate and oxygen for metabolism of D-glucose. D-Fructose can be used as a carbon source and an electron acceptor in these organisms (Zaunmuller *et al*., 2006) and in fact, they produce mannitol from the metabolism of D-fructose (Endo *et al*., 2009; Endo and Okada, 2008). Expression of *adhE* had a great impact on the metabolism of D-glucose, but not on the metabolism of other carbohydrates in *F. fructosus.* This is due to poor carbohydrate metabolic systems in *F. fructosus* NRIC 1058^{T}. Carbohydrate transport systems predicted present in the strain were only for D-glucose, D-fructose and Larabinose/D-ribose/D-xylose/D-galactose, although L-arabinose, D-ribose, D-xylose and D-galactose were not metabolized. In LAB, these pentoses and D-galactose are usually transported into cells and converted to specific intermediates of the phosphoketolase pathway (Cibrario *et al*., 2016; Ganzle *et al*., 2007; Kandler, 1983) but the enzymes needed for the conversion are not present in the genome of *F. fructosus*. Transport systems and enzymes needed for the metabolism of disaccharides are not found in the strain. This correlates with the previous findings that *Fructobacillus* spp. have lost genes specifically involved in carbohydrate transport and metabolism (Endo *et al*., 2015). *Lactobacillus kullabergensis* and *Lactobacillus kimbladii* (both honeybee symbionts) were reported to possess 87 and 88 genes, respectively, coding for 41 and 42 complete PTS transporters (Ellegaard *et al*., 2015). D-mannitol was slowly metabolized by *F. fructosus*, but a D-mannitol transport system was not predicted in the strain. This suggests that an unidentified D-mannitol transport system is present in the strain, or that other systems are used to transport mannitol.

Some LAB metabolize disaccharides such as cellobiose, lactose, maltose, melibiose, sucrose, etc. These sugars are transported across the cell membrane either as free sugars or phosphorylated, split into two monosaccharides or a monosaccharide and a monosaccharide phosphate, and metabolized via the glycolytic or phosphoketolase pathways. Some LAB prefer disaccharides rather than monosaccharides as growth substrates, even though disaccharide

fermentation seems more complicated compared to monosaccharide fermentation. Examples are lactose fermentation by dairy LAB and maltose fermentation by sourdough LAB.

In all of these pathways pyruvate serves is an intermediate electron acceptor and is if not converted to ethanol, as in the case of HE-F LAB and facultatively heterofermentative *Lactobacillus* spp., converted to diacetyl, acetoin or formate. The diacetyl-acetoin pathway is used by *Lactococcus lactis* subsp. *Lactis* biovar. Diacetylactis and converts citrate to pyruvate, oxaloacetate, diacetyl, and acetoin. Citrate metabolism increases cytoplasmic pH and is thus enhanced under acidic conditions (García-Quintáns *et al*., 2008). The pyruvate formate lyase pathway is only present in obligately homofermentative and facultatively heterofermentative LAB (Wagner *et al*., 2005). Pyruvate in converted to formate, acetate, and ethanol under anaerobic and substrate limiting conditions (Kandler, 1983; Thomas *et al*., 1979). Species of *Alkalibacterium, Marinilactibacillus* and *Trichococcus* metabolize glucose by using a combination of the glycolytic and pyruvate formate lyase pathways. Several heterofermentative LAB uses the pyruvate dehydrogenase pathway (under aerobic conditions) to produce acetate and CO_2 (Wagner *et al*., 2005). Ethanol production is suppressed under aerobic conditions and double the amount of acetate is produced. Pyruvate may also be degraded to acetate, CO_2 and H_2O_2 under substrate limiting conditions, as reported for *L. plantarum* (Goffin *et al*., 2006).

To conclude, LAB are classified as obligately homofermentative if they ferment glucose via the glycolytic pathway (key enzyme = FDP aldolase) and obligately heterofermentative if they lack FDP aldolase but contains phosphoketolase (Kandler, 1983). Facultatively heterofermentative LAB share characteristics of both groups, as they have enzymes from both metabolic pathways.

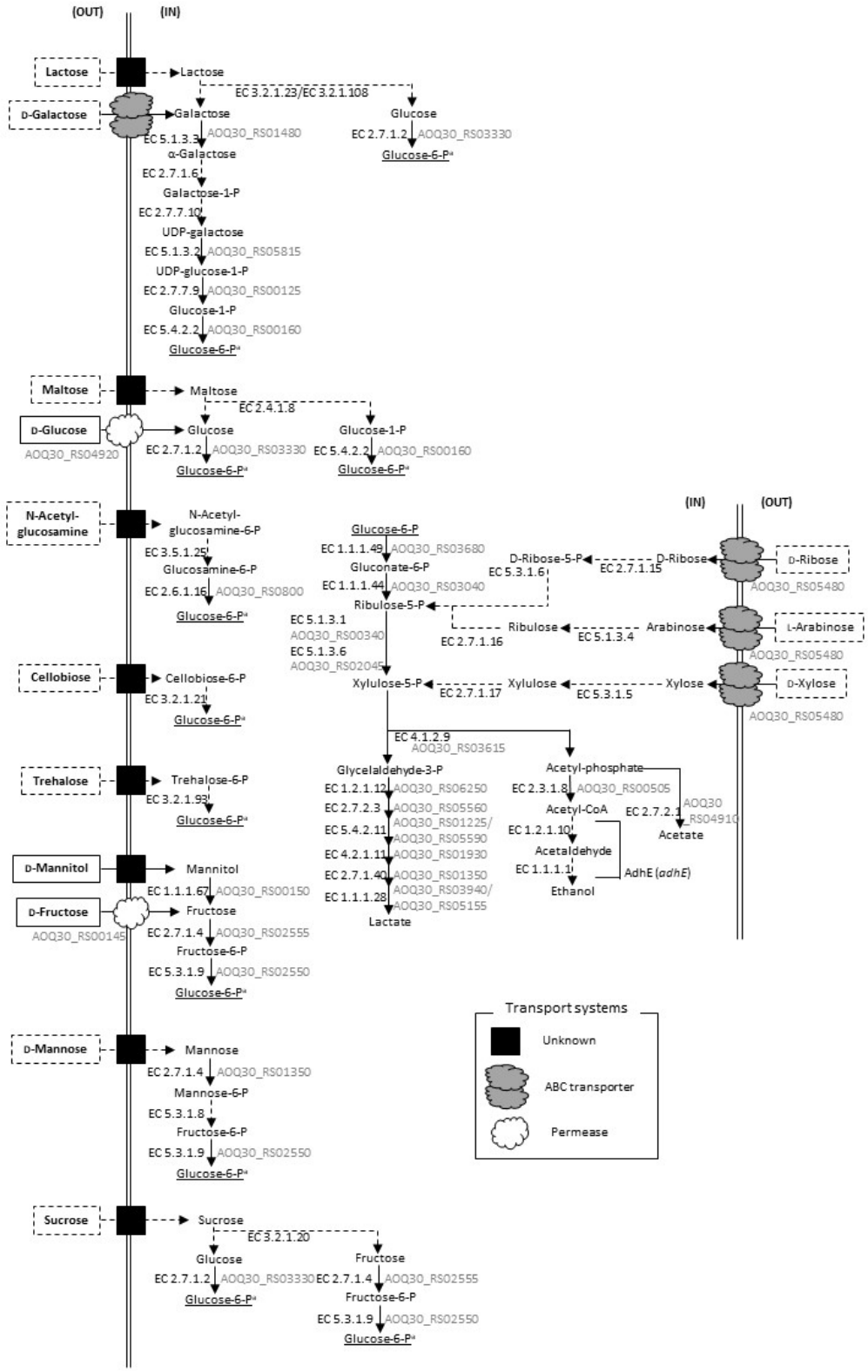

Fig. 2. Carbohydrate metabolic pathways predicted for *F. fructosus* NRIC 1058^{T}. Arrows with solid lines indicate proteins conserved in *F. fructosus* NRIC 1058^{T}. Arrows with dashed lines indicate unidentified proteins. Carbohydrates squared with solid lines were metabolized by strain NRIC 1058^{T}. Carbohydrates squared with dashed lines were not metabolized. Enzyme commission numbers are indicated for each enzymatic reaction. Locus tags to address proteins in genome of the strain NRIC 1058T (BBXR01000000) are also shown for the reaction, if available. [a]Generated glucose-6-phosphate in metabolism of each carbohydrate is metabolized in the phosphoketolase pathway. From Maeno *et al*. (2019).

Electron acceptors

Most LAB use oxygen and pyruvate as electron acceptors to oxidise NADH to NAD^+. Fructose, citrate and malate may also be used, but this is strain dependent. If obligately heterofermentative LAB do not use pyruvate as electron acceptor, acetic acid instead of ethanol, and double the amount of ATP (two moles), are produced from the fermentation of glucose. Oxygen serves as electron acceptor, but only in the presence of NADH oxidase. NADH oxidase 1 (NOX-1) produces H_2O_2, whereas NOX-2 forms H_2O, as summarised below.

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2 \text{ (Nox-1)}$$

$$2NADH + 2H^+ + O_2 \rightarrow 2NAD^+ + 2H_2O \text{ (Nox-2)}$$

The conversion of acetyl-CoA to ethanol is only suppressed if pyruvate is twice the level of glucose (Nuraida *et al*., 1992). The reasoning behind this is that one molecule of pyruvate oxidizes one molecule of NADH. Reduction of fructose leads to the formation of mannitol (Eltz and Vandemark, 1960). In co-fermentation of glucose and fructose, fructose is used mostly as an electron acceptor, whereas glucose is metabolized by the phosphoketolase pathway (Richter *et al*., 2003).

Wine LAB such as *Oenococcus oeni* uses L-malate as electron acceptor and converts it to L-lactate and CO_2. This decarboxylation process, referred to as malo-lactic fermentation (MLF) deacidifies the environment and proton pumps are used in the exchange of nutrients. L-malate entering the cell is converted by the malolactic enzyme (L-malate NAD^+ carboxylase) to L-lactic acid and carbon dioxide (Lonvaud-Funel, 1999). Studies with membrane vesicles prepared from *Lactococcus lactis* IL1403 have shown that L-malate enters the cell either via a uniport ($Hmal^-$) or a symport (mal^{2-}/H^+) system. L-lactate may also be exchanged for L-malate, either as $Hmal^-/Hlac$ or as mal^{2-}/lac^-. In both cases, a net negative charge is formed on the inside of the cell membrane. L-malate is deproteinated and it accumulates in the cytoplasm. L-$malate^{2-}$ is rapidly exchanged for L-$lactic^-$ acid, much faster than the symport of L-$malate^{2-}$ and H^+ takes place (Poolman *et al*., 1991). With the consumption of a proton, the pH of the cytoplasm increases (becomes more alkaline) and a pH gradient forms across the cell membrane. MLF forms a PMF strong enough to drive ATP synthesis via F0F1-ATPase (Poolman *et al*., 1991). *Oenococcus oeni* (Dicks *et al*., 1995) and a few LAB, e.g., *Enterococcus faecalis* and *Lactobacillus casei*, uses L-malate as energy source (London, 1990). Only L-malate (not D-malate), is converted to L-lactate.

Oenococcus oeni favors a more acidic pH environment than *L. lactis* and uses a PMF-generating uniport system to metabolise L-malate (Ramos *et al*., 1994; Salema *et al*., 1994). Malate is transported by a negatively charged mono-protonated ($Hmal^-$) mechanism and not by $Hmal^-$/Hlac (or mal^{2-}/lac^-) as in the case of *Lc. lactis*. Lactic acid is excreted by passive diffusion and generates a pH gradient. Since the process is driven by substrate (L-malate) levels, the cell has to keep the internal concentration of the substrate as low as possible. This is done by rapid MLF (Salema *et al*., 1994; 1996). Compared to MLF in *Lc. lactis*, the malate/lactate exchange in *O. oeni* is less efficient and the uniport system is favored.

Citrate serves as a precursor for an electron acceptor and may thus be defined as an indirect electron acceptor. Oxaloacetate, formed from citrate, is decarboxylated to pyruvate, and the pyruvate is used as an electron acceptor (Zaunmüller *et al*., 2006). Citrate metabolism in *O. oeni* occurs via transport mechanisms similar to that used in MLF (Ramos *et al*., 1994). The citrate carrier catalyses a one-directional transport of monovalent $Hcit^{2-}$ to generate a membrane potential. Metabolism of citrate increases the medium pH, i.e., protons are consumed in the metabolic breakdown inside the cell. Pyruvate is also an additional source of ATP (Hugenholtz, 1993; Lin *et al*., 1991). Fermentation of citrate leads to the production of aroma compounds such as diacetyl, acetoin and acetic acid, but is also important in establishing a proton-motive force, as shown for *Leuconostoc mesenteroides* and *Lc. lactis* (Magni *et al*., 1999; Marty-Teysset *et al*., 1996; Ramos *et al*., 1994). In the case of *Leuc. mesenteroides*, citrate enters the cell as D-lactate is excreted. The exchange is electrogenic, as the negative charge that forms in the cytoplasm generates a membrane potential. Metabolism of citrate leads to an increase in cellular pH and thus a pH gradient across the membrane.

pH control and energy production

LAB are omnipresent in environments rich in nutrients and have developed mechanisms to adapt to acidic conditions. Although lactate metabolism is unusual for LAB, *Lactobacillus bifermentans*, a facultatively heterofermentative species, converts the lactic acid it produces to acetic acid, ethanol, CO_2 and H_2 under nutrient limiting conditions, but only when the pH is above 4.0 (Kandler *et al*., 1983). Some strains of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* control their external pH by fermenting lactic acid to acetic acid, 1,2-propandiol and trace amounts of ethanol, but only under anaerobic conditions. This is done without the requirement of external electron acceptors and below pH 5.8 (Oude Elferink *et al*., 2001). Obligately heterofermentative LAB and a few facultatively heterofermentative LAB control

their pH variations by converting arginine to ornithine, CO_2 and two molecules of NH_3 (Arena *et al*., 2002; Champomier Vergès *et al*., 1999; Zaunmüller *et al*., 2006). This is usually done in the presence of a fermentable carbohydrate (Kandler & Weiss, 1986; Konings *et al*., 1989). A specific transporter (ATP independent) exchanges intracellular ornithine for extracellular arginine (Driessen *et al*., 1987; Poolman *et al*., 1987c). The ATP produced by the arginine-deiminase pathway (dephosphorylation of carbamoyl-P) may thus be used for other metabolic energy requiring processes. The arginine deiminase pathway is, however, strongly regulated (Konings *et al*., 1989; Poolman *et al*., 1987c) and is repressed in the presence of glucose or lactose. Increased levels of arginine-deiminase are observed when the pH decreases to below 5.0. During arginine/ornithine exchange protons are taken up from the cytoplasm, resulting in an increase of the cytoplasmic pH and the generation a transmembrane pH gradient. From a physiological point of view, the production of ammonia prevents a drastic decrease in internal and external pH (Marquis *et al*., 1987). Thus, at low pH the glucose PTS system glycolytic enzymes are inhibited, whereas the arginine deiminase pathway is still active (Poolman *et al*., 1987c). This derepression of the arginine deiminase pathway leads to an increase in pH, which in turn stimulates glycolysis. The increase in intracellular levels of ornithine ensures an outward migration. Both gradients therefore contribute to the driving force for the electroneutral arginine/ornithine exchange process.

As fermentation proceeds, lactic acid levels in the cytoplasm increases and must be secreted. The excretion of lactic acid generates an electrical potential across the cell membrane and may thus also lead to energy conservation in the form of proton motive force (PMF). Under normal physiological conditions the pH of the cytoplasm is above neutral (Poolman *et al*., 1987a). Since the pK of lactic acid is 3.9, most lactic acid is dissociated at neutral pH and present as anionic lactate (the pK of lactic acid is 3.9). The latter can only leave the cell with the assistance of a specific transport mechanism. Actively growing cells in exponential growth will thus export more lactate and generate a high membrane potential. More than one proton is translocated per lactate ion (ten Brink & Konings, 1982). In an acidic environment (low pH), the excretion of lactate does not generate a membrane potential and the process is described as electroneutral. In this case, only one proton is translocated per lactate ion. Thus, at high pH, at the onset of fermentation, lactate is excreted by a proton-driven symport system (Michels *et al*. 1979; ten Brink & Konings, 1982) with two or more protons (Konings and Booth, 1981). At low pH, towards the end of fermentation, excretion is facilitated by the same transport system, but with a proton/lactate stoichiometry of one and/or by passive diffusion of the undissociated lactic acid. The metabolism of glucose via the glycolytic pathway results in the

formation of lactate (one lactate anion and one proton, H^+). The excretion of lactate will thus only generate a PMF if this occurs in symport with two or more protons (Michels *et al*., 1979). The efflux of lactate via PMF symport is calculated to provide between 25% and 33% additional energy (Konings, 2006). A specific membrane-associated protein (carrier or permease) translocates the metabolite across the cell membrane together (in symport) with a proton. Most sugars, amino acids and peptides are transported using this system (Neves *et al*., 2005). Sugars translocated via PMF-driven symport are phosphorylated by kinases (Axelsson, 2004; Neves *et al*., 2005).

Some LAB, especially lactococci, transport carbohydrates across the cell membrane using a permease-mediated system combined with a phosphotransferase (PTS) system (Konings, 2006). This is also referred to as group translocation or sugar phosphotransferase (sugar PTS) as sugars are phosphorylated during transfer across the cell membrane. Energy is provided from the phosphate bond of phosphoenolpyruvate (PEP). The phosphoryl group is either transferred to EI and initiate the PTS cycle, or donated to pyruvate kinase to form ATP (Axelsson, 2004). In *Lc. Lactis* glucose is transported with a mannose-PTS (PTSman) system. The same system is also used to transport glucosamine and fructose (Neves *et al*., 2005). Glucose is phosporylated to glucose 6-phosphate by EIIA. A glucose-PTS system with specificity to glucose and α-methyl-glucoside has also been described (Neves *et al*., 2005). Glucose may be transported via a secondary transport system using sugar permeases. Fructose is transported via the PTSman system and then converted to fructose 6-phosphate, or via a fructose-PTS system yielding fructose 1-phosphate. Fructose 1-phosphate is then phosphorylated to FDP before it enters glycolysis. Both these transport systems are used by *Lc. lactis*. Some heterofermentative LAB, e.g. *Lactobacillus reuteri* and *Lactobacillus brevis*, also use PTS for fructose transport (Taranto *et al*., 1999). Sucrose is transported by sucrose-specific PTS, resulting in sucrose 6-phosphate. Sucrose 6-phosphate is hydrolyzed by sucrose 6-phosphate hydrolase, yielding glucose 6-phosphate and fructose. Glucose 6-phosphate enters glycolysis, whereas the fructose moiety is phosphorylated by an ATP-dependent fructokinase (Neves *et al*., 2005). Since PTS is tightly linked to glycolysis, this form of energy production is not common in heterofermentative LAB (Romano *et al*., 1979; Taranto *et al*., 1999). Obligate heterofermentative species prefer to transport carbohydrates using a PMF-driven transport mechanism (Taranto *et al*., 1999).

The uptake and excretion of solutes across the cell membrane is highly regulated by specific transport systems. In LAB chemical energy is used to generate proton gradients across the cell

membrane. This is done via primary transport systems such as the F_0F_1-ATPase and ATP-binding cassette (ABC)-transporters. Energy-rich ATP is hydrolyzed by membrane-bound F_0F_1-ATPases to generate electrochemical ion gradients (Konings, 2006). As protons leave the cell, an electrical potential ($\Delta\Psi$) and proton gradient (ΔpH) forms across the cell membrane. The difference in proton levels across the cell membrane leads to generation of the proton motive force (PMF), which in turn drives the transport of ions and metabolites from outside the cell across the membrane to the cytoplasm (Konings *et al*., 1997). Secondary transport systems, classified as uniporters, symporters and antiporters, are used to convert this (electro)-chemical energy to metabolites during their transport across the membrane. Uniporters catalyse the translocation of only one metabolite across the cell membrane. Symporters transport two or more metabolites in the same direction. Antiporters, on the other hand, exchange metabolites across the cell membrane in two different directions. It is thus important for LAB to maintain a relatively high ATP pool.

The direct exchange of a metabolic product for a substrate via the same transport system is possible if the two compounds are structurally similar. In this case the transport process is driven by both the precursor gradient ΔS and the product gradient ΔP, at no additional load on energy consumption. This antiport system may provide additional metabolic energy if the process is electrogenic, i.e. when the exchange process involves the translocation of positive charges from the cytoplasm to the outside or negative charges from outside the cell to the inside. Lactose transport by *Streptococcus thermophilus* is an interesting example of precursor-product antiport. Lactose enters the cell via an ATP-energized PMF-dependent system (Fox *et al*., 1990). Due to weak galactokinase activity (Thomas & Crow, 1984), only the glucose moiety of lactose is metabolized and galactose is excreted (Hutkins & Ponne, 1991). With each galactose leaving the cell, a lactose molecule is transferred into the cell (Hutkins & Ponne, 1991). Thus, PMF is not needed and ATP is spared to be used in other metabolic reactions. A similar phenomenon has been observed for some beer lactobacilli (Wood & Rainbow, 1961). Maltose is preferred to glucose, i.e. one molecule of glucose is rejected for every molecule of maltose utilized. This was the first evidence that maltose fermentation was initiated by maltophosphorylase, an activity that did not require ATP.

Lc. lactis may exchange L-lactate for L-malate, either as $Hmal^-$/Hlac or as mal^{2-}/lac^-. This is different from the MLF described for *O. oeni* that uses an electrogenic uniport step coupled to a proton consuming decarboxylation step. The reason for this may be that *Lc. lactis* prefers more alkaline growth conditions compared to *O. oeni* (Poolman *et al*., 1991). At high pH

(above 6.0), malate is mainly present as Mal^{2-}. For the same reason, lactate in the cell is mainly present as Lac^{-}. The lactate leaves the cell via the malate transporter. Thus, in *Lc. lactis* Mal^{2-}/Lac^{-} exchange is an electrogenic process in which negative charge is translocated from outside to inside and a proton is consumed in the decarboxylation process.

Decarboxylation of amino acids, producing biogenic amines such as histamine, cadaverine, putrescine, tyramine and tryptamine, occurs mainly at the end of fermentation when carbon and energy sources are low (ten Brink *et al*., 1990). Although these pathways have not all been studied with respect to their role in metabolic energy generation, it is very likely that these processes are important in the generation of PMF. Histidine (neutral charged) enters the cell via the histidine transporter, is decarboxylated to histamine with one positive charge and leaves the cell via the same transporter. Due to differences in charge, an electrochemical gradient form across the cell membrane (Molenaar *et al*., 1993) and a pH gradient is generated by the decarboxylation process, similar to that observed for MLF.

Undissociated glutamate (glutamic acid) is transported across the cell membrane, but only at low external pH (Poolman & Konings, 1988). At high pH (alkaline conditions), glutamate is not transported and cell growth is retarded. Glutamine, on the other hand, is transported independent of pH gradient (Poolman & Konings, 1988). The requirement for glutamate can be nullified by the supply of additional glutamine.

Requirement for amino acids, vitamins and minerals

LAB usually require complex amino acids and vitamins for growth, and synthetic medium is necessary for the confirmation of specific requirements. L-glutamic acid, L-isoleucine, L-leucine and L-valine are required by almost all LAB. L-Methionine, L-tyrosine and L-tryptophan are required by many LAB (Garvie, 1967; Ledesma *et al*., 1977). Pantothenate and niacin are essential as precursors for HSCoA and NAD. Biotin is also required by many LAB (Garvie, 1967; Rogosa *et al*., 1961; Simpson & Taguchi, 1995). Several metals, e.g. Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, are used as minerals for culturing of LAB. Although Mn^{2+} stimulates the growth of LAB in general, enterococci has no requirement for the mineral (Efthymiou & Joseph, 1972). The minerals are usually required for enzymatic reactions (Archibald, 1986). On the other hand, most LAB do not require iron ions (Archibald, 1986; Imbert & Blondeau, 1998; Pandey *et al*., 1994). Requirements are basically strain dependent and results sometimes differ when different synthetic growth medium is used.

Proteolysis and lipolysis

As free amino acids are scarce in milk, some dairy LAB have proteolytic activities to obtain amino acids from milk casein. In general, the exploitation of casein by LAB is initiated by a cell-envelope proteinase that degrades the protein into oligopeptides that are subsequently taken up by the cells via specific peptide transport systems for further degradation into shorter peptides and amino acids by a concerted action of various intracellular peptidases (Savijoki *et al*., 2006). Several types of proteinase genes were cloned from dairy LAB, including *Lc. lactis, Lactobacillus helveticus, Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Savijoki *et al*., 2006). Proteolysis is also of industrial importance due to its contribution to organoleptic properties of fermented milk products (Meyer & Spahni, 1998; Sridhar *et al*., 2005).

Lipolysis is important in the developing of flavor in dairy products, especially in cheese ripening. Several dairy LAB, including *Enterococcus faecalis, E. faecium, E. durans, Lb. casei, Lb. plantarum* and *Lb. rhamnosus*, have been reported to have lipolytic activity (Abeijón Mukdsi *et al*., 2009; Centeno *et al*., 1999; Di Cango *et al*., 2006). Lipolysis in cheese usually occurs via esterase/lipase systems of LAB, propionic acid bacteria, molds and yeasts.

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Chapter 4

The Walks of Life – From a Microbial Perspective

The gastro-intestinal tract (GIT) of an adult human with a body mass of 70 kg is home to approximately 4 trillion microorganisms

More than 90% are bacteria in the GIT are represented by the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes

Firmicutes are in the majority, with lactic acid bacteria dominated by *Lactobacillus* spp. The other major group is Gram-negative Bacteroidetes

Gut microbiota supersedes the number of cells in our bodies 10-fold and contains 100 times more genes than the human genome

Seconds after birth the responsibility of developing a gut microbiome sits square on your shoulders

Your gut microbiome is yours for life and if treated right, has an infinite shelf life

The Walks of Life – From a Microbial Perspective

Most of the information in this chapter was published in a review by Dicks *et al*. (2018), Our Gut Microbiota: A Long Walk to Homeostasis. *Benef. Microbes* 9: 3–20.

With Love from Mother

The gastro-intestinal tract (GIT) of an adult human with a body mass of 70 kg is home to approximately 4 trillion microorganisms (Huttenhower *et al*., 2012; Nelson *et al*., 2010; Qin *et al*., 2010; Li *et al*., 2014), of which more than 90% are bacteria represented by the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. (Li *et al*., 2014; Hugon *et al*., 2015; Bilen *et al*., 2018). Firmicutes are in the majority (Giovannini *et al.*, 2021). Fusobacteria and Verrucomicrobia make up the remaining 10% of the gut microbiome (Eckburg *et al.,* 2005). Gut microbiota are protected by more than 32 m^2 epithelial cells and several layers of mucus (Helander and Fandriks, 2014). It is generally believed that gut microorganisms supersedes the number of cells in our bodies 10-fold and contains 100 times more genes than the human genome (Guarner and Malagelada, 2003; Shreiner *et al*., 2015).

Translocation of bacteria from the mother to the unborn remains an area of dispute. Evidence of *Enterococcus faecium* transferred from pregnant mice to their offspring (Jiménez *et al*., 2005; Martín *et al*., 2008) and the presence of *Escherichia*, *Enterococcus*, *Staphylococcus* and *Propionibacterium* in blood sampled from mice suggests that bacteria may reach the fetus via the placenta and bloodstream (Jiménez *et al*., 2008; Borre *et al*., 2014). Bacteria in the mother's bloodstream are most likely captured by dendritic cells (DCs) in the gut wall and then translocated to lymphoid tissue (Vazquez-Torres *et al*., 1999; Rescigno *et al*., 2001). Bacteria may also be transferred through breast milk, as hypothesized with studies on rodents (Perez *et al*., 2007). Following a completely different approach, Dasanayake *et al*. (2005) have shown that *Actinomyces naselundii* from human oral cavity may reach the uterus via the circulatory system. This finding was supported by high cell numbers of oral microbiota in the placenta of healthy mothers (Aagaard *et al*., 2014). It is also possible that bacteria in the placenta (Aagaard *et al*., 2014) and amniotic fluid (Bearfield *et al*., 2002; Jiménez *et al*., 2008; Rautava *et al*., 2012), are transferred across epithelial cells in the umbilical cord. These findings and reports of bacteria in fetal membranes of healthy newborns (Jiménez *et al.*, 2005; Rautava, 2012; Steel

et al., 2005), suggests that the intestinal tract of the fetus is colonized when still in the womb. Reports of *Streptococcus mitis* and *Lactobacillus plantarum* found in the meconium of fetuses, and staphylococci, enterococci, *Escherichia coli*, *Klebsiella pneumoniae* and *Serratia marcescens* from the first stool of newborns (Hu *et al*., 2013; Moles *et al*., 2013; Rodríguez *et al*., 2015), supports this hypothesis.

The first three months of pregnancy is characterized by an increase in butyrate-producing *Faecalibacterium* and *Eubacterium* spp. During this time, the bacteria from the foetus simulates the microbial development in the gastro-intestinal tract (GIT) of the expecting mother Mor and Cardenas, 2010). It is thus not abnormal to observe an increase in cytokine levels in the placenta during the first three months of pregnancy, as pointed out Mukhopadhya *et al*. (Mukhopadhya *et al*., 2012). The last three months of pregnancy is characterized by higher cell numbers of *Enterobacteriaceae, Streptococcus* spp. and proteobacteria. *Enterobacteriaceae* is known to promote inflammatory responses, but is kept under control with increased cytokine levels at the placental interface (Mor and Cardenas, 2010; Mukhopadhya *et al*., 2012). What is astounding is that the development of a foetus is not affected by changes in the mother's gut microbiome during pregnancy. However, should the placenta become infected with *Prevotella* and *Gardnerella*, new-borns stand a chance developing distinctive inflammatory responses (Fichorova *et al*., 2011; Stout *et al*., 2013). It is, however, generally accepted that the transfer of microorganisms from the mother to the foetus, and colonization of the GIT of the unborn, is directly influenced by the mother's health. Changes in physiological conditions, stress, the abuse of alcohol and nicotine, and the taking of medication prescribed during pregnancy has a profound influence on the development of the foetus's gut microbiome (Fichorova *et al*., 2011; Stout *et al*., 2013).

Making Mother's Gift Your Own

Now that the infant is born, the responsibility shifts from two people to an individual. How and when the gut microbiome develops has lasting effects on the health of an infant and continues throughout life (Arrieta *et al*., 2014). Alterations in gut microbiota during early infancy has an effect on childhood obesity (Koleva *et al*., 2015), type 1 diabetes (Kostic *et al*., 2015), non-alcoholic fatty liver disease (NAFLD) (Nash *et al*., 2017), asthma (Arrieta *et al*., 2015), and allergies (Low *et al*., 2017; Candy *et al*., 2018). A better understanding of the

development of intestinal microbiota in infants is also important in understanding immune development and disease prevention (Niu *et al*., 2020).

The mode of birth (vaginal versus Caesarean) has a major influence on the composition of the infant's gut microbiome (Dominguez-Bello *et al*., 2010; Palmer *et al*., 2007; Tsuji *et al*., 2012). A metagenomic study conducted on 98 infants and their mothers have shown that one-year-old infants delivered via Caesarean I-section hosted *Enterobacter hormaechei*, *Enterobacter cancerogenus, Haemophilus parainfluenzae*, *Haemophilus aegyptius*, *Haemophilus influenza*, *Haemophilus haemolyticus, Staphylococcus saprophyticus*, *Staphylococcus lugdunensis*, *Staphylococcus aureus*, *Streptococcus australis*, *Veillonella dispar*, *Veillonella parvula* and a few *Bacteroides* spp. (Bäckhed *et al*., 2015). The GIT of same-age infants vaginally delivered contained mostly *Bacteroides*, *Bifidobacterium*, *Parabacteroides*, *Escherichia* and *Shigella* (Bäckhed *et al*., 2015).

The GIT of a new-born is aerobic, at least for the first two days. It thus makes sense that most bacteria that first colonizes the GIT have an oxidative metabolism, i.e., lactose in breast- or formula milk is converted to organic acids by aerobic bacteria. Infants delivered by C-section have less of a bacterial diversity and some studies indicated that the microbial population in these infants may remain imbalanced for months or even years (Jakobsson *et al*., 2014; Salminen *et al*., 2004). Although many reports have been published favouring natural birth, more in-depth studies are required to claim an infant is microbially aggrieved by Caesarean birth. It is generally accepted that lactobacilli, which comprises the largest component of bacteria in the vagina (Aagaard *et al*., 2010), plays a major role in the manifestation of the gut microbiome. However, not all bacteria are transferred to the infant during birth, as reported by Bäckhed *et al.* (2015). Findings from this study have shown that at least 52 MetaOTUs (metagenomic operational taxonomic units) from a group of mothers were either not transferred to their infants or did not colonize the GIT (Bäckhed *et al*., 2015). The opposite have also been reported. *Propionibacterium acnes*, *Streptococcus agalactiae* and *Veillonella* spp. were detected in more than 10 new-borns but not in their mothers (Bäckhed *et al*., 2015). Irrespective of the birth method, the GIT of all pre-weaned infants are colonised by Actinobacteria. Approximately 4 days after birth, *Bifidobacterium* becomes dominant (Bezirtzoglou, 1997; Marques *et al*., 2010). After 3 years the composition of the gut microbiome resembles that of healthy adults (Koenig *et al*., 2011). The exact role

Actinobacteria, specifically *Corynebacterium*, *Propionibacterium*, *Rothia*, *Actinomyces*, and *Bifidobacterium*, play in the early development of the gut microbiome is unknown.

Development of an infant's gut microbiome is largely influenced by the mother's handling, as the first major changes in the gut microbiome is observed as early as 3 days after birth (Grönlund *et al*., 2011; Vaishampayan *et al*., 2010; Avershina *et al*., 2014). The composition of the gut microbiome undergoes vast changes during the first two years of an infant's life (Avershina *et al*., 2014**)**. These changes occur in conjunction with several physiological changes, e.g. increase in mucus production, the rate at which mucin is glycosylated, changes in bile secretion (Martin *et al*., 2007) and variations in production of antimicrobial peptides (Salzman *et al*., 2003). Binding of microbiota to glycosylated mucus is selective, i.e., only cells with a complementary set of adhesins will adhere (Juge, 2012). Changes in the composition or structure of glycan will thus influence colonization. Mice lacking the enzyme α-1,2-fucosyltransferase encoded by the *FUT2* gene hosted a less diverse population of gut microbiota (Kashyap *et al*., 2013). On the other hand, an increase in mucus glycosylation may lead to microbial infections, overgrowth of pathogens and, in severe cases, dysbiosis, the development of inflammatory bowel disease (IBD) (Larsson *et al*., 2011; Rausch *et al*., 2011; Tong *et al*., 2014.) and autoimmune diseases (Buisine *et al*., 1998; Reid and Harris, 1998). Control over mucus production is important, especially during infancy, as mucosal surfaces, along with the innate immune system, represents the first line of defence against pathogens. Mucosal-associated lymphoid tissue (MALT) generate immune responses against antigens. The proximal small intestine is the least covered with mucosa and has the lowest microbial diversity, rendering it more susceptible to microbial invasion. However, high levels of bile salts and acids secreted in this area limits the colonization and survival of pathogens (Maynard *et al*., 2012). Mucosal layers in the stomach and colon consists of an outer loose and an inner thick layer close to the epithelium and is thus more populated (Dongarrà *et al*., 2013). It is thus important for the immune system to have mechanisms in place to restrict the negative impact of inflammation (Ayres, 2013). Intestinal epithelial cells (IECs) release signals that induce immune responses by recruiting pro-inflammatory leucocytes to activate immune cells (Artis, 2008). This innate immune system is part of the initial recognition and sensing of the microbial environment and relies heavily on the support of healthy IECs and the integrity of the epithelial barrier. The latter depends on the formation of tight junctions, regulated by transmembrane proteins positioned between adjacent IECs, and the secretion of mucus, immunoglobin A (IgA) and antimicrobial proteins (Fig. 1).

Mucin genes are expressed from the early mid-trimester of pregnancy and are regulated by bacteria colonizing the mucus, metabolic compounds produced by bacteria and several inflammatory mediators (Ashida *et al*., 2011). The expression of mucus is controlled at transcriptional level where pathogen-associated molecular patterns (PAMPs) initiates signaling pathways via transmembrane toll-like receptors (TLRs) and NOD-like receptors (NLRs), which in turn induces the transcription of genes responsible for mucus production (Macia *et al*., 2012). Binding of cells to mucus stimulates the release of more mucus. This is considered a protective mechanism administered by epithelial cells (Ashida *et al*., 2011). Mucus production is a finely controlled process, as mucosal layers also protect the host from over-immune stimulation that may be caused by commensal microorganisms (Macia *et al*., 2012).

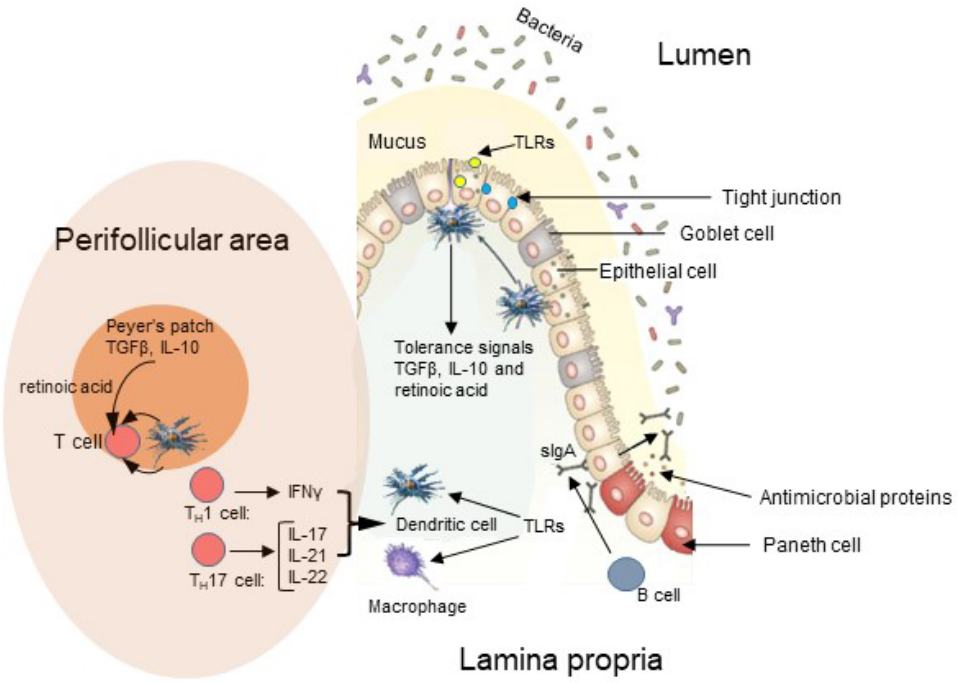

Figure 1. Regulatory mechanisms induced by epithelial and immune cells to protect the GIT from pathogens (modified from Didierlaurent *et al.*, 2002). TLR = transmembrane toll-like receptors; IL = interleukin; TGβF = transforming growth factor β; IFNγ = interferon γ; SigA = secretory immunoglobulin A. From Dicks *et al*. (2018).

Necrotizing enterocolitis (NEC), a condition associated with a decrease in mucin production, is the second most common cause of morbidity in premature infants (Clark *et al*., 2006). The *MUC2* gene of damaged goblet cells in necrotic tissue is not expressed and the microbiome is drastically altered (Caplan and Jilling, 2001; Martin *et al*., 2011). Human milk, with

oligosaccharides similar in structure to mucin glycans, may reduce the risk of developing NEC (Lucas and Cole, 1990; McGuire and Anthony, 2003), putting breast-fed infants in an advantageous position. A literature survey by The Cochrane Neonatal Group, published by AlFaleh and Anabrees (2014), has shown that the use of probiotics significantly reduced the incidence of NEC and death in preterm infants. The probiotics included in the comparison were different *Lactobacillus* spp., *Bifidobacterium* spp. and *Saccharomyces boulardii* (AlFaleh and Anabrees, 2014). The authors did not identify a species with superior probiotic properties but did indicate that the administration of a combination of species superseded the use of single-species probiotics that contained only *Lactobacillus, Bifidobacterium* or *S. boulardii.* The conclusion was that extensive research is required to develop a more effective probiotic formulation, and dose level, for preterm infants (AlFaleh and Anabrees, 2014).

Sialic acid Neu5AC, produced by intestinal epithelial cells (IECs), regulates the texture of mucus and is thus indirectly responsible for the adhesion of microorganisms. It is thus not surprising to find high levels of sialic acid Neu5AC in the mucus of fetuses and concentration variations thereof is normally only observed in the GIT of infants (Bergström *et al*., 2012; Khailova *et al*., 2009; Wrzosek *et al*., 2013). This once again emphasizes the critical role commensal bacteria play in stimulation of mucus production, more specifically mucin glycan production, and the formation of new goblet cells. Apart from keeping the GIT in a homeostatic state, mucus also prevents the apoptosis of IECs and helps to maintain the integrity of the gut wall (Macia *et al*., 2012). An increase in fucosylated and sialylated glycans stimulated the adhesion of *Bacteroides* spp. to mucus (El Aidy *et al*., 2013; Meng *et al*., 2007). Concurrent with this, an increase in the colonization of lactic acid-producing species, such as *Bifidobacterium adolescentis*, was observed (Deplancke *et al*., 2000; El Aidy *et al*., 2012, 2013).

Acetate produced by *Bifidobacterium* spp. prevents the growth of enteropathogenic hemorrhagic *E. coli* (EHEC) and the release of toxins. Acetate also stimulate receptors that regulate host inflammatory responses. Butyrate prevents pathogenesis by directly affecting the expression of virulence genes, stimulates the expression of antimicrobial peptide LL-37, and provides repair injuries to epithelial cells (Ashida *et al*., 2011). Production of SCFAs need to be controlled, as elevated levels may increase the luminal pH (Laurin *et al*., 1994; Woodmansey *et al*., 2004), which in turn decreases the absorption of minerals, especially Ca^{2+} and Mg^{2+} (Abrams *et al*., 2005).

Over time, the *Bifidobacterium*-dominated microbiota of an infant changes into a Bacteroidetes- and Firmicutes-dominated community, which is characteristic of an adult GIT (Ottman *et al*., 2012). Of interest are the sequential changes recorded in the relative abundance of *Bacteroides*, Lachnospiraceae and *Bifidobacterium* during childhood. The abundance of Enterobacteriaceae in infants is indicative of an immature intestinal barrier not fully developed (Odamaki *et al*., 2016). Breast feeding has several advantages. Immunoglobulin A (IgA) in breast milk protects the infant against infection (Cacho and Lawrence, 2017). Although the gut microbiota of breastfed infants is less diverse, higher levels of bifodobacteria (e.g., *Bifidobacterium breve, Bifidobacterium bifidum* and *Bifidobacterium longum*) have been reported. This is largely due to galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) in human milk (Stewart, *et al.,* 2018; Laursen, *et al.,* 2016). Four glycosyl glycosidase genes (sialidase, fucosidase, *N*-acetyl-β-hexosaminidase and β-galactosidase), and transport-related genes (solute-binding proteins and ABC transporters) have been identified in one localized human milk oligosaccharide (HMO) metabolism cluster (Sela *et al.,* 2008). Of interest is that *B. longum* subsp *infantis* varies across populations, with highest presence in Russian infants (23%), followed by Estonial (20%) and Finnish infants (10%) (Vatanen *et al.,* 2019). Gut microbiota that develops during breast feeding has the ability to metabolise plant-derived glycans, including starch (Koenig *et al.,* 2011). This is important, as it prepares the infant to degrade plant-derived foods. Bacterial load and diversity typically increases with the intake of solid food (Koenig *et al.,* 2011). The increase in Bacteroidetes and Firmicutes results in more production of short-chain fatty acids (Laursen *et al.,* 2016). A Swedish study conducted over the first year of an infant's development has shown that amino acids and vitamins are produced from as early as 4 months (Bäckhed *et al.,* 2015). At the age of 12 months, the gut microbiota of these infants degraded complex sugars and starch and had a higher abundance of *Bacteroides thetaiotaomicron* that expresses a number of glycan-degrading enzymes (Xu *et al.,* 2003). The gut microbiome of a 3-year-old infant have the ability to biosynthesise folate (vitamin B9). The biosynthesis of cobalamin (vitamin B12), however, develops at a later stage (Yatsunenko *et al.,* 2012).

All evidence indicates that the first 5 years of a child is the most critical in developing a core group of microorganisms (Cheng *et al*., 2016). Much more research is required to understand the microbial interactions in a genetically diverse environment such as the GIT. Establishing of a gut microbiome with beneficial bacteria at an early age and maintaining the balance in

microbial populations is of critical importance. An abnormal, disturbed or imbalance gut microbiome is a contributing factor to the pathophysiology of various neurological and psychiatric diseases, including anxiety and depression, major depressive disorder (MDD), schizophrenia, bipolar disorder, autism and obsessive-compulsive disorder (OCD). The role gut bacteria play in neuropsychiatric disorders is reviewed by Dicks *et al*. (2021).

Later in life, closer to puberty, the gut microbiome changes with fluctuations in hormone levels (Begley *et al*., 2006; Dicks *et al*., 2018) and an increase in stress (Lozupone *et al*., 2012; Gevers *et al*., 2012). This is the stage in life when the gut microbiome is forming a select group of species represented by strains with unique phenotypic and genotypic characteristics (Yassour *et al*., 2018; Garud *et al*., 2019; Browne *et al*., 2016). Strains within species adapt or mutate as their environment changes. Strains within the same species isolated from different individuals have at least one variation in every hundred base pairs (Schloissnig *et al*., 2012; Costea *et al*., 2017; Truong *et al*., 2017; Garud *et al.*, 2019). Species that cannot adapt or compete are replaced by novices that are able to regulate their own gene expressions, or alter their genetic composition (Schloissnig *et al*., 2012; Maurice *et al*., 2013). Once adapted to the GIT, strains are not easily replaced (Costea *et al*., 2017) *Bacteroides fragilis* adapted so well to humans that the species is represented by a single strain (Verster *et al*., 2017) *Helicobacter pylori, Mycobacterium tuberculosis* (Linz *et al*., 2007, 2003; Comas *et al*., 2013), *Eubacterium rectale* (Karcher *et al*., 2020) and *Prevotella copri* are host-specific and contain strains that are associated with individuals from specific geographic regions (Tett *et al*., 2019).

Diet plays a big role. *Bacteroides* spp., for example, are associated with high-fat or high-protein diets, *Prevotella* spp. with high-carbohydrate diets and Firmicutes with a high-fiber diet (Wu *et al*., 2011). A protein-rich diet stimulates the growth of microorganisms with a putrefactive metabolism, especially in the colon (Woodmansey, 2007). When this occurs, the levels of short chain fatty acids (SCFA), such as butyrate, decrease and are replaced by higher levels of branched fatty acids, ammonia, phenols (Woodmansey, 2007), indoles, *N*-nitroso compounds and sulphides (Hughes *et al*., 2000) that increases the risk of developing colon cancer. A diet that favors the growth of saccharolytic bacteria in the colon is more beneficial to the elderly (Hughes *et al*., 2000). Higher levels of SCFAs produced by these bacteria keeps the intestinal barrier healthy and prevents the growth of pathogens (Suzuki *et al*., 2008).

A healthy GIT is characterized by a balanced gut microbiome with a core population of beneficial microbiota. Strain-level structures are maintained by keeping genetic changes under control, as illustrated in the studies on *Escherichia coli*. Genome sequences of 24 intestinal isolates of *E. coli* ED1a, studied over a year, showed a mutation rate of only 6.9×10^{-7} per base (Ghalayini *et al*., 2018). This was, however, complemented by a reduction in population size (Ghalayini *et al*., 2018), suggesting that external stress factors have a profound influence on the survival of bacteria in the GIT. Roodgar *et al.* (2022) have shown that strains exposed to stress, such as antibiotics, develop resistance rapidly and change the overall composition of the gut microbiome. Although the outcome of the reports by Ghalayini *et al*. (2018) and Roodgar *et al*. (2022) were different, the two studies illustrated that the frequency at which strains adapt to an ever-changing and stressful environment, such as the GIT, is unpredictable. Whatever changes takes place in the microbial population, these will affect the synthesis and degradation of neurotransmitters and, ultimately, communication with the CNS.

With shifts in the microbiome over time, whether due to changes in diet, the taking of medication or the development of intestinal abnormalities, changes in microbial interactions and responses from the immune system is unavoidable. Structural and physiological changes, e.g., the thickness of mucus layers, number of healthy epithelial cells and receptor sites has an impact on the microbe-host interactions, regulation of the intestinal barrier integrity and the immune system. Drastic changes in gut homeostasis may lead to inflammation caused by normal commensal microorganisms. Probiotics may correct a microbial imbalanced GIT, but we need to have a better understanding of the metabolic compounds, such as bacteriocins and other antimicrobial peptides, produced by probiotic bacteria. We also need to have a much clearer understanding of the communication between microorganisms and epithelial cells, especially reactions involved in stimulating IECs to produce defensins and other antimicrobial peptides. This topic is addressed in the review by Dicks (2022). The ultimate challenge is to develop a combination of probiotic bacteria that will live in complete harmony with the other intestinal microorganisms, keep the microbiota in the GIT in a balanced state and regulate the challenges the immune system is confronted with. Keeping a fine balance between pro- and anti-inflammatory cytokines is critical to maintain a healthy intestinal barrier. For further research, the reader is referred to the review by Dicks (2021).

Despite many studies on the development of a gut microbiome, several intriguing questions remain unanswered. For example, how long does the gut microbiome of children remain stable,

given adequate feeding and a healthy lifestyle? Do infants and teenagers under stress experience the same changes in their gut microbiome compared to adults or are they more resilient to changing physiological and environmental factors? Are these changes, driven by variations of external factors the same across all populations? Is there an ultimate gut microbiome profile that needs to develop from infancy to ensure a healthy adulthood? What do we need to do to maintain a healthy, balanced, gut microbiome throughout life? Do we need to accelerate the establishing of an adult-like gut microbiome earlier in life? How do we prevent the proliferation of non-beneficial gut microbiota associated with the developing of disease, including cancer? Would it be possible to delay the development of these unwanted microorganisms in the GIT? What effect does fungi and bacteriophages have on bacterial development in the GIT? Would it be possible to cure diseases with microbial interventions and if so, what scale of intervention is required? What effect would an intervention early in life have later in life? Would it be possible to develop a synthetic gut microbiome? Could this be the next generation of probiotics?

The Last Few Years of Life

Changes in the gut microbiota of adults and the elderly have been studied in segmented age groups, with "elderly" defined as "over 60" (Mueller *et al*., 2006), "over 65" (Claesson *et al*., 2011), "over 70" (Mariat *et al*., 2009) and "older than 70" (Biagi *et al*., 2010). Odamaki *et al.* (2016) shed some light on the changes of the gut microbiome from birth to old age. The authors sequenced the *16S rRNA* genes (V3-V4 regions) of more than 1.8 million bacteria isolated from 371 samples collected from Japanese subjects just after birth up to 104 years old. Analyses of the sequences revealed close to 6 000 operational taxonomic units (OTUs), representing 186 genera. Four predominant phyla were identified, i.e. Actinobacteria (predominant before weaning, but decreasing with age), Firmicutes (predominant after weaning), and Bacteroidetes and Proteobacteria, with increasing numbers in subjects older than 70 years (Fig. 2). Of interest was the decline in Firmicutes in comparison to the relatively strong increase in Proteobacteria in subjects older than 70 years, especially in subjects older than 90. To provide a better picture of the changing gut microbiota, the authors grouped the bacteria into co-abundance groups. This revealed a significant abundance of *Bacteroides*, *Eubacterium* and Clostridiaceae in the elderly, Enterobacteriaceae in infants and the elderly, *Bifidobacterium* in infants and children and Lachnospiraceae in adults. *Megamonas* and *Peptoniphilus* were predominant in the elderly. *Dorea* were the least abundant of all groups and remained so throughout life. The

abundance of Enterobacteriaceae in the elderly suggests that intestinal epithelial cells are more susceptible to infections. The abundance of butyrate-producing bacteria such as Lachnospiraceae, *Blautia*, *Coprococcus*, *Roseburia* and Faecalibacterium may stimulate Treg cell differentiation (Furusawa *et al*., 2013). The reduction of *Bifidobacterium* in adults and the elderly may be age-related, as shown by Odamaki *et al.* (2016), but is also influenced by diet as shown from studies conducted on the Hadza tribe of Tanzania (Schnorr *et al.*, 2014). The authors concluded that the complete absence of *Bifidobacterium* in individuals from this tribe may be linked to their foraging lifestyle. Bifidobacteria are known to down-regulate pro-inflammatory responses in the gut epithelium (Bermudez *et al*., 2013; Groeger *et al*., 2013; Sagar *et al*., 2014). Furthermore, production of acetate by bifidobacteria stimulates the growth of butyrate-producing bacteria and increases butyrate levels in vitro (Falony *et al*., 2006; Mahowald *et al*., 2009). Wu *et al*. (2015) and Pérez *et al*. (2014) have shown that lowering of Enterobacteriaceae numbers stimulates the growth of *Bifidobacterium* spp.

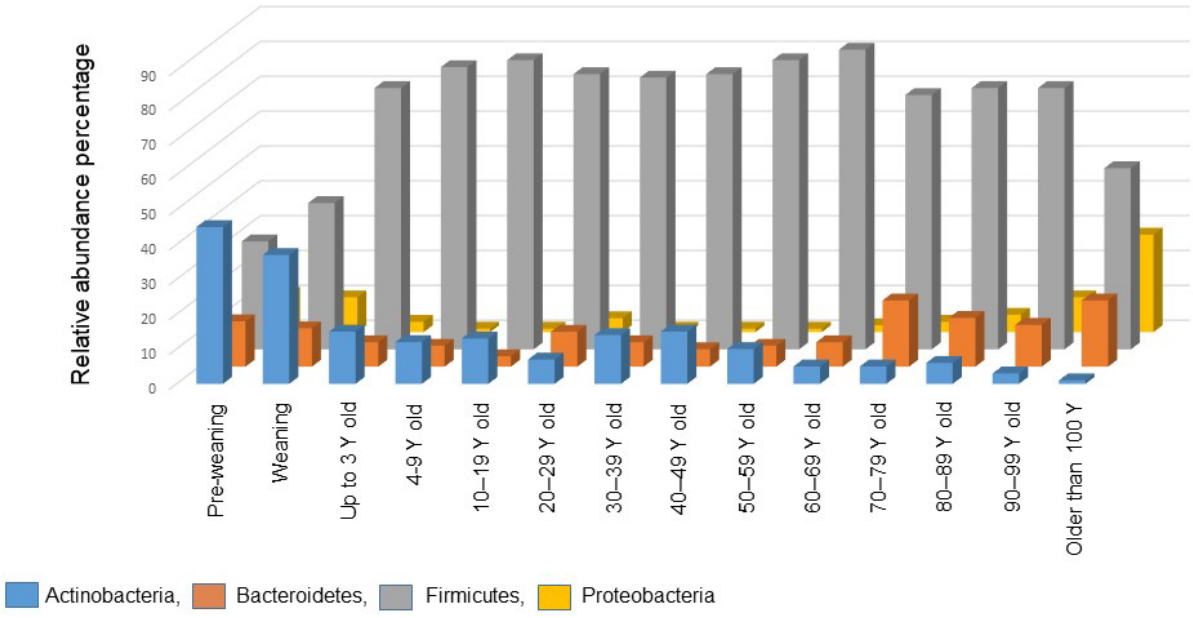

Figure 2. Age-related sequential changes in gut microbiota composition (modified from Odamaki *et al.*, 2016).

Benefits from a Healthy Gut Microbiome – Summarised

The most profound benefit gained from a healthy and well-balanced gut microbiome is a sound immune system. A normal and well-balanced microbiome (a "homeostatic GIT") stimulates the immune system and recruit immune cells to mucosal layers surrounding the gut wall. This in turn stimulates the generation and maturation of gut-associated lymphoid tissue (GALT) situated beneath the epithelium and closely associated with T- and B-cell lymphocytes, and macrophages. When in contact with antigens, the neutrophils, macrophages, T-and B-cells, and DCs in GALT react by inducing innate and adaptive inflammatory responses (Dongarrà *et al*., 2013; Ivanov, 2012; Purchiaroni *et al*., 2013). DCs in and below the epithelial cells constantly sample the surrounding environment for viruses and bacteria. This is accomplished through pattern recognition receptors (PRRs) such asTLRs that recognize specific chemical structures, also referred to as signature molecules, of a pathogen. Peptidoglycans and lipopolysaccharides (LPS) in cell walls of Gram-negative bacteria (teichoic acids in cell walls of Gram-positive bacteria) are common signature (immune responsive) molecules (Didierlaurent *et al.*, 2002). These signature molecules, unique to each microbial cell, give rise to distinct membrane-associated molecular patterns (MAMPs), also known as microbe-associated molecular patterns or PAMPs. In short, DCs use MAMPs to identify the nature of the potentially threatening microbe (Didierlaurent *et al.*, 2002). Once DCs have made contact with an antigen, they become activated, develop into mature DCs, migrate to the lymph node and interact with T- and B-cells to shape the adaptive immune response. The "cross-talk" between gut epithelial cells and intestinal microbiota, performed with the assistance of specific signature molecules, helps the host to differentiate between commensal and pathogenic bacteria (Purchiaroni *et al*., 2013). For further information on gut homeostasis and the immune system the reader is referred to Dicks *et al*. (2018).

The epithelial barrier integrity is maintained by inflammasome-microbial interactions. These multi-protein complexes are involved in the sensing of microbial-derived molecules and host-derived products through NLRs, with adapter proteins leading to the production and maturation of pro-inflammatory cytokines IL-1β and IL-18 (Ubeda and Pamer, 2012; reviewed by Dicks *et al*., 2018). The gut microbiome also plays an intrinsic part in intestinal permeability, enteric reflex, and entero-endocrine signaling. A vast number of bacterial signals generated in the GIT reaches the brain via the vagus nerve. Many papers have been published on the link between

the gut–brain axis (GBA), digestion of food and satiety. Less papers have been published on the role gut microbiota play in mood, cognitive behavior and neuropsychiatric disorders such as autism, depression and schizophrenia. For a review on this topic, the reader is referred to Dicks et al. (2021). The more we discover about the gut microbiome and the more we learn about the GBA, the greater the chance of developing novel therapeutics, probiotics and psychobiotics to treat gastro-intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), but also improve cognitive functions and prevent or treat mental disorders. The reader is also referred to a review by Dicks (2022) on quorum sensing amongst gut microbiota and its effect on health and mental status.

Another important function of the gut microbiome is maintaining homeostasis, i.e. keeping a healthy balance between beneficial and non-beneficial species, and repressing pathogens. This role is fulfilled by the production of antimicrobial compounds such as SCFAs and a range of antimicrobial peptides. Antimicrobial proteins (AMPs) in the small intestine and colon regenerate REGIIIγ, a C type lectin that targets Gram-positive bacteria and contributes to intestinal barrier function (Chu and Mazmanian, 2013). REGIIIγ plays a protective role against the infection by *Citrobacter rodentium* and other Gram-positive bacteria (Qiu *et al*., 2012; Satoh-Takayama *et al*., 2008). Commensal bacteria are also directly involved in stimulating the expression of AMPs that in turn leads to REGIIIγ expression by the activation of TLRs and the myeloid differentiation primary response gene 88 (MyD88)-dependent signaling in IECs. Pore forming defensins target Gram-positive and Gram-negative bacteria and C-type lectins target peptidoglycans in the cell wall of Gram-positive bacteria (Peterson and Artis, 2014). Paneth cells in the small intestine secrete AMPs such as defensins and lysozyme. β-defensin prevents the adhesion and invasion of bacteria and are largely responsible for maintaining an antimicrobial environment (Didierlaurent *et al*., 2002). Bacteriocins produced by probiotic LAB prevented the colonization and growth of pathogenic bacteria (Turroni *et al*., 2014). An increase in the transcription of bacteriocin genes was observed following adherence to IECs. Bacteriocin production also plays a role in immunomodulation by inducing the production of an anti-inflammatory cytokine, IL-10 (Turroni *et al*., 2014). Most E. coli strains have genes encoding colicins. These proteins are expressed when cells experience stress and usually leads to self-destruction due to co-expression with lysis protein (Alvarez-Sieiro *et al*., 2016).

A research area receiving a lot of attention is the role of gut bacteria, including LAB, in the treatment of cancer. The first report of a bacteriocin crude extract displaying anticancer properties was published in the late 1970s (Cornut *et al*., 2008). Since then, several reports of bacteriocins with anticancer properties (also from our own group, unpublished), have been reported (Cotter *et al*., 2013). For the latest information of the topic the reader is referred to the review by Dicks and Vermeulen (2022).

The human body does not produce vitamins and have to rely on exogenous sources (LeBlanc *et al*., 2013). Some gut microbiota synthesize vitamin K and most of the water-soluble B vitamins such as biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin and thiamine (Hill, 1997). Most of these vitamins are produced in the colon (Ichihashi *et al.*, 1992; Said and Mohammed, 2006). *De novo* synthesis of folate (vitamin B9) requires 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and para-aminobenzoic acid (pABA). Strains harbouring the genes encoding *de novo* pABA synthesis are thus expected to synthesize folate. This seems to be species- and strain-specific, as certain strains of *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis* produce higher levels of folate than stains of *Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum* and *B. adolescentis* (Pompei *et al*., 2007). *Bifidobacterium animalis* subsp. *lactis* is auxotrophic for DHPPP and does not produce folate, even in the presence of pABA (Pompei *et al*., 2007). Amongst the lactobacilli, *L. plantarum* WCFS1 seems to be the only strain having the genetic determinants for *de novo* pABA synthesis (Kleerebezem *et al*., 2003), suggesting that the majority of lactobacilli cannot synthesize folate in the absence of pABA.

A diverse group of microorganisms in newborns have the ability to synthesize folate (Bäckhed *et al*., 2015). Similar observations have been recorded for pyridoxal (vitamin B6) and biotin (vitamin B7) biosynthesis (Bäckhed *et al*., 2015). Microorganisms with the ability to produce thiamine (vitamin B1), pantothenate (vitamin B5) and cobalamin (vitamin B12) were present in lower numbers, but their cell numbers increased in older children (Yatsunenko *et al*., 2012). Microbiota with strong Vitamin B12 transport systems are present in newborns, but this decreases with age (Bäckhed *et al*., 2015). Similarly, transporters for iron, hemin, and heme, which are linked to vitamin B12 synthesis and important for iron metabolism, are also present in higher levels in the microbiome of newborns (Bäckhed *et al*., 2015).

The synthesis of cobalamin by gut microorganisms is not well documented. A reuterin-producing strain of *L. coryniformis* produced a cobalamin-type compound (Martin *et al.*, 2005). *L. reuteri* CRL1098 has at least 30 genes involved in the *de novo* synthesis of cobalamin (LeBlanc *et al*., 2013). The genetic organization of the *cob* and *cbi* genes are very similar to those of *Salmonella enterica* and *Listeria innocua* (Santos *et al*., 2007). Other strains of *L. reuteri*, DCM 20016 (Santos *et al*., 2007), JCM1112 (Santos *et al*., 2008), CRL 1324 and CRL 1327 (Vannini *et al*., 2008) are also able to produce cobalamin. Since *L. reuteri* is a normal inhabitant of the human GIT, the species may be responsible for synthesis of part of the vitamin B12 requirements. The genetic pathway for *de novo* synthesis of vitamin B12 by *L. reuteri* has been defined (Saulnier *et al.,* 2011). Little is known about the *de novo* synthesis of riboflavin (vitamin B2) by gut microorganisms. Genes encoding the synthesis of riboflavin seems to be absent amongst *Bifidobacterium* spp. (Ventura *et al*., 2007).

Bacteroides, *Escherichia* and *Shigella* spp. are known vitamin K2 producers (Wang *et al*., 2013). Concluded from the study conducted by Bäckhed and co-workers (2015), vaginally-delivered infants have an advantage regarding vitamin production. Since vitamin K2 plays an important role in bone and heart development (Sjögren *et al*., 2012), one may conclude that vaginally delivered infants are in general healthier than C-section delivered infants.

Vitamin production by the gut microbiota is complex and much more research is required. We also need to understand how vitamins influence the gut microbiome. A recent study conducted by Bashir *et al*. (2016) has shown that vitamin D_3 modulates the gut microbiome of the upper GIT by decreasing the numbers of Gammaproteobacteria, including *Pseudomonas* spp., *Escherichia* spp. and *Shigella* spp. With the oral intake of vitamin D_3, Bacteroidetes and Firmicutes were the dominating phyla and less Proteobacteria were recorded in the mucus of the upper GIT than in the lower GIT. Vitamin D_3 had no major effect on the microbial population in the lower GIT. Of even greater interest is the finding that vitamin D_3 lowered the cell numbers of *Helicobacter* spp. in the *Helicobacter pylori*-positive subgroup.

The GIT of elderly people have increased numbers of facultative anaerobes such as proteobacteria and bacilli, and lower numbers of *Faecalibacterium prauznitzii, Clostridium* spp., *Bacteroides* spp., *Bifidobacterium* spp. and Enterobacteriaceae (Drago *et al*., 2012). More research needs to be done to understand the implications of these changes in the general health of elderly people, especially vitamin K2 production. As databanks with genome sequences

expand, more vitamin-producing gut microbiota will be discovered and perhaps genetically engineered to produce required levels of specific vitamins.

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Chapter 5

Probiotic Properties and Health Benefits of Lactic Acid Bacteria, and their Bacteriocins

A good probiotic is worth trillions and may

Modulate your immune system

Keep serum cholesterol levels at bay

Alleviate lactose intolerance

Normalise stool transit

Repair liver damage (hepatic encephalopathy)

Sooth or prevent peptic ulcers

Relief neuropathic pain

Regulate ion channels (important in neurotransmission)

Fight virus infections

Fight tumour growth and growth of cancerous cells

Sustain a healthy mental status

etc…….

Probiotic Properties and Health Benefits of Lactic Acid Bacteria, and their Bacteriocins

Introduction

Probiotics have been defined in as "a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance" (Fuller, 1989), "microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being" (Marteau *et al*., 2002), "living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (Gorbach, 2002) and "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Although lactic acid bacteria (LAB) are generally regarded as safe (GRAS status) and do not cause immunological side effects (Salminen *et al.,* 1998), *Lactobacillus casei* has been associated with symptoms of fever, arthritis, and hepatobiliary lesions (Schwab, 1993). Symptoms such as these may be caused by a cytokine response elicited by peptidoglycan and other cell wall components (Miettinen *et al.,* 1996). This is usually only possible when cells invade epithelial cells, migrate through mucus (Tang *et al.,* 1993) or degrade mucus (Salminen *et al.,* 1998). Medical practitioners from the University of Utrecht, The Netherlands, announced that a probiotic supplement of six strains was responsible for the death of 24 patients (Vogel, 2008). It is, however, important to note that the patients suffered from acute pancreatitis and that they were immune compromised when treated. In another report, *L. casei* and *Lactobacillus rhamnosus* have been associated with bacteremia and endocarditis (Cannon *et al.,* 2005). This is extremely rare (Salminen *et al.,* 1998), as such strains would have to metabolize glycoproteins and lyse fibrin clots (Oakley *et al.,* 1995). A few strains of LAB have been associated with urinary tract infections, wound, tissue and other infections (Vankerckhoven *et al.,* 2008). In immunocompromised patients some lactobacilli have been associated with arthritis and meningitis (Vankerckhoven *et al.,* 2008). Beneficial properties of probiotic LAB is summarized in Table 1.

Of specific concern is the safety of *Enterococcus* spp. In a few cases *Enterococcus* infections have been associated with abnormal physiological conditions, underlying disease, and immunosuppression (Franz and Holzapfel, 2004). This is rather surprising, as these organisms are closely associated with the human environment and gastro-intestinal tract (GIT) and one would assume this minimizes the chance of causing diseases, at least in healthy individuals. Clinical trials have shown that *Enterococcus faecium* SF68 is effective in the prevention and treatment of diarrhea (Lewenstein *et al.,* 1979) and lowers the cholesterol levels in serum (Agerbaek *et al.,* 1995). Another strain of *E. faecium*, CRL 183, lowered cholesterol levels by 43% in *in vitro* tests (Rossi *et al.,* 1999). *E. faecium* Fargo 688® alleviated the symptoms of irritable bowel syndrome and was successfully used in the production of cheddar cheese (Allen *et al.,* 1996; Gardiner *et al.,* 1999). Despite these findings, controversy still exists in the use of enterococci as probiotics (Vankerckhoven *et al*., 2008). The Scientific Committee on Animal Nutrition classified *E. faecium* DSM 7134, NCIMB 10415, CECT 4515, NCIMB 30098, NCIMB 1181, DSM 5464, DSM 3520, NCIMB 10415, DSM 4788, DSM 4789, and *Enterococcus mundtii* CNCM MA 27/4E as safe animal probiotics. In our own group, the probiotic *E. mundtii* ST4SA caused no haematological and histological abnormalities when tested on mice (Botes *et al.,* 2008). The ability of *E. mundtii* ST4SA to colonize the GIT and effectively compete with pathogens, without side effects, was demonstrated in several follow-up studies (Van Zyl *et al*., 2015a,b,c; 2018; 2019). Strain ST4SA adheres to CaCo-2 cells (Botes *et al*., 2008), colonizes the GIT of mice (Granger et al., 2008; Van Zyl *et al*., 2015), and survived conditions simulation that of the human GIT (Dicks *et al*., 2010). Probiotic properties of strain ST4SA were first evaluated using a gastro-intestinal model (Botes *et al*., 2008), and later in mice where it alleviated symptoms of *Salmonella* infection (Dicks and Ten Doeschate, 2010; Van Zyl et al., 2018, 2019) and excluded *Listeria monocytogenes* from the GIT of mice (Van Zyl *et al*., 2016). Safety studies were first done in mice (Ramiah *et al*., 2009), and later in a clinical trial with human volunteers (unpublished, CiplaMedpro, Bellville, South Africa). *E. mundtii* ST4SA is included with *L. plantarum* 423 in the probiotic product entiro™, marketed and distributed by CiplaMedpro, SA.

Most studies on probiotic LAB are performed *in vitro* (Lin *et al.,* 2006). Adhesion to mucus, glycoproteins and epithelial cells are studied using human cell lines such as Caco-2, HT-29 and HT29-MTX (Sambuy *et al.,* 2005). *In vitro* models, simulating the GIT in humans, have been developed to represent *in vivo* conditions. Examples of such models are the GIT model (GIM) developed by Botes *et al.* (2008), the stomach and duodenum (upper gastro-intestinal tract)

model (Mainville *et al.,* 2005), an anaerobic three-vessel continuous-flow culture system (Payne *et al*., 2003), a three-stage compound continuous culture system simulating the proximal colon (Macfarlane *et al*., 1998), an upper gastro-intestinal model representing the stomach, duodenum, jejunum and ileum (Minekus *et al.,* 1995), a similar model mimicking the colon (Minekus *et al.,* 1999), and a simulated human intestinal microbial ecosystem (SHIME) developed by Molly *et al.* (1993). A flow-cell model of the GIT was developed by Klopper *et al*. (2019, 2020) that allowed studies on biofilm formation by commensal microbiota in the presence and absence of a probiotic.

Table 1. Health effects of probiotic bacteria and main pathogens targeted (from Van Zyl *et al*., 2020).

Probiotic strains	Pathogen(s)[a]	Reported effects
Lactobacillus rhamnosus GG	*Helicobacter pylori, rotavirus, C. difficile*	Reduced diarrhoea and nausea in a human trial. Immune enhancement. Used for alleviation of atopic dermatitis in children, stabilisation of intestinal permeability
L. johnsonii La1	*H. pylori*	Regular ingestion modulated *H. pylori* colonization in children
L. casei DG	*H. pylori*	Increased eradication rate of *H. pylori* infection when supplemented with first-line therapies
L. casei CRL431	*Salmonella enterica* serovar Typhimurium	Preventative administration protected mice against infection
L. rhamnosus HN001	*Salmonella enterica* serovar Typhimurium	Conferred immune enhancement and protection against *Salmonella* infection in mice
Bifidobacterium longum Bb46	*Salmonella enterica* serovar Typhimurium	Protective effect against *Salmonella* challenge in gnotobiotic mice
L. plantarum 423 and *Enterococcus mundtii* ST4SA	*Salmonella enterica* serovar Typhimurium	Alleviated symptoms of *Salmonella* infections in challenge study using rats
L. casei BL23 and *L. paracasei* CNCM I-3689	*Listeria monocytogenes*	Decreased pathogen systemic dissemination in orally infected mice
L. salivarus UC118	*L. monocytogenes*	Protected mice from pathogenic infection in liver and spleen
L. plantarum 423 and *E. mundtii* ST4SA	*L. monocytogenes*	Excluded the pathogen from the intestinal tract of mice after daily administrations of probiotic strains
Lactococcus lactis MM19 and *Pediocin acidilactici* MM33	Vancomycin resistant enterococci (VRE)	Modulated intestinal microbiota and reduced pathogen intestinal colonization in mice.
L. rhamnosus R0011 and *L. acidophilus* R0052	*Citrobacter rodentium*	Pre-treatment with the probiotic strains attenuated pathogen infection in mice
L. reuteri	*C. rodentium*	Attenuated *C. rodentium*-induced colitis in mice. Significantly decreases diarrhoea symptoms in infants and children.

Table 1. Continued.

B. breve	*Escherichia coli* O157:H7	Protected mice from Shiga toxic-producing *E. coli.*
Pediococcus pentasaceus NB-17	*n/a*	Effectively stimulated immune cell activities and allergic inhibitory effects
Oenococcus oeni 9115	*n/a*	Significantly decreased acid-induced colitis in mice. Modulated the immune response of immunocompetent cells *in vitro*.
B. infantis UCC 36524	*Clostridium*	Reduced clostridia levels and increased lactobacilli and bifidobacteria. Increased blood phagocytic activity. Reduced inflammation in mice.
Saccharomyces boulardii	*C. difficile*	Used for prevention and treatment of antibiotics associated and acute diarrhoea in children, treatment of *C. difficile* colitis, prevention of diarrhoea in critically ill tube-fed patients
B. adolescentis	*Bacteroides thetaiotaomicron*	Significantly modulated both systemic and intestinal immune response in germ-free rats.
L. acidophilus	*n/a*	Reduced the severity of Irritable Bowel Syndrome.

[a]**Pathogen (s):** n/a, not applicable

Although studies with simulated GIT-models have provided valuable insights into the adherence of probiotic cells, it remains an *in vitro* approach with limitations portraying the complex multicellular nature of the GIT. *In vivo* pharmacokinetic studies (Marteau and Vesa, 1998; Vesa *et al*., 2000) on the transit of probiotic strains through the GIT, and intestinal intubation and pyxigraphy (Marteau and Vesa, 1998) provides more information on the beavior of cells *in situ*. Differentiation of probiotic strains from commensal gut microbiota can be monitored by using antibiotic markers (Marteau and Vesa, 1998; Van Zyl *et al*., 2015c). In our own group, the migration of the probiotic entiro^{TM} (*L. plantarum* 423 and *E. mundtii* ST4SA) through the GIT of mice was followed using genetically modified strains. The strains were made bioluminescent by cloning of the firefly luciferase gene (*ffluc*) from *Photinus pyralis* onto a plasmid (Van Zyl *et al*., 2018) and genes encoding the *mCherry* fluorescent protein into the genomes (Van Zyl *et al*., 2015a). This allowed monitoring of the strains in real time. The technique was sensitive enough to detect 10^4 CFU of the two strains in fecal sample sizes of 100 mg, using a high-resolution *in vivo* imaging system (Van Zyl *et al.*, 2015a,b; 2018). The technique also allowed us to study the state of viability of the strains in the GIT, competition

amongst strains for adhesion to the gut wall, and the exclusion of pathogens from the GIT (Van Zyl *et al*., 2016, 2019). For a review on the application of optical imaging systems in *in vivo* tracking of LAB, the reader is referred to Van Zyl *et al*. (2015b).

Information on the viability of transgressing cells is important, as some reports have suggested that non-viable and non-colonizing probiotics may also confer certain health benefits to the host (Kullen *et al*., 1997; Marco *et al*., 2006; Ouwehand and Salminen, 1998; Plaza-Diaz *et al*., 2019; Roy *et al*., 2011; Xiao *et al*., 2003). Non-viable *L. rhamnosus* CRL1505 administered to mice displayed similar immunomodulatory effects than viable cells of the same strain (Taverniti and Guglielmetti, 2011). These findings raised some confusion over the assumption that a probiotic must be in direct contact with a pathogen to prevent direct binding to the gut wall, and that only viable cells display probiotic properties. Fujiwara *et al*. (1997, 2001) have shown that direct contact between cells are not always required, at least for some bifidobacteria. The authors have shown that certain bifidobacteria produce a 100 kDa peptide that prevents the adhesion of pathogenic *E. coli* to intestinal mucosal cells. The mode of function is by blocking the binding of *E. coli* to the glycolipid-binding receptor gangliotetraosylceramide. Strains with such abilities could be classified as paraprobiotics, defined as "inactivated microbial cells or cell fractions conferring a health benefit to the consumer" (Taverniti and Guglielmetti, 2011). Probiotic strains with the ability to produce bacteriocins in the GIT may thus be defined as paraprobiotics. This description is, however, seldomly used and perhaps so because little is known about the survival of bacteriocins in the GIT. In our own group, we have shown that nisin F, intraperitineally injected into mice, stabilized the bacterial population in the GIT (Van Staden *et al*., 2011) and that bacteriocins may cross the gut-blood barrier (Dreyer *et al*., 2019). The fate of bacteriocins in the human GIT is discussed in a review by Dicks *et al*. (2018) and possible toxicity addressed in the review by Dicks *et al*. (2017). Bacteriocins with activity against bacterial pathogens are listed in Table 2.

Table 2. Bacteriocins with activity against bacterial pathogens (from Dicks *et al*., 2017).

Bacteriocin	**Producer**	**Application**
Nisin A	*Lactococcus lactis* subsp. *lactis*	Treatment of dental plaque and gingivitis
		Prevention of intramammary streptococcal and staphylococcal infections
		Treatment of diarrhea caused by *Clostridium botulinum, Clostridium tyrobutyricum* and *Clostridium difficile*
		Treatment of stomach ulcers, caused by *Helicobacter pylori*, and oral mucositis
		Treatment of *S. pneumoniae* intravenously
		Inactivation of sperm
		Coating of biomedical implants
Nisin A in combination with polymyxin E and clarithromycin		Treatment of *Pseudomonas aeruginosa* infections
Nisin A in combination with lysostaphin		Prevention of intramammary infections caused by *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis*
Nisin F	*Lactococcus lactis* subsp. *lactis*	Treatment of intranasal infections caused by *S. aureus*
		Controls subcutaneous infections of *S. aureus*
		Suppresses the growth of *S. aureus* in the intraperitoneal cavity
Lacticin 3147	*Lactococcus lactis* subsp. *lactis*	Prevention of dental decay caused by *Streptococcus mutans*
		Treatment of *S. aureus* and MRSA infections
		Treatment of bovine mastitis caused by mastitic staphylococci and streptococci
Salivaricin A2 and B	*Streptococcus salivarius*	Treatment of bad breath
Macedocin ST91KM	*Streptococcus gallolyticus* subsp. *macedonicus*	Treatment of mastitis in dairy cows, caused by *S. agalactiae, Streptococcus dysgalactiae* subsp. *dysgalactiae, S. uberis, S. aureus* and *Staphylococcus epidermidis*
Mutacin 1140	*Streptococcus mutans*	Prevention of tooth decay caused by *S. mutans*
Mutacin B-Ny266	*Streptooccus mutans*	Controls *S. aureus* infection in the peritoneal cavity
Abp118	*Lactobacillus salivarius*	Treatment of *Listeria* infections
Peptide ST4SA	*Enterococcus mundtii*	Prevention of middle ear bacterial infections

Table 2. Continued.

E50-52	*Enterococcus faecium*	Inhibits the intracellular growth of *Mycobacterium tuberculosis*
Pediocin PA-1	*Pediococcus acidilactici*	Treatment of *Listeria monocytogenes* infections
Piscicolin 126	*Carnobacterium piscicola*	Treatment of *Listeria* infections
Divercin V41	*Carnobacterium divergens*	Treatment of *Listeria* infections
Bacteriocin Bcn5*	*Clostridium perfringens*	Treatment of tuberculosis caused by *Mycobacterium tuberculosis*
Subtilosin*	*Bacillus subtilis* and *Bacillus amyloliquefaciens*	Treatment of bacterial vaginosis. Inactivation of sperm
Mersacidin*	*Bacillus* spp.	Treatment of MRSA infections in the nasal cavity
Cinnamycin-like lantibiotics*	*Streptomyces cinnamoneus*	Regulation of blood pressure and fluid balance
Ancovenin*	*Streptomyces* spp.	Regulation of blood pressure and fluid balance Lantibiotics may thus have potential for treating high blood pressure
Bacteriocin Pep5* and epidermin	*Staphylococcus epidermidis*	Prevents growth of staphylococci and/or enterococci in and on catheter tubing
Planosporicin*	*Planomonospora* sp.	Controls *S. pyogenes*-induced septicaemia
Philipimycin*	*Actinoplanes philippinensis*	Controls *S. aureus* infection
Thiazomycin*	*Amycolatopsis fastidiosa*	Controls *S. aureus* infection
Nosiheptide*	*Streptomyces actuosus*	Controls methicillin-resistant *S. aureus* infection
Microcin J25*	*Escherichia coli*	Decrease *Salmonella* numbers in the liver and spleen

*Not produced by lactic acid bacteria

Little is known about the role FLAB play in the human gut. An increase in the production of [GAR+] prions in *S. cerevisiae* when cells were cultured in the presence of *Apilactobacillus kunkeei* was noted (Ramakrishnan *et al.,* 2016). This resulted in the suppression of glucose fermentation in *S. cerevisiae* in favour of the fermentation of a larger variety of sugars (Ramakrishnan *et al.*, 2016). It may be that a similar change in carbohydrate fermentation occurs amongst microorganisms in the human gut, but this must be confirmed. Dead cells of *A. kunkeei* stimulated the secretion of immunoglobulin A (SIgA) in human saliva (Asama *et al.*, 2015). This may initiate further research interests in non-viable probiotics. A follow-up study (Asama *et al.*, 2016) have shown a decrease in viable cell numbers of *B. fragilis* in the GIT of individuals that received *A. kunkeei*. Production of β-glucosidase, β-galactosidase and leucine arylamidase by *A. kunkeei* (Vergalito *et al.*, 2020) may play an important role in the

hydrolysis of glycosidic bonds, lactose and proteins, respectively (Son *et al.*, 2018). *A. kunkeei* produces extracellular polymeric substances (EPS) that may lower cholesterol levels in humans (Sakandar *et al.*, 2019; Son *et al.*, 2018; Vergalito *et al.*, 2020). Fillanino *et al.* (2019) suggested that the degradation of fructose could provide relieve to individuals suffering from fructose-mediated irritable bowel syndrome (IBS). Added to these beneficial properties, is the low antibiotic resistance reported for the strains the authors studied, and the ability to inhibit the growth of *Pseudomonas aeruginosa* and *Enterococcus faecalis*, at least *in vitro.* The ability of *A. kunkeei* to inhibit pathogenic strains of *P. aeruginosa* could be due to the formation of biofilms (Berrios *et al.*, 2018). Survival of *A. kunkeei* through the human GIT was similar to, and in some instances better, than reported for *Lacticaseibacillus rhamnosus* GG (Vergalito *et al.*, 2020). Further research is required on the probiotic properties of FLAB in humans, especially since FLAB are used as starter cultures in fermented food (Pruckler *et al.*, 2015). Applications for using *A. kunkeei* in food and parapharmaceutical products have been filed in Canada and the USA (Matsuura *et al.*, 2018; Olofsson and Vasquez, 2016).

Immune modulation

The mucosal immune system plays a key role in the innate and systemic (adaptive) immune responses of humans and serves as first line of defence against antigens (Helbert, 2017). LAB modulate the immune system by altering the functions of dendritic cells (DCs), monocytes/macrophages, and T and B lymphocytes (Bermudez-Brito *et al*., 2012; Corr *et al*., 2009; Lebeer *et al*., 2008; Marco *et al*., 2006; Mathipa and Thantsha, 2017; Yan and Polk, 2011), thereby enhancing phagocytosis (Viaşu-Bolocan *et al*., 2013; Yan and Polk, 2010) and displacing pathogens from the GIT (He *et al*., 2000; Malin *et al*., 1996; Meydani and Ha, 2000; Yan and Polk, 2010). The triggering of anti-inflammatory responses from the innate immune system send signals to dendritic cells (DCs) to secrete cytokines such as interleukin 10 (IL-10) (Hutchins *et al*., 2013; Mirpuri *et al*., 2012). Enteric pathogens stimulate nuclear factor-kappa B (NF-ĸB) and mitogen-activated protein kinase (MAPK)-signaling pathways to produce pro-inflammatory cytokines (e.g. IL-8) and recruit inflammatory immune cells (e.g. neutrophils) to the infected area. This results in severe inflammation, tissue damage and may escalate to the development of other diseases (Llewellyn and Foey, 2017; Saxelin *et al*., 2005). Probiotic cells interfering with NF-ĸB and MAPK down-regulates pro-inflammatory cytokine secretion from immune cells (Finamore *et al*., 2014; Llewellyn and Foey, 2017; Saxelin *et al*., 2005; Takanashi *et al*., 2013; Yan and Polk, 2011). Finamore *et al*. (2014) have shown that *Lactobacillus*

amylovorus DSM 16698 has the ability to protect intestinal epithelial cells (IECs) against pro-inflammatory responses induced by enterotoxigenic *E. coli* (ETEC) K88 through suppression of IL-8 and IL-1ß. Another study demonstrated the suppression of ETEC-induced pro-inflammatory responses by *L. casei* OLL2768 inhibiting NF-ĸB and MAPK pathways (Takanashi *et al*., 2013).

Probiotics also play a role in the stimulation and production of antibodies in the gut, particularly immunoglobulin A (IgA) (Viaşu-Bolocan *et al*., 2013; Yan and Polk, 2011). Antibodies released in the intestinal lumen inhibits the adherence of pathogens to IECs by interfering with adhesive cell receptors on the pathogen's cell membrane. *Saccharomyces boulardii* and *L. rhamnosus* GG increased the secretion of IgA in the GIT (Kaur *et al*., 2002; Parvez *et al*., 2006). Oral administration of probiotic lactobacilli also increased IgA levels and shortened the duration of diarrhea in children (Isolauri *et al*., 2001; Guarino *et al*., 1997; Roberfroid, 2000; Viaşu-Bolocan *et al*., 2013). Several probiotic strains modulate host immune mechanisms by stimulating phagocytosis (Cross, 2002; Tien *et al*., 2006). The inhibition of enteropathogenic *Pseudomonas aeruginosa* and *L. monocytogenes* in mice by *L. casei* correlated to an increase in abundance of macrophages (Driessen and De Boer, 1989). Evidence of probiotic bacteria stimulating phagocytosis has also been reported for healthy individuals (Gill *et al*., 2001a,b; Roessler *et al*., 2008).

Probiotic strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus* used in a post-infectious IBS model, supressed the expression of pro-inflammatory cytokines IL-6 and IL-17, and stimulated the expression of TJ proteins (claudin-1 and occluding), leading to enhanced barrier stability (Wang *et al*., 2014). *L. rhamnosus* GG decreased inflammation in an IL-10 receptor-dependent manner in immature murine colons (Mirpuri *et al*., 2012). The increase in expression of the IL-10 receptor was also associated with reductions in pro-inflammatory TNF-α and MIP-2. *Bifidobacterium* decreased production of IL-6 and TNFα in LPS-coated CaCo-2 monolayers and supressed the expression of zonulin responsible for dismantling TJs. The latter altered the permeability of epithelium cells, whilst upregulating the expression of occluding, claudin-2, and ZO-1 (Ling *et al*., 2016). The stabilizing effect *Bifidobacterium* has on CaCo-2 monolayers is due to the *in vivo* environment of the gut (Ling *et al*., 2016). Probiotic *Streptococcus* and *Lactobacillus* strains reversed the negative effects caused by entero-invasive *E. coli* on epithelial cell lines (Resta-Lenert and Barret, 2003). Probiotic strains of *L. acidophilus* and *S. thermophiles* increased trans-epithelial resistance with pre-treated

monolayers and protected the cells from damage caused by *E. coli.* Probiotic administration was also associated with phosphorylation of cytoskeletal (actin and actinin) and TJ proteins (ZO-1 and occludin), all of which provides stability to TJs. Furthermore, *Lactobacillus* spp. are capable of stabilizing AJs through the increased expression of E-cadherin, as well as strengthening the E-cadherin/β-catenin complex through enhanced phosphorylation of β-catenin (Hummel *et al*., 2012).

Extracellular proteins secreted by *B. breve* C50 interacted with TLR-2 on the surface of immature DCs and induced several functional and physiological changes. Some of the effects included prolonged survival of DCs, earlier maturation of DCs, and an increase in IL-10 and IL-12 production (Hoarau *et al*., 2008). Proteins secreted by probiotic lactobacilli are involved in maintenance of the mucosal barrier, mainly through MAPK-dependent mechanisms (Schlee *et al*., 2008). Proteinaceous compounds secreted by *Lactobacillus acidophilus* PZ 1138, *Lactobacillus fermentum* PZ 1162 and *Lactobacillus paracasei* subsp. *paracasei* LMG P-17806 stimulated the production of b-defensin 2 (hBD2) in human epithelial cells. The signal of these proteins was transduced to the nucleus through the MAPKs ERK, p38 and c-Jun terminal kinase (JNK). Synthesis of hBD2 increased through modulation of NF-kB and activator protein 1 (AP-1). This resulted in increased IL-8 production (Schlee *et al*., 2008).

Lactobacillus rhamnosus GG interacted with intestinal cells and maintained the integrity of the gut-blood-barrier (GBB) (Bajaj *et al*., 2015). Several *Lactobacillus* spp. induce gene-regulation pathways that lead to upregulation of IL-1β, resulting in the transcription of genes involved in B-cell maturation and lymphogenesis, which contributes towards enhanced barrier stability and function. *L. plantarum* regulated human epithelial TJ proteins *in vivo* and conferred protective effects against chemically induced disruption of the epithelial barrier in an *in vitro* model (Karczewski *et al*., 2010). Administration of *L. plantarum* into the duodenum of healthy human volunteers significantly increased ZO-1 and occludin in the vicinity of TJ structures (Karczewski *et al*., 2010). These results suggest that administration of *L. plantarum* can enhance the stability of TJ complexes in humans and may attenuate their disruption by cytokines, toxins and pathogens. Several studies have shown that LAB can trigger an immune response that results in a rapid and efficient antiviral reaction (Hirose *et al*., 2006; Sugimura *et al*., 2013). A daily intake of heat-killed *L. plantarum* L-137 cells led to a significant decrease in upper respiratory tract infections by stimulating a Th1-type immune response in healthy adults with high physiological stress (Hirose *et al*., 2006). *Lactococcus lactis* JCM5805

displayed positive immunomodulatory effects on plasmacytoid dendritic cells (pDCs) *in vitro*, which decreased the morbidity attributed to the common cold (Sugimura *et al*., 2013). Laiño *et al*. (2016) addressed the ability of probiotics to beneficially modulate IFN and inflammatory signalling pathways in IECs and immune cells, thus decreasing RV symptoms.

The serine protease inhibitor (serpin) produced by *Bifidobacterium longum* subsp. *longum* NCC2705 interact directly with host factors (Ivanov *et al*., 2006). Extracellular serpin is also produced by other bifidobacterial species, including *Bifidobacterium breve*, *Bifidobacterium dentium* and *B. longum* subsp. *infantis*. Serpin inhibits pancreatic and neutrophil elastases (Ivanov *et al*., 2006). Neutrophils are recruited in the intestinal mucosa from blood vessels by means of the secretion of inflammatory cytokines. Serpin produced by bifidobacterian act on enzymes directly involved in the inflammatory response and might thus mediate some of the anti-inflammatory effects reported for bifidobacteria (Ivanov *et al*., 2006).

Proteinaceous compounds secreted by probiotic strains of *L. plantarum*, *L. acidophilus*, *L. casei* and *L. delbrueckii* subsp. *bulgaricus* stimulated the expression of the muc2 gene and increased the production of mucin by murine colonic epithelial cells (Caballero-Franco *et al*., 2007). Extracellular proteins produced by *L. rhamnosus* GG increased production of the heat-shock proteins HSP25 and HSP72 in murine colon cells (Tao *et al*., 2006). Protein p40, produced by *L. rhamnosus* GG, is homologous to an uncharacterized surface antigen of *L. casei* ATCC 334 (gi|116493594) and protein p75 to a cell wall-associated hydrolase of strain ATCC 334 (gi|116493849) (Yan *et al*., 2007). Both these proteins induced the proliferation of murine colonic epithelial cells and reduced injuries to colonic cells caused by tumour necrosis factor alpha (TNFα) (Yan *et al*., 2007). Proteins p40 and p75 inhibited TNF-α-induced apoptosis in the KSRI2/2 MCE cell line (Yan *et al*., 2007) and attenuated TER decrease induced by hydrogen peroxide. Concluded from these results, proteins p40 and p75 play an important role in cell proliferation, apoptosis, and maintenance of the mucosal barrier.

Little is known about the effect bacteriocins have on the immune system. It is generally believed that the peptides will be destroyed by macrophages once they cross the gut wall. The lantibiotics gallidermin, Pep5 and nisin induced the release of multiple chemokines at levels similar to that of the human cationic antimicrobial peptide LL-37, with nisin seemingly able to activate multiple signalling pathways, including ERK/MAPK, PKC and PKA (Kindrachuk *et al*., 2013). Nisin administered prophylactically to mice confers protection to mice challenged

with Gram-positive (*S. aureus*) and Gram-negative (*S. enterica* and *E. coli*) bacteria. This is significant, as nisin is ineffective against Gram-negative bacteria, suggesting that nisins' interaction with the host's immune response provides a selective advantage. At high concentrations, nisin activates neutrophils, resulting in formation of neutrophil extracellular traps (Begde *et al*., 2011). Neutrophil extracellular traps are known for trapping and killing bacteria (Zawrotniak and Rapala-Kozik, 2013). Furthermore, loci harbouring genes involved in bacteriocin production and secretion modulate the immune response of dendritic and peripheral blood mononuclear cells (Meijerink *et al*., 2010; van Hemert *et al*., 2010). By enhancing the hosts' immune system, bacteriocins indirectly provide protection against infectious microbial agents. These effects are not that surprising as host cationic defence peptides also have immune modulatory effects.

Drissi and co-workers (2015) explored the role bacteriocins may have in the GIT. In a genome mining project, the authors retrieved 641 genomes (307 whole genomes and 334 draft genomes) from microorganisms in the human GIT. The genomes represented 199 bacterial genera, including *Lactobacillus*, *Streptococcus*, *Clostridium* and *Bacillus.* A bidirectional protein BLAST, compared to bacteriocin sequences listed in the BUR database, revealed that 317 of the genomes encoded putative bacteriocins of classes I (44%), II (38.6%) and III (17.3%). This supports the hypothesis that bacteriocins are widespread across the GIT. Of the 317 putative bacteriocins, 175 were from Firmicutes (which includes LAB), 79 from Proteobacteria, 34 from Bacteroidetes, and 25 from Actinobacteria. The high number of bacteriocins being (hypothetically) produced by Proteobacteria may explain why they are so persistent and virulent. The study also suggested that bacteriocins produced by gut bacteria are generally smaller in size and differ in amino acid composition compared to most other bacteriocins. Furthermore, these (putative) bacteriocins contained less aspartic acid, leucine, arginine, and glutamic acid but more lysine and methionine. Based on their α-helical structure, charge, and hydrophobicity they may have a broad spectrum of antimicrobial activity (Dathe and Wieprecht, 1999; Giangaspero *et al*., 2001; Zelezetsky and Tossi, 2006). Considering these findings, the bacteriocins produced by gut bacteria, especially Firmicutes and Proteobacteria, may render them a competitive advantage over other bacteria in the GIT (Schuijt *et al*., 2013). Drissi and co-workers (2015) speculated that bacteriocins in the GIT may have low levels of antimicrobial activity and may thus not have such a drastic effect on microbial populations. This makes sense, as the GIT supports the existence of a large variation of gut bacteria, thus a balanced population. If bacteriocins play a lesser role in population

dynamics, they may have a greater role to play in quorum sensing, or possibly in host immune modulation.

The lantibiotics Nisin, gallidermin and Pep5 have immune-regulatory properties (Kindrachuk *et al*., 2013). Nisin modulates multiple signaling pathways, resulting in the release of several chemokines (Kindrachuk *et al*., 2013). In mice pretreated with nisin, modulation of the immune system protected the animals against *Salmonella enterica* serovar Typhimurium and *E. coli* infections (Kindrachuk *et al*., 2013). Treatment with Nisin also activated the release of neutrophils in neutrophil extracellular traps (NETs) (Driouich *et al*., 2019) and may play a role in providing immunity to fungal infections (Urban and Nett, 2019). Although antimicrobial properties associated with NET formation can be advantageous, chronic NET formation, which entails cellular release of large amounts of free radicals and nuclear material such as histones, is also associated with chronic inflammation and increases the risk of developing autoimmune diseases such as rheumatoid arthritis (reviewed by Apel *et al*., 2018). This must be taken into consideration when using nisin (or other lanthipeptides) to control infections. Nisaplin (a commercially available nisin preparation) administered to mice resulted in a short-term increase of CD41 and CD81 cells and T lymphocytes (Pablo *et al*., 1999). The significance of these findings warrants further research. Cinnamycin-like lantibiotics suppressed the production of phosphatidylethanolamine (PE), which is the precursor for phospholipase A2. Decreased levels of phospholipase A2 mediates an inflammatory response (Märki *et al*., 1991). Modulation of phospholipase A2 activity also results in the production of higher levels arachidonic acid. Oxidation of arachidonic acid leads to the formation of eicosanoids such as prostaglandins and leukotrienes, which are strong mediators of the immune system. The cinnamycin-like lantibiotic ancovenin inhibits angiotensin-converting enzyme (ACE) (Kido *et al*., 1983) which destabilises angiotensin II and leads to hypertension, diabetic inflammation, and fibrosis (Bernstein *et al*., 2018). Peptidase-resistant lanthionine-stabilized angiotensin-(1–7) alleviated diabetic nephropathy and cerebral stroke (Cassis *et al*., 2019; Kuipers *et al*., 2020). Streptocollin, similar in structure to cinnamycin-like lantibiotics, does not inhibit phospholipase A2 (Iftime et al., 2015). Streptocollin partially inhibits protein tyrosine phosphatase 1B (PTP1B), a regulator of various signaling pathways, including insulin signaling, but also plays a role in immune cell signaling (Través *et al*., 2014). Treatment with Streptocollin may thus alleviate insulin sensitivity (Ricke *et al*., 2020). These results suggest that lantibiotics can interact and modulate the immune system, potentially using similar

mechanisms employed by human and other ctAMPs. Additional cell biology research is required to fully understand how lantibiotics/lanthipeptides interact with the immune system.

If permeability is severely changed, gut microbiota may enter the blood stream and cause bacteraemia. Certain pathogens can disrupt intracellular junctions by interacting with cell receptors. Enteric pathogens often gain access to the body by altering the structure and function of tight junctions to increase permeability of the barrier via the secretion of proteases, which can cleave tight junction proteins or by altering the cytoskeleton (Berkes *et al*., 2003). Inflammatory cytokines such as TNFα and IFNγ, which are induced during infection and in IBD, increase intestinal permeability in general, although single inflammatory models yielded different results (Corridoni *et al*., 2012). Probiotics and commensal microbiota can reverse such inflammatory dysfunctions in human intestinal epithelial cells. This is done by improving barrier functions or by inhibition of pathogen adherence (Anderson *et al*., 2010; Moorthy *et al*., 2009; Resta-Lenert *et al*., 2006). Synergistic effects between sIgA and probiotics have been published (Mathias *et al*., 2010).

Anticarcinogenic and antitumour activity

Microbial enzymes such as azoreductase, β-glucuronidase and nitroreductase may convert procarcinogens into carcinogens and cause colon cancer (Fernandes and Shahani, 1990; Goldin, 1990). Goldin and Gorbach (1977) have shown that *L. acidophilus* could decrease nitroreductase, azoreductase and β-glucuronidase activities in carnivorous animals. In a subsequent study, Goldin *et al.* (1992) have shown that *L. rhamnosus* GG could lower bacterial β-glucuronidase activity in the large intestine. LAB may also retard or prevent the initiation and promotion of tumours. *L. acidophilus* and *Lactobacillus bulgaricus* and/or *L. casei* suppressed Ehrlich ascites tumour or Sarcoma 180 in mice (Goldin *et al.*, 1996). Tumour suppression is associated with intact viable cells, intact dead cells, and cell wall fragments of lactobacilli and bifidobacteria. *Lactobacillus* GG positively affected the initiation or promotion of DMH-induced tumours in rats on a high-fat diet. Orally administered strains of *L. casei* were effective in preventing the recurrence of superficial bladder cancer (Aso *et al.*, 1995).

Nitrites used in food processing are converted to carcinogenic nitrosamines in the GIT. Cellular uptake of nitrites by lactobacilli and bifidobacteria has been shown *in vitro* (Grill *et*

al., 1995). Bile salts have been implicated in the initiation of colon carcinogens (Lewis and Gorbach, 1972). *L. acidophilus* reduced the biotransformation of primary to secondary bile salts, thus reducing the possible initiation of cancer (Fernandes and Shahani, 1990). Modler *et al.* (1990) suggested that the reduction of intestinal pH, through metabolic activities of LAB, could inhibit the growth of putrefactive bacteria and thus prevent large bowel cancer.

Aflatoxins produced by moulds are known to cause cancer. At least 13 aflatoxins, of which B1, B2, G1, G2, M1 and M2 are the best known, have been described for *Aspergillus* spp. (Groopman *et al.*, 2008). Aflatoxin B1, the best studied of all, causes liver cancer in humans. Gourama and Bullerman (1995) reported the inhibition of mould growth and aflatoxin production by LAB. *L. casei* subsp. *pseudoplantarum* inhibits the biosynthesis of aflatoxins B1 and G1 (Gourama and Bullerman, 1997). *L. rhamnosus* GG binds aflatoxin B1, and to a lesser extent aflatoxins B2 and G1 (El-Nezami *et al.,* 1996).

Several studies have shown that some bacteriocins have anticancer properties (Kaur and Kaur 2015). Bacteriocins have a higher affinity for cancer cells due to the general negative charge of cancer cells. Treatment of head and neck squamous cell carcinoma (HNSCC) cells with nisin induced DNA fragmentation and apoptosis on three different cancer cell lines (Kamarajan *et al*., 2015; Joo *et al*., 2012). Apoptosis in NHSCC cells, caused by nisin, is associated with calcium influx and upregulation of CHAC1 (cation transport regulator and apoptosis mediator) (Joo *et al*., 2012). The size of tumours in mice with oral cancer were reduced when treated with nisin (Joo *et al*., 2012). The authors concluded that the selective action of nisin was due to structural differences in the composition of the plasma membranes between HNSCC cells and primary keratinocytes. The class IIc human defensins-like bacteriocin, laterosporulin 10, displays cytotoxic effects against several cell lines and causes necrotic and apoptotic cell death at high and low concentrations, respectively. At high concentrations (10 µM), more than 95% of normal prostate epithelial cells remained viable, whereas 80% of cancer cells lost their viability. As with cytotoxicity against normal cells, the concentrations used to be effective against cancerous cells may be higher than the levels crossing the GBB. However, the higher affinity for cancerous cells may result in bacteriocins targeting these cells. Immune priming by bacteriocins may also assist in the elimination of cancer cells.

Nisin (nisins A and Z) has been shown to be effective in vitro and in vivo against head and neck squamous cell carcinoma (HNSCC) (Joo *et al*., 2012; Kamarajan *et al*., 2015). These

peptides induce apoptosis in HNSCC cells in a dose-dependent manner and has a minimal effect on primary keratinocytes. This may be due to the structural differences in the plasma membrane, specifically the phospholipid content, of the different cell types. This is supported by the observation that nisin binds to phosphatidylcholine, which is known to be increased in cancer cells (along with PE) (Cheng *et al*., 2016; El Jastimi *et al*., 1999; El Jastimi and Lafleur, 1999). The mechanism by which nisin induces apoptosis has been proposed to be calcium dependent (influx of calcium) (Joo *et al*., 2012). The subsequent influx of calcium results in the activation of calpain-1, resulting in caspase 3-independent apoptosis (Kamarajan *et al*., 2015). This is further supported by the observation that nisin affects plasma membrane integrity through the release of lactose dehydrogenase (LDH) (Dreyer *et al*., 2019).

The potency of nisin *in vitro* and *in vivo* can be further increased in combination with 5-fluorouracil or doxorubicin (Rana *et al*., 2019; Preet *et al*., 2015). The involvement of calcium in this context and the increased efficacy by addition of doxorubicin, which is known to exert anticancer effects via induction of free radical damage in cancer cells, again suggests that lantibiotics may have a role in the modulation of redox status, although the nature of this modulation varies between lantibiotics. Duramycin has also shown potential in the treatment of cancer, as it induces apoptosis and reduces the proliferation of tumor cells (Broughton *et al*., 2016; Yates *et al*., 2012). Due to its high affinity for PE, duramycin may be more selective towards cancerous cells. An interesting application of duramycin as an anticancer treatment is its fusion to IgG, generating a new duramycin-IgG variant (He and Thorpe, 2004; Thorpe *et al*., 2016). Fusion of IgG to duramycin does not influence its PE binding capability and has the advantage of reducing duramycin cytotoxicity. The IgG fused to duramycin helps guide the host immune cells to apoptotic cells, resulting in enhanced phagocytosis. Furthermore, tumor growth (MethA tumors) is inhibited in mice after treatment with duramycin-IgG (He and Thorpe, 2004). Since duramycin binds to PE and the Fc region on (fused) IgG antibodies, it may be cleared from cancer cells soon after the induction of apoptosis. This would explain the lower cytotoxicity of duramycin to non-cancerous cells.

The urokinase plasminogen activator (uPA) is a serine protease responsible for the conversion of plasminogen to plasmin. The urokinase plasminogen activator system has been implicated in activities associated with tumor progression and metastasis and is a potential target for anticancer therapy (reviewed by Mahmood *et al*., 2018). Using this system, the authors identified several novel lanthipeptides capable of inhibiting the catalytic ability of uPA. These

peptides have not been evaluated for cancer treatment, but they do show potential. More importantly, the study has highlighted techniques that could vastly increase the efficiency with which potential candidates may be screened for anticancer activity. For further information on the anticarcinogenic properties of LAB the reader is referred to the review by Dicks (2022), included in Chapter 6 of this dissertation.

Reduction of cholesterol

Excessive caloric intake with a high-fat diet is associated with the development of obesity and complications such as non-alcoholic fatty liver disease (NAFLD) and insulin resistance (Softic *et al.*, 2020). Recently, focus changed to the effect sugar-sweetened beverages, including those containing fructose, have on obesity and hepatic insulin resistance (Softic *et al.*, 2020). High levels of fructose also lead to an increase in intestinal permeability due to loss of tight junction proteins. This may lead to an increase in the translocation of endotoxins and damage to hepatocytes, resulting in the activation of Kupffer cells, and the release of inflammatory cytokines and oxygen radicals. These changes are associated with the induction of toll-like receptors (TLRs) in the liver (Jegatheesan *et al.*, 2016), an increase in serum lipopolysaccharide levels and an increase in the expression of TLR4 in the liver, all of which are physiological reactions leading to NAFLD and possible weight gain (De Sousa Rodrigues *et al.*, 2017). Studies conducted on mice fed a high fat diet supplemented with high fructose showed an increase in lymphocyte infiltration in the liver (De Sousa Rodrigues *et al.*, 2017), accompanied by increased inflammation and an increase in the expression of tumour necrosis factor alpha (Spruss *et al.*, 2012). For further reading on the effect high fructose levels may have on the liver, the reader is referred to Dicks and Endo (2022).

Specific strains of *S. thermophilus* and *L. acidophilus* reduced cholesterol levels in rats (Grunewald, 1982). Milk fermented with LAB and *Streptococcus cerevisiae* led to lower serum cholesterol levels, phospholipids, and bile acids in the faeces of mice (Tamai *et al.,* 1996). Similar findings were reported by Fukushima and Nakano (1996) and Tortuero *et al.* (1997). Zacconi *et al.* (1992) showed that serum cholesterol levels were lower in axenic mice colonised with *E. faecium* and *L. acidophilus*. Gilliland and Walker (1990) found that the consumption of *L. acidophilus* reduced serum cholesterol levels in pigs that have been fed a high-cholesterol diet. Studies conducted by de Rodas *et al.* (1996) supported these findings. However, the serum lipoprotein levels of 334 individuals remained unchanged when they were

treated with *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* (8×10^6 CFU/day) (Lin and Chen, 2000). *E. faecium* administered over six weeks to adults resulted in an initial increase in total cholesterol and LDL, followed by a sharp decrease two weeks after termination of treatment (Mikeš *et al.,* 1995). The decrease corresponded with an increase in the reduction of iodonitrotetrazolium and superoxide production by peripheral neutrophils and an elevated production of IgG.

In all studies conducted thus far, the real factor responsible for a reduction in cholesterol levels remains unknown. Klaver and Van der Meer (1993) suggested that the reduction of cholesterol is not due to assimilation or to a direct interaction between the bacteria and cholesterol, but to the co-precipitation of cholesterol with deconjugated bile salts at pH values below 6.0. This would not explain reduction of cholesterol *in vivo* as the pH of the lower GIT is neutral to alkaline. Marshall and Taylor (1995) also observed co-precipitation of cholesterol with deconjugated bile salts, but also reported cholesterol removal in the absence of bile. The authors suggested a physical association between cholesterol and the cell surface.

Bile salt deconjugation may play a role in the reduction of cholesterol. Cholesterol and bile salt metabolism are interlinked, where cholesterol is the precursor for synthesis and bile salts the water-soluble excretory product. Bile salts are deconjugated during enterohepatic circulation (EHC) by bile salt hydrolase (BSH) (E.C.3.5.1.24). The free bile acids as well as glycine and taurine, are not so easily reabsorbed and are excreted in the faeces (De Smet *et al.,* 1994). The loss in bile salts increases the catabolism of cholesterol to bile acids, resulting in lower cholesterol levels (De Rodas *et al.,* 1996; Driessen and De Boer, 1989). BSH activity has been shown in *Lactobacillus, Enterococcus, Peptostreptococcus, Bifidobacterium, Clostridium* and *Bacteroides* spp. (Bateup *et al.,* 1995; Grill *et al.,* 1995). The BSH hypothesis has not definitely been proved. Recent observations indicate that free bile acids are less effectively absorbed by the active transport system in the ileum but are more effectively reabsorbed in the intestine and colon by passive diffusion (Marteau *et al.,* 1990).

De Smet *et al.* (1998) have shown that feeding pigs with cells of *Lactobacillus reuteri* containing active BSH resulted in significant lowering of serum total and LDL-cholesterol concentrations, accompanied by a gradual increase in *Lactobacillus* cell numbers. No change in HDL cholesterol concentration was observed. The authors have also shown that during the final three weeks of changing from a high fat diet to a regular diet, the cholesterol levels

significantly decreased and the differences in total and LDL-cholesterol concentrations between the treated and untreated animals largely disappeared. *L. reuteri* cells were gradually washed out and they failed in permanently colonising the intestinal tract. Taranto *et al.* (2000) have shown that administration of *L. reuteri* CRL 1098 (104 cells/day) to mice for 7 days effectively prevented hypercholesterolemia. A 17% increase in the ratio of high-density lipoprotein to low-density lipoprotein was observed. The total serum cholesterol and triglyceride levels decreased by 22 and 33%, respectively, in mice that received *L. reuteri*.

Alleviation of lactose intolerance

Many individuals, especially of Asian and African descent, lack the intestinal mucosal enzyme β-galactosidase (lactase) or suffer from a reduction in lactase activity caused by intestinal infection (e.g., rotavirus gastroenteritis). *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* produce high levels of β-galactosidase. Both species are sensitive to bile salts, leading to the release of high levels of β-galactosidase in the GIT. *L. acidophilus* is bile resistant and has lower levels of β-galactosidase compared to *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* but grows in the GIT and may produce β-galactosidase over a longer period (Driessen and De Boer, 1989). Lactose from yoghurt and milk containing the probiotic *L. acidophilus* were better absorbed by subjects with low β-galactosidase activity (Sanders, 1993). There were fewer symptoms of lactose intolerance and bacterial fermentation of undigested lactose was also evident in breath hydrogen concentrations.

Normalisation of stool transit

Lactobacillus GG accelerated the recovery from acute watery diarrhoea in young children (Colombel *et al.,* 1987). In another study (Siitonen *et al.,* 1990) volunteers with diarrhoea and on erythromycin treatment reacted positively when they received *Lactobacillus* GG. Symptoms of diarrhoea, stomach pain, abdominal pain and nausea were less frequent and recovery much quicker. Similar findings have been reported by Isolauri *et al.* (1991) and Majamaa and Isolauri (1997). *E. faecium* SF68 proved as effective in children with paediatric diarrhoea (Bellomo *et al.,* 1980). In other studies *E. faecium* SF68 also reduced the duration of diarrhoea in adults (Buydens and Debeuckelaere, 1996). Another form of diarrhoea more difficult to treat is that caused by *Clostridium difficile*. Symptoms usually occur after antibiotic

treatment, which makes treatment of this disorder with antibiotics less optimal. Treatment with *L. rhamnosus* GG improved symptoms of intestinal disorders (Bennett *et al.,* 1996).

Salminen *et al.* (1988) studied the effect of *L. acidophilus* NCFB 1748 on 21 female cancer patients that received pelvic radiotherapy. Patients who consumed milk fermented by the strain experienced less diarrhoea. The effect of different LAB on different types of diarrhoea has been reviewed by Gorbach (2002). Further research is needed to determine which mechanisms LAB use to relieve diarrhoea. *L. acidophilus* and *Bifidobacterium* spp. have been shown to relieve constipation, but more conclusive data are needed (De Vrese and Marteau, 2007; Fuller, 1989). A study conducted by Koebnick *et al.* (2003) has shown that *L. casei* strain Shirota (LcS) of Yakult resulted in a significant improvement in self-reported severity of constipation and stool consistency, from the second week of treatment onwards. Eighty-nine percent of the patients that received LcS versus 56% of the placebo group reacted positively to the treatment. Marteau *et al.* (2002) have shown that a fermented milk product containing *Bifidobacterium animalis* strain DN-173 010 of Danone shortened the colonic transit time in healthy women.

Hepatic encephalopathy

Patients with liver failure have higher levels of ammonia, leading to encephalopathy. Ingestion of *L. acidophilus* lowered levels of faecal urease and blood ammonia (Salminen *et al.,* 1993). *E. faecium* SF68 also proved effective in lowering blood ammonia levels (Loguercio *et al.,* 1987). Probiotic preparations containing *E. faecium* SF68 proved to be as effective as lactulose in lowering blood ammonia and in improving mental state and psychometric performance (Loguercio *et al.,* 1987). The effects of strain SF68, contrary to that of lactulose, persisted longer after treatment withdrawal. The review by Dicks and Endo (2022) addresses the role fructophilic lactic acid bacteria (FLAB) may play in liver damage. To the best of my knowledge, no reports have been published on the presence of FLAB in the human GIT.

Treatment of peptic ulcers

Lactic acid produced by *L. acidophilus* inhibited the growth of *Helicobacter pylori* in *in vitro* tests (Bhatia *et al.,* 1989). Only strains of *L. acidophilus* and *L. rhamnosus,* obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) starter culture collection, Australia, inhibited the growth of *H. pylori* (Midolo *et al.,* 1995). Metabolic

products other than lactic acid may also play a role. *Lactobacillus salivarius* inhibited the attachment of *H. pylori* and interleukin-8 release *in vitro* (Kabir *et al.,* 1997). Probiotic lactobacilli are acid tolerant and may survive conditions in the stomach. They may thus be good candidates for treatment of peptic ulcers. For further information on research concerning peptic ulcers the reader is referred to the paper published by Asgari *et al*. (2020).

Neuropathic pain relief

Labyrinthopeptin-like lantibiotics have limited antibacterial activity. However, labyrinthopeptin A2 and NAI-112 have been shown to have antiallodynic and antinociceptive properties in mice (Meindl *et al*., 2010; Iorio *et al*., 2014). Labyrinthopeptin A2 administered intravenously at concentrations ranging from 0.01 to 3.0 mg/kg resulted in significant attenuation of tactile allodynia (ED50, 50mg/kg). Efficacy remained stable over 6 h posttreatment, with loss of efficacy after 24 h (Meindl *et al*., 2010). Similarly, NAI-112 was also able to significantly reduce allodynia and hyperalgesia 2 h after administration, albeit at much higher doses (0.10 mg/kg) (Iorio *et al*., 2014). Significant antinociceptive effects could be observed at somewhat lower doses (from 3 mg/kg). At this point, due to differences in experimental procedures, the efficacies of these two lanthipeptides cannot be directly compared. More research is required, as the mechanisms of action of these peptides have not been elucidated, although a potential interaction with the vanilloid pathway has been suggested for NAI-112 (Iorio *et al*., 2014). For further information on neuropathic pain relief the reader is referred to the paper by Lin *et al*. (2020).

Ion channel regulation

Duramycin has potential in the treatment of cystic fibrosis, which is caused by abnormal chloride ion transport into cells. It has been demonstrated in tissue culture that the efflux of chloride observed after duramycin treatment is associated with a decrease in intracellular calcium levels (Oliynyk et al., 2010). It was therefore proposed that the efflux of chloride from epithelial cells is likely due to the interaction duramycin has with cell membranes. This is supported by the interaction that duramycin has with PE, whereby it can be deposited into the cell membrane and indirectly affect ion channel function (Oliynyk *et al*., 2010; Zebedin *et al*., 2008; Sheth *et al*., 1992). Duramycin has undergone phase I and II clinical trials, with phase II clinical trials reporting it to be safe, with overall positive results on the pulmonary function

of cystic fibrosis patients (Grasemann *et al*., 2007; Steiner *et al*., 2008; Zeitlin *et al*., 2004). This effect of duramycin to lower cellular calcium levels may also have broader application in health, given the known association between intracellular accumulation of calcium and a variety of diseases linked to cumulative oxidative damage and chronic inflammation, such as neurodegenerative disease, cancer, accelerated aging, and type II diabetes (reviewed by Smith, 2018).

Antiviral activity

Various lantibiotics, including nisin, labyrinthopeptin, and duramycin, have been evaluated for their antiviral properties (Blockus *et al*., 2020; Férir *et al*., 2013; Małaczewska *et al*., 2019; Prochnow *et al*., 2019; Richard *et al*., 2015; Tabata *et al*., 2016). Of interest are the antiviral properties of labyrinthopeptin A1 and duramycin, which show antiviral activity through their ability to bind PE. The PE binding capability of duramycin has proven useful once again, with duramycin being able to inhibit the entry of filo- and flaviviruses into host cells (Richard *et al*., 2015; Tabata *et al*., 2016). Phosphatidylethanolamine is a ligand for the T-cell Ig mucin domain (TIM) protein TIM1, and together they are involved in phosphatidylserine (PS)-dependent phagocytosis of apoptotic cells (Richard, 2015). Additionally, TIM family proteins have also been shown to promote infection of enveloped viruses because of virion lipid content, specifically PS and PE (Richard *et al*., 2015). Duramycin was therefore evaluated for its ability to inhibit TIM1-mediated virus entry through blocking virus attachment to TIM1 (Richard *et al*., 2015). Duramycin was reported to be most effective at the entry phase of the viral infection, with no effect observed when duramycin was administered post infection and was effective in inhibiting viral entry into human TIM1-expressing cells (hTIM1-293T) as well as cells naturally expressing TIM1 (Vero and A549 cells). This inhibitory effect was shown to be specific for TIM1-mediated entry of viruses through interaction of duramycin with PE present in the viral membrane (Richard *et al*., 2015). Through inhibition of Zika virus binding to TIM1, duramycin has also been shown to be effective in reducing infection of placental cells and explants (Tabata *et al*., 2016). Labyrinthopeptin A1 has shown promising antiviral activity against several viruses, including human immunodeficiency virus (HIV) and herpes simplex virus (HSV), with activity against laboratory-adapted strains and clinical isolates (including drug-resistant strains) (Férir *et al*., 2013). Labyrinthopeptin was able to inhibit cell-free viral infection as well as inhibit cell-to-cell spread of HIV in vitro. This inhibitory activity was dependent on time of drug administration and was only effective if the drug was administered

within 1 h after infection. Like duramycin, these results suggest that labyrinthopeptin A1 likely also interferes with the viral entry process. Labyrinthopeptin A1 was shown to interact with the virus (interaction with envelope protein gp120) and not receptors on the host cell. However, binding to the virus is highly likely to be via interaction with lipids (specifically PE) in the viral membrane (Prochnow *et al*., 2019). A moderate degree of synergism when combined with other commonly used antiretroviral therapies was seen (Férir *et al*., 2013). An advantage of not having significant antibacterial activity is that labyrinthopeptin A1 does not have a negative effect on host microbiota, such as vaginal lactobacilli, reducing the risk of dysbiosis (Férir *et al*., 2013). Importantly, labyrinthopeptin A1 did not stimulate targeted immune cells (peripheral blood mononuclear cells [PBMCs]), as expression of CD69 and CD25 remained unchanged and did not result in significant induction of inflammatory cytokine secretion from these cells (Férir *et al*., 2013). Additionally, labyrinthopeptin A1 was not cytotoxic against vaginal epithelial cells or other nonepithelial cells at effective antiviral concentrations (Férir *et al*., 2013). Labyrinthopeptin A1 and A2 have also been tested against a variety of other enveloped viruses, with labyrinthopeptin A1 being the most effective, conferring broadspectrum antiviral activity (Prochnow *et al*., 2019). Of interest is the observation that labyrinthopeptins bind to PE and may be responsible for labyrinthopeptin binding to viral membranes. Furthermore, it was shown that the antiviral effect was a result of virolysis (viral membrane disruption), although similar effects on TIM1-mediated entry to those reported for duramycin cannot be excluded (Richard *et al*., 2015; Prochnow *et al*., 2019). Additionally, labyrinthopeptins are effective against respiratory syncytial virus (RSV) *in vitro* and have shown promising results in vivo (Blockus *et al*., 2020). Moreover, the labyrinthopeptins are not affected by resistance mutations usually detrimental toward RSV entry inhibitors. The mode of action against RSV is similar to that reported for other viruses (i.e., interaction with the virus-associated PE) (Blockus *et al*., 2020). Although promising results were reported using an in vivo murine model, treatment was not as effective compared to in vitro models and requires additional research (Blockus *et al*., 2020). Efforts at generating lanthipeptides new to nature have also shown promise for generating lanthipeptides capable of inhibiting HIV budding from cells (Yang *et al*., 2018). A bacterial reverse two-hybrid (BRTH) system was used to screen potential lanthipeptide analogues for their ability to inhibit the interaction of the HIV p6 protein with the ubiquitin E2 variant (UEV) domain of human TSG101 (important for budding of HIV from infected cells). Using prochloricin A2.8 as a backbone, the amino acids between the two rings were randomized and modification performed by ProcM (LanM). In vitro testing using the BRTH system resulted in the identification of one peptide, XY3-3,

capable of disrupting the interaction between HIV p6 and UEV. Further in vitro testing revealed that the lanthipeptide had more than 10-fold increase in activity compared to a previously identified inhibitor and specifically binds to UEV. Both lanthionine bridges were also shown to be crucial for activity. To access the peptides' ability to prevent Gag-mediated budding of virus-like particles in cell-based assays, the cell-penetrating Tat peptide was fused to the N terminus of XY3-3. The newly generated XY3-3-Tat was not toxic to cells at concentrations up to 500nM and inhibited viral budding by 65% at 100 nM. The peptide interfered with the degradation of the epidermal growth factor receptor (at 500 nM), which is mediated by the UEV domain of TSG101, further supporting binding of XY3-3-Tat to UEV. Although further testing is required, lanthipeptides such as XY3-3-Tat and labyrinthopeptins may prove useful in antiviral therapy. Furthermore, methods based on BRTH and phage display systems provide a platform for identifying and testing novel lanthipeptides (Yang *et al*., 1989; Urban *et al*., 2019).

A further example of immunoregulation and beneficial antiviral modulation of a human host was described by Tonetti *et al*. (2020). The authors reported that by nasally administering *L. rhamnosus* CRL1505 to mice that had been infected with influenza virus, the levels of influenza virus-specific IgA and IgG, as well as IFN-, in the serum and respiratory tract of infected mice increased significantly in comparison to the control group that did not receive strain CRL1505. Probiotics with the ability to increase the immune response of individuals infected by viruses are referred to as "immunobiotics" (Hirose *et al*., 2006; Sugimura *et al*., 2013). DCs are critical to the activation and corrective functioning of the innate immune system. This is due to DCs displaying phagocytotic capabilities as well as being able to produce type-I IFNs (IFN-α and IFN-β). IFNs serve as the first line of viral defence by blocking viral replication (Swiecki and Colonna, 2015). According to Kanauchi *et al*. (2018) most microbial specimens used in their study showed insignificant IFN upregulation. However, the study did elucidate *Lactococcus lactis* strains that showed significant modulation of DCs by directly stimulating DC cells to produce type-1 and -3 IFNs (Kanauchi *et al*., 2018). *L. lactis* induces TLR9/MyD88 signalling and IFN when engulfed by DCs (Kawasaki and Kawai, 2014). Although direct DC stimulation by *L. lactis* is considered the most probable antiviral mechanism (Kanauchi *et al*., 2018), some strains activate NK cells *in vivo* and *in vitro* (Suzuki *et al*., 2016). *L. lactis* subsp. *lactis* JCM5805 induced the cytotoxicity of NK cells, contributing to host defence against infection by parainfluenza virus particles (Suzuki *et al*., 2016). A review by Kitazawa and Villena (2014)

commented on the immunomodulatory effects probiotics have in treatment of viral infections and focused on L. rhamnosus CRL1505 in the treatment of respiratory syncytial virus (RSV) infections. Recent research has shown that immunobiotics could be used in the prophylactic treatment of respiratory viral infections (Ekitazawa *et al*., 2014). Yasui *et al*. (1989) showed that orally administered immunobiotics had a stimulatory effect on the mucosal and the anti-viral humoral immune systems. *B. breve* YIT4064 augmented the production of anti-viral antibodies by directly activating B cells to produce antibodies (Yasui *et al*., 1989). The immunoglobulins included those that identified polio, influenza, and rotaviruses. The resulting antibodies neutralise an infecting virion by blocking the glycoprotein spikes used by the virus to bind to a host target cell (Greenspan et al., 2019). This binding subsequently interferes with target cell viral uptake, in turn diminishing the ability of the virus to replicate (Rhorer *et al*., 2009).

For further reading on the antiviral properies of LAB, the reader is referred to the reviews by Dicks and Grobbelaar (2021) and Tiwari *et al*. (2020).

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Chapter 6

Do Bacteria Provide an Alternative to Cancer Treatment and What Role Does Lactic Acid Bacteria Play?

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Review

Do Bacteria Provide an Alternative to Cancer Treatment and What Role Does Lactic Acid Bacteria Play?

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Abstract: Cancer is one of the leading causes of mortality and morbidity worldwide. According to 2022 statistics from the World Health Organization (WHO), close to 10 million deaths have been reported in 2020 and it is estimated that the number of cancer cases world-wide could increase to 21.6 million by 2030. Breast, lung, thyroid, pancreatic, liver, prostate, bladder, kidney, pelvis, colon, and rectum cancers are the most prevalent. Each year, approximately 400,000 children develop cancer. Treatment between countries vary, but usually includes either surgery, radiotherapy, or chemotherapy. Modern treatments such as hormone-, immuno- and antibody-based therapies are becoming increasingly popular. Several recent reports have been published on toxins, antibiotics, bacteriocins, non-ribosomal peptides, polyketides, phenylpropanoids, phenylflavonoids, purine nucleosides, short chain fatty acids (SCFAs) and enzymes with anticancer properties. Most of these molecules target cancer cells in a selective manner, either directly or indirectly through specific pathways. This review discusses the role of bacteria, including lactic acid bacteria, and their metabolites in the treatment of cancer.

Keywords: bacteria; lactic acid bacteria; cancer treatment

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1. Introduction

Cancer is one of the leading causes of death, according to 2022 statistics published by the American Cancer Society [1]. At the 17th World Health Assembly [2], the WHO passed the resolution "cancer prevention and control in the context of an integrated approach" and predicted that the number of cancer cases world-wide could increase to 21.6 million by 2030. The American Cancer Society predicted 1.9 million new cancer cases and 609 360 deaths in the United States of America in 2022, rating cancer as the second leading cause of death [1]. According to the latest statistics from the South African National Cancer Registry (NCR), published by the National Institute for Communicable Diseases (NICD), 85 302 new cancer cases have been reported in 2019 [3]. Of all cancers, breast, lung, prostate, colon, rectum, bladder, kidney, renal, pelvis, pancreatic, thyroid and liver cancer are most common [4].

Cancer is normally treated with radiotherapy or chemotherapy and tumours are surgically removed. Less severe cancers are treated with hormone-, immune- and antibody-based therapies [5]. Most of these treatments, however, do not discriminate between normal cells and cancer cells, and vary concerning the ability to infiltrate tumours. Patients that receive radiotherapy or chemotherapy complain of adverse side effects such as flu-like symptoms, heart problems, diarrhoea, nausea, lockjaw (trismus) and chronic bladder spams [6–9]. Radiotherapy of the neck and head may elicit difficulty in swallowing (dysphagia), dry mouth feel (xerostomia), necrosis, inflammation of the spinal cord, and even permanent trismus or neurological damage [6,10]. Approximately 90% of patients with advanced cancer experience severe pain after surgery [11] and need to take opioids. This may lead to drug abuse [12]. In some cases, cancer cells have developed resistance to conventional treatments [13], which emphasizes the need to search for alternative treatments and anticancer drugs. This led to the search for anticancer compounds produced

by plants, marine organisms, fungi, algae, and bacteria [14]. Several toxins, antibiotics, bacteriocins, non-ribosomal peptides, polyketides, phenylpropanoids, phenylflavonoids, purine nucleosides, short chain fatty acids (SCFAs) and enzymes with anticancer properties, mostly produced by bacteria, have been described [15]. The challenge is to identify compounds that only target cancer cells and not normal cells.

Cancer cells differ from normal cells by having more fluidic cell membranes [16], a net negative charge (due to elevated levels of phosphatidylserine, O-glycosylated mucins, sialylated gangliosides and heparin sulfates), and more microvilli, thus a larger surface area [17–20]. Because of these characteristics, cationic peptides such as bacteriocins may find it easier to adhere to cancer cells than normal cells [21,22].

The aim of this review is not to discuss various cancers and symptoms, nor the advantages and disadvantages of conventional therapies, but to provide the reader with the latest developments in the use of bacterial toxins, antibiotics, bacteriocins, non-ribosomal peptides, polyketides, phenylpropanoids, phenylflavonoids, purine nucleosides, SCFAs and enzymes in cancer treatment. The possibility of using lactic acid bacteria or their bacteriocins as anticancer agents is investigated.

2. Bacterial-Mediated Cancer Therapy

Experiments with viable or attenuated microorganisms to treat cancer dates back more than a century [7,23,24]. The first cancer vaccine, composed of viable *Streptococcus pyogenes* cells, was developed by Dr Coley in 1891 [23–25]. The cells activated macrophages and lymphocytes, and stimulated production of tumour necrosis factor α (TNFα) that regulates inflammatory responses required to attack malignant neoplasm [26,27]. The "Coley-toxin", that consisted of heat-treated culture supernatants of *S. pyogenes* and *Serratia marcescens*, was rejected by the USA Food and Drug Administration (FDA) in 1962 due to lack of unequivocal scientific support and reports of organ damage [23,28]. Subsequent reports of *Clostridium*, *Corynebacterium*, *Bacillus* Calmette-Guérin, *Salmonella*, *Escherichia coli*, *Bifidobacterium* and *Listeria* associated with cancer cells [29–33] led to renewed interest in the search for bacterial cells with anticancer properties, especially species autochthonous to the human gut. Several papers have been published advocating the use of *Salmonella* in cancer treatment.

Salmonella typhimurium VNP20009 was made less toxic by deleting *msbB* encoding lipopolysaccharide (LPS) production [34]. By deleting *purI* the strain became auxotrophic for adenine [35]. In a later study, King et al. [36] engineered strain VNP20009 to express cytosine deaminase (CD) that converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Fluorouracil, commercially known as Adrucil, is a cytotoxin used in treatment of colorectal, oesophageal, stomach, pancreatic, breast, and cervical cancers [37]. In murine models 5-FU formed at tumour sites, resulting in a dramatic repression of cell growth [36,38]. A CD-expressing strain of *Salmonella enterica* yielded similar results when tested in mice [39], raising the hope that engineered strains of *Salmonella* may be used to activate cytotoxic drugs within tumour cells. Another strain of *S. typhimurium* (TAPET-CD, also referred to as VNP20029) expressed genes encoding CD and colonized tumour cells for at least 15 days. The strain converted 5-FC to 5-FU in 2 of the 3 patients treated [40]. No reports of follow-up clinical trials with any of these strains have been reported.

Inducible promoters responding to specific conditions in tumour cells (e.g., hypoxia) were used to design delivery systems for anti-cancer treatment [41]. The hypoxia-inducible promoter (HIP) and a fluorescent marker were cloned into *S. typhimurium* VNP20009 [42]. A 15-fold increase in fluorescence was recorded in HCT116 human colorectal carcinoma cells. Non-cancerous cells did not fluoresce [42], which suggested the *Salmonalla* delivery vector is very specific. Other HIPs experimented with included those regulating the expression of *pflE* and *ansB* [43]. Swofford et al. [44] transformed the luxI/luxR quorum-sensing system of *Vibrio fischeri* into *S. typhimurium* VPN200010. These reporter genes were expressed in 4T1 mammary tumours, but only when cell densities were above a certain threshold.

Other strains of *S. typhimurium* tested for anti-cancer therapy included strain AR-1, an arginine and leucine auxotroph defective in ppGpp synthesis (strain ΔppGpp) [45], and strain SF200 with mutations in lipid A and flagella synthesis [46]. Clinical trials on metastatic cancer patients intravenously injected with maximum tolerable cell numbers of strain VNP20009 (3×10^8 cfu/m^2) proved ineffective [47]. Higher cell numbers led to anaemia, low platelet counts, bacteraemia, high blood bilirubin, nausea, vomiting, diarrhoea, hypophosphatemia, and an increase in alkaline phosphatase [47]. A genetically engineered strain of VNP20009 that produced TNF-related apoptosis-inducing ligand (TRAIL) under control of a γ-irradiation-inducible RecA promoter, stimulated caspase-3-mediated apoptosis in 4T1 mammary carcinoma cells [48]. *S. typhimurium* ΔppGpp that expressed tissue inhibitor of metalloproteinases 2 (TIMP-2) reduced the size of glioma brain tumours in BALB/c mice and increased survival by 60% [49]. Modification of *S. typhimurium* ΔppGpp to express the mitochondrial targeting domain of Noxa (MTD), fused with the cell-penetrating peptide DS4.3 (DS4.3-MTD), led to the complete destruction of colon carcinoma tumours [50]. An engineered strain of *Salmonella choleraesuis* that expressed the angiogenesis inhibitor thrombospondin-1 inhibited the growth of B16F10 melanoma cells in mice [51]. No clinical trial data have been published on any of these genetically engineered strains.

A strain of *S. typhimurium* genetically engineered to produce truncated human interleukin-2 (SalpIL2), reduced adenocarcinoma metastases of the liver [52–55]. Sorensen et al. [56] reported that metastatic osteosarcoma in a mouse model could be treated with a single oral dose of attenuated SalpIL2-producing *S. typhimurium*. Barnett et al. [57] have shown that SalpIL2 reduced the volume and mass of retroperitoneal neuroblastoma tumours in a murine model. In vitro experiments have shown that SalpIL2-producing strains invade and divide within K7M2 osteosarcoma cells [58], suggesting that SalpIL2 may persist for long periods in malignant tissue.

Significant lysis of tumor cells in mice were recorded with *Clostridium histolyticum* treatment [58]. Shrinking of tumor cells were observed when these cells were exposed to *Clostridium tetani* [59]. *Clostridium novyi*, made non-pathogenic by deleting the gene encoding α-toxin NT, destroyed tumours and secreted liposomase [60]. The latter has been experimented with in enhancing the release of liposome-encapsulated drugs within tumours [61]. Endospores of *C. novyi*-NT colonized the hypoxic regions of tumours, elicited cell lysis and an immune inflammatory response that resulted in immunogenic cell death [60,62]. In a phase I study, injection of *C. novyi*-NT into tumour cells led to a decrease in the size of the cells, resulted in a systemic cytokine response and enhanced systemic tumor-specific T-cell responses [63]. Bettegowda et al. [64] reported a long-term remediation effect when tumours in a mice model were treated with a combination of *C novyi*-NT endospores and radiation therapy. The authors suggested that a combination of radioactive iodine with *C novyi*-NT might enable patients to be treated with lower doses of radiolabeled antibodies, which limits injury to normal tissue such as bone marrow.

Nuyts et al. [65] has shown that the *recA* and *recN* genes in *Clostridium acetobutylicum* DSM792 could be activated with a radiation dose of 2 Gy. The authors argued that the activation of the recA promoter could increase TNFα production in recombinant clostridia. In a later study, Jiang et al. [66] showed that *E coli* K12, harbouring plasmid pAClyA, produced higher levels of cytolysin A, which enhanced the therapeutic effects of radiation.

Cloning the genes encoding nitroreductase from *E. coli* to *Clostridium beijerinckii* resulted in the activation of CB1954 (nontoxic) into a drug with anticancer properties. Intravenous injection of activated CB1954 into mice destroyed tumours [67]. *Clostridium* spp. engineered to deliver CD to tumours [68] have also been modified to secrete TNFα [69] and may, in future, be used in cancer therapy. Li et al. [70] suppressed the growth of Heps mouse liver cancer cells in vivo by using a genetically engineered strain of *Bifidobacterium adolescentis*. *Bifodobacterium* spp. have also been used to deliver active CD enzymes to hypoxic regions of solid tumours in mice [71–74].

The first report using *Listeria monocytogenes* to direct an immune response to tumours was published by Pan et al. [75]. The authors have genetically engineered *L. monocytogenes*

to secrete influenza virus nucleoprotein and have shown that the protein could repress tumours in colon and renal cancer models. Several other model studies were published using recombinant strains of *Listeria* to repress cervical, head and neck, breast, skin, and renal cancers; reviewed by Guirnalda et al. [76] and Cory and Chu [77]. An immunotherapy-based treatment for cervical cancer was developed based on live attenuated *L. monocytogenes* that secretes the fusion protein *Lm*-LLO-E7 [78]. The protein, referred to as ADXS11-001 (ADXS-HPV), targets human papillomavirus (HPV)-associated tumours. The immune response elicited by the ADXS11-001 vaccine against HPV oncoprotein E7 led to the reduction in tumour cells in animal models. Phase I and II clinical studies were later conducted [79–81]. Clinical trials are being conducted to evaluate another genetically engineered vaccine, protein ADXS-504, for treatment of biochemically recurrent (early) prostate cancer (https://tinyurl.com/2p8zcac7; https://tinyurl.com/yunwkcdx, assessed on 15 August 2022).

E. coli Nissle 1917 was genetically engineered to convert NH3 to L-arginine (L-arg). The recombinant strain, referred to as SYNB1020, reduced systemic hyperammonemia in mouse models [82]. A phase 1 clinical study showed that SYNB1020 was well tolerated at daily doses of up to 1.5×10^{12} cfu (colony-forming units) administered for up to 14 days. An increase in urinary nitrate, plasma ^{15}N-nitrate and urinary ^{15}N-nitrate was reported, suggesting that SYNB1020 could be used to treat hyperammonemia, including urea cycle disorders and hepatic encephalopathy [82]. Another genetically engineered strain of *E. coli* Nissle 1917, strain SYNB1618, yielded promising results when tested for the ability to alleviate phenylketonuria (PKU), a disorder caused by defective phenylalanine hydrolase, thus the inability to convert phenylalanine (Phe) to tyrosine [83]. Dose-responsive increases were observed in plasma (trans-cinnamic acid) and urine (hippuric acid) levels of Phe metabolites, suggesting that genetically engineered *E. coli* may be used in the treatment of rare metabolic disorders [83].

Intestinal bacteria influence various inflammatory and immune processes, many of which are implicated in tumour etiology, such as in colorectal cancer (CRC) [84]. *Bacteroides fragilis* and *Fusobacterium* spp. are directly associated with tumours, including CRC [85,86]. *Fusobacterium nucleatum* suppresses the immune response that leads to the induction of chronic inflammation [85]. *Bacteroides fragilis* alters (damages) the DNA of host cells, increases cell proliferation, and induces pro-inflammatory processes through the production of toxins [86]. A gene encoding *B. fragilis* toxin detected in colonic mucosa is associated with late-stage CRC [86]. Further research is required to determine if these species could be genetically modified to prevent the proliferation of cancer cells.

Although gut microbiota may prevent CRC, they also pose a risk of inducing CRC. This is mostly diet related. High levels of secondary bile acids (BAs) are produced from a high fat content diet [87,88]. Abnormal high levels of BAs in the colon induces inflammation [89,90] and forms reactive oxygen species that disrupts cell membranes and mitochondria [88]. Species primarily responsible for production of BAs are *Clostridium scindens, Clostridium hiranonis, Clostridium hylemonae* and *Clostridium sordellii* [91]. A diet rich in proteins and low in carbohydrates my also cause CRC, as reported by Russel et al. [92]. Fermentation of proteins in the distal colon leads to the production of toxic ammonia, amines, phenols and sulfides [93]. Lithocholic acid (LCA), a derivative of cholic acid, is an exception to the rule, as it inhibits the growth of human prostate cancer cells LNCaP and PC-3 by induction of caspase-3, 8 and 9 mediated apoptosis [94]. LCA not only induces endoplasmic reticulum (ER) stress that triggers the unfolded protein response (UPR) activating cell death [95], but transforms growth factor-β in HepG2 liver cancer cells, and suppresses the growth of breast cancer cells [96]. In addition, LCA induces oxidative phosphorylation, inhibits epithelial-mesenchymal transition and expression of vascular endothelial growth factor A, and stimulates antitumor immunity [96].

The anticarcinogenic properties of lactic acid bacteria (LAB) is addressed in far fewer publications and focuses mainly on exopolysaccharides (EPS), peptidoglycan, nucleic acid, bacteriocins, and S-layer proteins [97]. Viable cells of *Lactobacillus casei* BL23, intranasally

administered using the human pappilomavirus (HPV)-induced model, reduced tumour growth [98]. *Lactobacillus reuteri* BCRC14652, tested in vitro, damaged the cell membranes of colon carcinoma HT29 cells [99], suppressed tumor necrosis factor (TNF)-induced NF-κB activation, and repressed the growth of cancer cells by apoptosis [100]. EPS produced by *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* repressed the growth of HT-29 cells by inducing the activity of Beclin-1 (an autophagy protein) and GRP78 (an endoplasmic reticulum chaperone) directly, and indirectly by regulating apoptosis through stimulation of *Bcl-2* (B-cell lymphoma 2) and *Bak*, a pro-apoptotic gene of the Bcl-2 family [101]. A combination of *L. acidophilus* and *L. casei*, used with 5-FU, induced apoptosis of LS513 cancer cells [102], suggesting that these species may be used as adjuvants in anticancer chemotherapy.

Anti-tumor activities were also reported for cell-free supernatants of LAB, and irradiation-inactivated and heat-killed cells of LAB [99,102–105]. Exopolysaccharides (EPS) produced by *L. casei* 01 reduced the cytotoxicity of 4-nitroquinoline N-oxide (4-NQO), a pro-mutagen [106]. EPS isolated from *L. acidophilus* 606 repressed the growth of cancerous cells [103] and EPS from *Lactobacillus plantarum* and *L. acidophilus* significantly reduced tumour growth [107–109]. An interesting observation is that the repression of tumour growth exerted by an EPS-producing strain of *L. acidophilus* (strain LA1) may be associated with the suppression of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). Inhibition of LDH in the glycolytic pathway of cancer cells results in lower ATP production, hence slower growth [110,111]. EPS116, produced by *L. plantarum* NCU116, binds to TLR2 and activates the TLR2/MyD88/TRAF6/MKK7 pathway, which, in turn, activates JNK/c-Jun that upregulates the transcription and translation of *Fas* and *Fasl*. The Fas/Fasl signaling pathway activates FADD of caspase-8 and caspase-3. Activated Caspase-3 facilitates apoptosis by upregulating the expression of cellular target proteins PARPs and Rock1, followed by cleavage of PARP1 and inhibition of CT26 growth [112]. Many probiotic LAB produce EPS and may, in future, be used as alternative or complementary treatment of cancer. The anticancer properties of EPS are reviewed by Wu et al. [113].

Several reports highlighted the importance of LAB in the prevention of CRC, reviewed by Zhong [114]. The health and quality of life of patients that underwent surgical resection of CRC were significantly improved when administered *L. acidophilus* and *Bacillus natto* [115]. Despite this, there is no consensus on the role LAB play in CRC treatment. It is, however, certain that a select few LAB activate mechanisms involved in the killing or repression of cancer cells, and that they regulate immune response [116]. This includes neutralizing free radicals [117] and inactivation of reactive oxygen species (ROS) by NADH oxidase/peroxidase and catalase [118,119]. Strains of *Bifidobacterium longum* and *L. acidophilus* displayed antioxidative activity by inhibiting linoleic acid peroxidation [120]. Heat-killed cells of *L. acidophilus* 606 and EPS produced by the strain has potent antioxidative activity [103]. According to Kumar et al. [121] and Annuk et al. [122], obligate homofermentative lactobacilli display high antioxidant activity, but is highly strain-dependent among facultative and obligate heterofermentative lactobacilli.

LAB play an important role in stimulating the immune system, especially anti-inflammatory cytokine IL-10 [123]. Lipoteichoic acid, present in the cell walls of all LAB, stimulate DCs through Toll-like receptor 2, resulting in the release of cytokines [124,125]. Some lactobacilli stimulate DCs to produce IL-12 and IL-10 [126,127]. Disruption of LTA in *L. acidophilus* resulted in the production of IL-10 by DCs and the downregulation of IL-12, which led to T cell-mediated colitis in mice [128]. It thus seems possible to treat CRC by altering the cell surface components of *L. acidophilus*. A strain of *L. acidophilus* deficient in LTA (strain NCK2025) repressed the growth of colonic polyps by downregulating IL-12, TNF-α and IL-10. This activated CD4+ T-cells, as observed in a mice model [129]. In vivo studies have shown an increase in cytoplasmic levels of TNF-α, interferon-γ (IFN-γ) and IL-10 in animals administered *L. casei* and *B. longum* [130]. This correlated with an increase in T cells, NK cells and MHC class II^+ cells, and $CD4\text{-}CD8^+$ T cells in a murine model [131]. *L. casei* Shirota (LcS) suppressed chemically induced carcinogenesis [131], supported by an increase in IFN-γ, interleukin-β (IL-1 β) and TNF-α levels [132]. A butanol extract prepared

from the cell-free supernatant of *B. adolescentis* significantly increased the production of TNF-α and NO, which regulated immune modulation and repressed tumor growth [133].

Messenger RNA (mRNA) and interferon gamma (IFN-γ) levels of leukemia KHYG-1 cells increased when treated with a combination of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *L. Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *L. plantarum*, *Leuconostoc mesenteroides* subsp. *cremoris*, and *L. casei* [134]. The six LAB enhanced the cytotoxicity to human chronic myelogenous leukemia K562 cells and colorectal tumor HCT116 cells [134]. *L. reuteri* ATCC-PTA-6475 reduced the growth of mammary tumors in Swiss mice by blocking NFκ-B-p65 nuclear translocation and the expression of c-jun, an oncogenic transcription factor [135]. Phenyllactic acid (PLA), hydroxyphenyllactic acid (OH-PLA), lactic acid, and indole lactic acid (ILA) produced by *L. plantarum* UM55 inhibited the growth of *Aspergillus flavus* and thus production of carcinogenic aflatoxins in food products [136].

Peptidoglycan isolated from *Bifidobacterium infantis* ATCC 15,697 repressed the growth of Meth A fibrosarcoma in BALB/c mice [137]. A cell wall-derived polysaccharide-peptidoglycan complex (PSPG) from *L. casei* Shirota prevented the activation of IL-6/STAT3 signalling and repressed ileal cancer [138]. Peptidoglycan from *Lactococcus* and *Bifidobacterium* inhibited the growth of bladder cancer HT-1376, colon cancer DLD-1 and SNUC2A cells, and kidney cancer A498 cells [139]. S-layer proteins from *L. acidophilus* CICC 6074 up-regulating the expression of p53, p21, and p16 and down-regulated the expression of CDK1 (cyclin-dependent kinase) and cyclin B in colon cancer HT-29 cells [140].

RNA extracted from the cell-free supernatant of *Lactobacillus* DM9811 inhibited the growth of colon cancer HT-29 cells and mouse ascites hepatoma cells [141]. The authors ascribed the anticarcinogenic activity to increased activity of NK and $CD4^+$ T cells and the upregulation of cellular immunity. DNA fragments of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* promoted mitosis in mouse spleen B and Pierre spot cells, resulting in enhanced immune functions [141]. These studies may be the first towards nucleic acid-based vaccines in cancer therapy.

Probiotic LAB and bifidobacteria inhibit signaling of epidermal growth factor receptor (EGFR) pathways [142], leading to an increase in phosphorylation of cytoplasmic tyrosine kinase domains. This causes the activation of cell proliferation, apoptosis, migration and differentiation [143].

Jackson Laboratory (JAX) mice, known to be easily colonized by commensal microbes, had higher cell numbers of *Bifidobacterium* in their colons. These mice showed reduced growth of skin cancer cells and had higher levels of antitumor cytotoxic T lymphocytes (CTL). Species linked to antitumor immune responses were *Bifidobacterium breve*, *B. longum* and *B. adolescentis* [144]. Mice devoid of these species recovered from melanoma and showed an increased in tumour specific CTLs when administered *B. breve* or *B. longum* [144]. Once administered, bifidobacteria proliferate in the nutrient-rich environment created by cell death and necrosis [144]. The specific mechanism by which bifidobacteria or other intestinal bacteria stimulate antitumor immune responses is unknown. They may stimulate the maturation of dendritic cells (DCs) that, similar to antigen-presenting cells (APC), play a role in T-cell activation. *B. longum* BB536 stimulate the development and maturation of interferon γ (IFN-γ) secreting cells. Newborn infants showed an increase in the ratio of IFN-γ/IL-4 secreting T helper (Th) cells (Th1/Th2) when they received *B. longum* BB536 [145].

Enterococcus faecalis downregulates the expression of the FIAF (angiopoietin-like protein 4) gene associated with the development of some cancer types [146]. In a mouse model of ulcerative colitis, *E. faecalis* inhibited inflammation by suppressing T helper (Th)-1 and Th17 responses [147]. Heat-killed cells of *E. faecalis* YM-73 enhanced immune modulation by increasing Th1 and reducing Th2-associated cytokines [148].

Gut bacteria could also be used to reduce normal tissue damage during or after radiotherapy. Several studies have shown that probiotic strains of *Lactobacillus* and *Bifidobacterium* reduce radiotherapy side effects [149–153].

LAB are of paramount importance in the prevention of CRC. Insights into the cellular and molecular mechanisms which include apoptosis, antioxidant, immune responses, and epigenetics opened the door for the development of novel therapeutic approaches. Although a wide range of studies has shown the remarkable potential of LAB strains in interfering with colorectal carcinogenesis, conclusive clinical evidence supporting the role of probiotics in CRC treatment is still lacking. More epigenetic studies on LAB are required to demonstrate their effects in cancer prevention. Although several mechanisms of action of LAB in carcinogenesis have been described in in vitro and animal model studies, we are still far from pinpointing the exact cellular signals.

3. Bacterial Toxins

Bacterial toxins with medicinal applications have been well studied, especially those produced by *Bacillus thuringiensis* [154], *Clostridium* spp. and *Bacillus* spp. [155]. Botulinum neurotoxin (BoNT), produced by *Clostridium botulinum*, is widely used in ophthalmology, dermatology, and neurology [156]. To date, 108 clinical trials have been registered to evaluate the anticancer properties of bacterial toxins and an additional 98 trials have been registered to study the anticancer properties of immunotoxins (https://www.clinicaltrials.gov/, accessed on 1 June 2022).

3.1. Diphtheria Toxin

In 1884, Loeffler injected a pure culture of *Clostridium diphtheriae* into rabbits and pigeons and described the formation of lesions in several organs [157]. Follow-up studies have shown that these lesions were caused by diphtheria toxin (DT), encoded by the *tox* gene located on the genome of corynebacteriophage β [157,158]. The toxin targets elongation factor II (aminoacyl transferase II) and inhibits peptide synthesis [159,160]. The mode of action of DT is summarised in Figure 1.

In many human cancer cells, including hepatocarcinoma, melanoma, and colon, breast, myeloma, prostate, bladder and oral tumours, the *HBEGF* gene, encoding the membrane-anchored precursor of proHB-EGF, is significantly upregulated [161]. Increased levels of proHB-EGF and HB-EGF have been implicated in resistance to chemotherapeutic agents [162]. Cross-reactive material 197 (CRM197), a non-toxic variation of DT, binds to pro-HB-EGF and HB-EGF and inhibits the mitogenic action of HB-EGF by preventing its binding to ErbB (epidermal growth factor) receptors [163]. CRM197 has been used in the treatment of oral squamous cell carcinoma [161]. Tumour formation was completely inhibited in vivo when CRM197 was used in combination with cisplatin, a chemotherapeutic drug [164]. In humans, CRM197 led to an increase in neutrophil and TNF α levels, and a decrease in lymphocyte numbers. The drug, also referred to as BK-UM, proved effective in the treatment of resistant cancers [162,164]. Nam et al. [164] tested the efficacy of intravenously administered BK-UM against the triple-negative breast cancer cell line MDA-MB-231. The size of tumours treated with BK-UM decreased after two weeks. Furthermore, no adverse side effects such as weight loss were reported. In phase I clinical studies, BK-UM was tested against recurrent ovarian and peritoneal cancer cells [162]. Patients were administered four dose levels (1.0, 2.0, 3.3 and 5.0 mg/m^2), of which 2.0 mg/m^2 was the most effective. Those treated with 3.3 mg/m^2 complained of nausea, hypotension, fever, and irritation of the peritoneum [162]. A truncated version of DT, in which the cell receptor-binding domain was replaced by proteins that selectively binds to the surface of cancer cells (referred to as DT385), inhibited angiogenesis and decreased tumour growth in chick chorioallantoic membranes [165]. DT385 also inhibited the growth of Lewis lung carcinoma (LLC) tumours in mice models [165]. Although the results were promising, DT385 entered cells without its R-domain, which triggered non-specific toxicity. To circumvent the problem, immunotoxins that target specific receptors on cancer cells and biochemical processes were developed and approved by the FDA in 1978 [166].

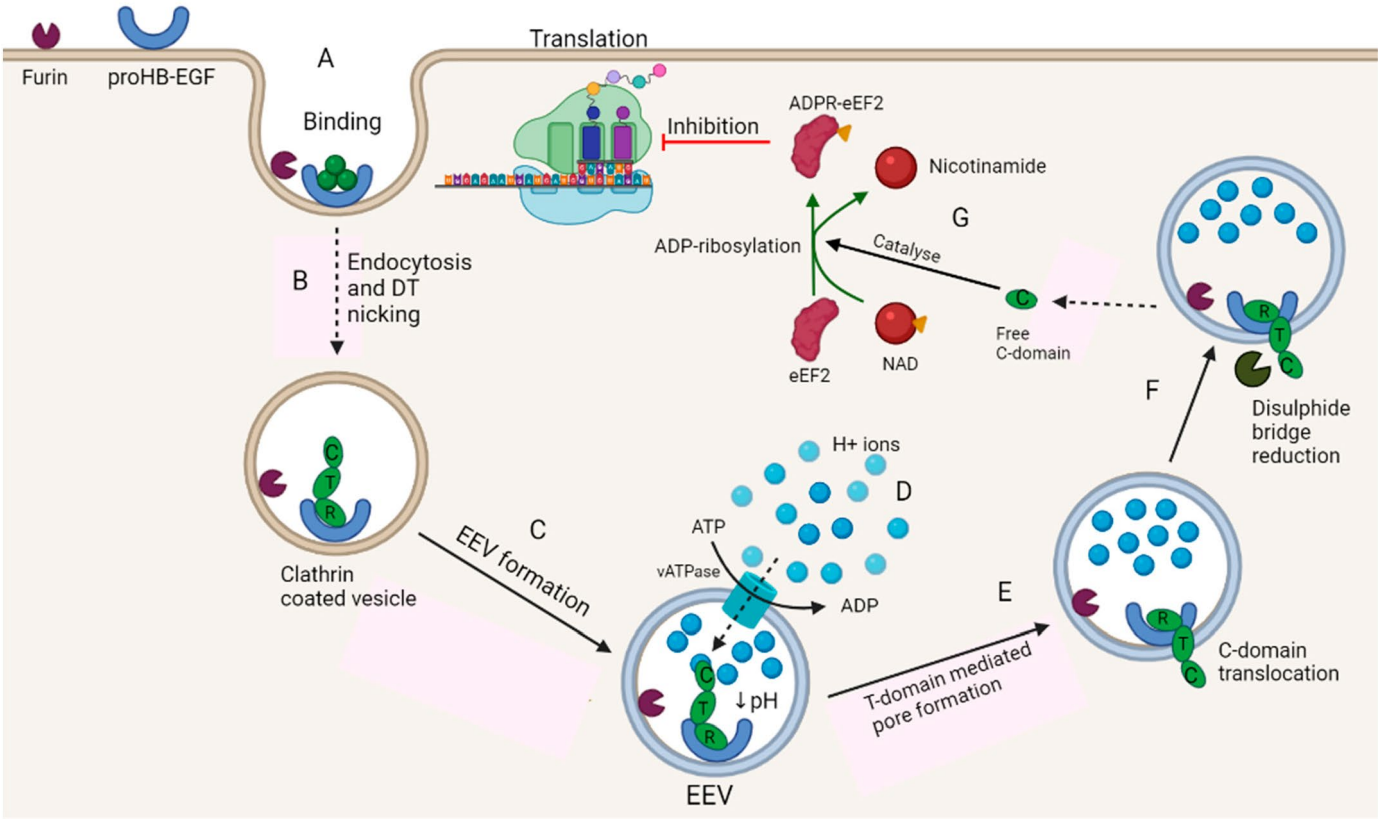

Figure 1. Mode of action of diphtheria toxin (DT). (A) The receptor (R domain) of DT, shown here as green speres, binds to the membrane-anchored precursor of heparin-binding epidermal-like growth factor (proHB-EGF). (B) DT is nicked and the DT-HB-EGF enters a clathrin-coated vesicle through endocytosis. Furin or furin-like proteases converts DT to mature form. (C) An early endosomal vesicle (EEV) is formed by replacing clathrin proteins with the GTPase Arf-1 and coat protein COPI (not shown). (D) EEV is acidified by the transport of protons (H^+) across the membrane, instigated by vacuolar adenosine triphosphatase (vATPase). (E) The T-domain is translocated across the membrane, exposing the C-domain to the cytosol. (F) The disulphide bridge is reduced to liberate the catalytic C-domain. (G) The free C-domain catalyses the ADP-ribosylation of eukaryotic elongation factor 2 (eEF2) to ADPR-eEF2, which inhibits translation. This illustration was made using BioRender (https://biorender.com/, accessed on 12 May 2022).

First-generation immunotoxins developed were antibodies randomly linked to non-binding toxins or derivatives thereof [167]. These immunotoxins were non-specific and targeted several receptors on cell surfaces. Second-generation immunotoxins were without cell surface-receptor domains and were much larger, which made penetration of solid tumours more difficult. Both generations of immunotoxins caused severe side effects, such as vascular leak syndrome, haemolytic uremic syndrome and pleuritis [166]. Progress in recombinant DNA technology led to the development of third-generation immunotoxins with less side-effects and improved tumour-penetrating properties. In these immunotoxins the non-specific receptor-binding domain is replaced by the Fv domain of an antibody, either genetically or chemically. Despite being more specific in the targeting of cancer cells, third-generation immunotoxins were not effective in patients vaccinated against diphtheria [168]. Attempts to suppress immune responses with anti-monoclonal antibodies [169] and binding to polyethylene glycol (PEGylation) were unsuccessful [170]. This led to the developing of humanized immunotoxins, i.e., immunotoxins containing a human protein with anti-cancer properties [168]. The first FDA approved humanized immunotoxin, *Denileukin Diftitox* (Ontak, DAB389 IL-2, Eisai Medical Research, Inc., Tokyo, Japan), was constructed by fusing human interleukin-2 (IL-2) with fragment A of DT [171]. IL-2 was genetically fused

to the first 388 amino acids of DT, thereby replacing the R-domain [167]. Success rates with Ontak ranged from 30% to 50% [172]. Side effects reported were nausea, diarrhoea and vascular leak syndrome [173]. Despite difficulties encountered in purification of the first recombinant protein expressed by *E. coli* [173], several humanized immunotoxins were developed, all based on DT (Table 1).

Table 1. Humanized immunotoxins developed from fusion to diphtheria toxin (DT).

Immunotoxin	Toxin/Fragment	Targeting Moiety	Cancer or Cell Line	Result	Reference
Ontak	DT_{389}	IL-2	Adult T-cell leukaemia and CTCL	Significant activity. FDA approval for CTCL treatment	[171]
mVEGF-DT	DT_{386}	mVEGF	TC1-induced solid tumour	Tumour regression and an increase in survival rate	[174]
DTAT	C- and T-domains of DT	N-terminal of uPA	Glioblastoma cells	Selective killing and regression in tumour growth	[175]
DT_{386}-BR2	DT_{386}	Buforin II (BR2)	MCF-7 and HeLa cells K-562	Specific and significant reduction in survivability and apoptosis	[176,177]
Tagraxofusp (Elzonris™)	DT_{388}	IL-3	BPDCN, AML, CMML, & MM	FDA approval for BPDCN treatment	[178]
DT_{389}GCSF	DT_{389}	GCSF	HL-60	Specific apoptotic death and nuclease activity	[179]
hDT_{806}	DT_{390}	HuBiscFv806	4 HNSCC cell lines	Apoptosis, tumour size reduction and EGFR signalling disruption	[180]
PD1-DT	DT_{386}	PD1	C57BL/6 tumorous mice	67% decrease in tumour volume	[181]
DT_{389}-YP7	DT_{389}	hYP7 scFv	HepG2 HCC	Decreased cell viability and specific toxicity	[182]

CTCL: cutaneous T-cell lymphoma; mVEGF: mouse vascular endothelial growth factor; DTAT: DT fused to the amino (N)-terminal of uPA; uPA: urokinase-type plasminogen activator; BPDCN: blastic plasmacytoid dendritic cell neoplasm; AML: acute myeloid leukaemia; CMML: chronic myelomonocytic leukaemia; MM: multiple myeloma; GCSF: granulocyte colony-stimulating factor; HuBiscFv806: humanized bivalent single-chain variable fragment of monoclonal antibody 806; HNSCC: head and neck squamous cell carcinoma; EGFR: epidermal growth factor receptor; PD1: programmed cell death protein-1; hYP7 scFv: humanized YP7 single-chain variable fragment; HCC: hepatocellular carcinoma.

3.2. *Clostridium perfringens* Enterotoxin

Clostridium perfringens enterotoxin (CPE) is a polypeptide of 319 amino acids [183], arranged in three domains. Domain I represents the binding domain (residues 162 to 309) and domains II and III the cytotoxic domains [184,185]. CPE is produced intracellularly and is only released during endospore germination [183]. Mutations in the TM1 region of CPE (amino acids 81 to 106) resulted in loss of membrane insertion, indicating that it plays a role in pore formation. The mode of action of CPE is illustrated in Figure 2. The amphipathic TM1 region forms a β-hairpin and a β-barrel pore once inserted into the membrane [186]. Amino acids at positions 45 to 53, located upstream of the TM1 region, are responsible for CPE oligomerization [187]. Domains II and III consist of eight β-sheets (two of which span the entire length of the module), two α-helices, and two 3_{10} helical segments [185]. Domain I is a nine-stranded β-sandwich [188]. Briggs et al. [189] reported only two domains. Despite the discrepancy in the number of domains, both studies agreed on the structure of CPE and that the N-terminal domain is divided into two sections.

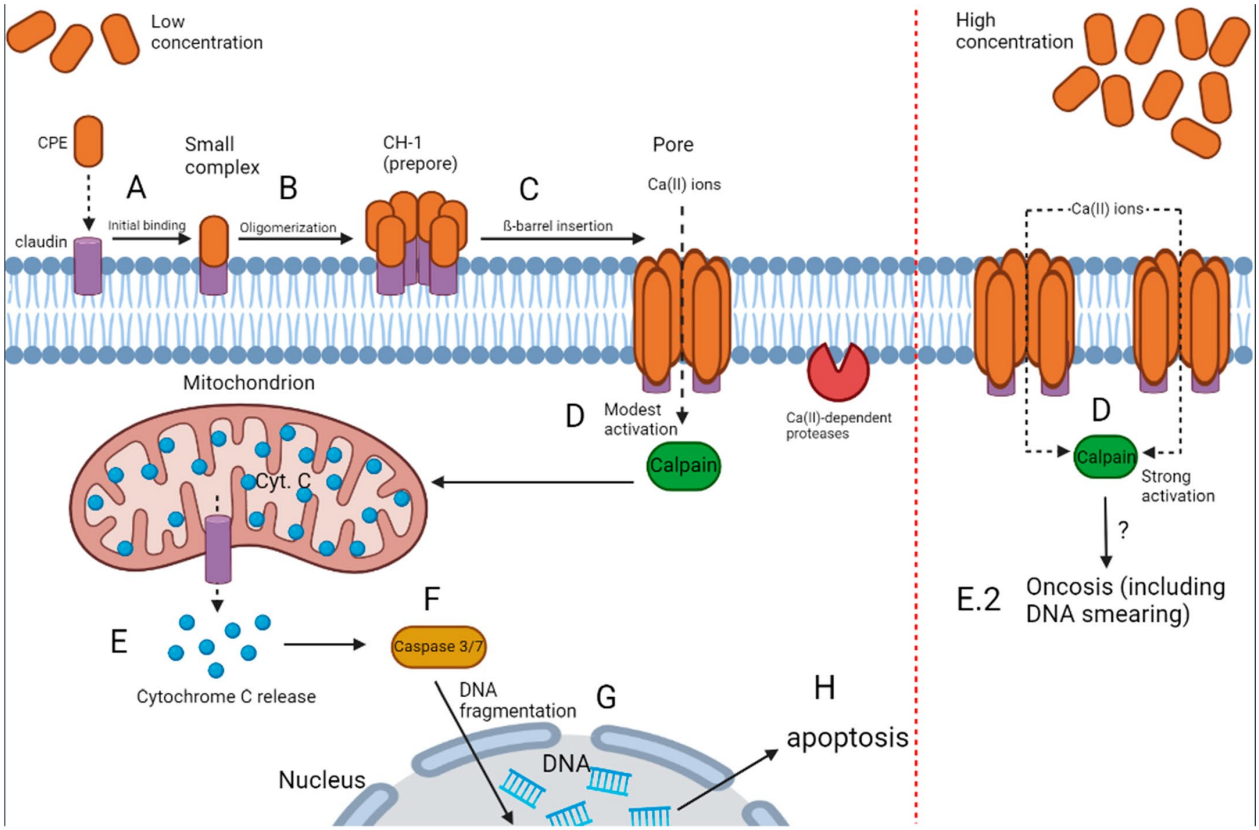

Figure 2. *Clostridium perfringens* enterotoxin (CPE) mode of action. (A) Tyrosine residues in the C-terminal of CPE interact with the second extracellular loop (ECL-2) of a receptor claudin (CLDN) and form small complexes. (B) Approximately six small complexes oligomerize to form a larger CPE hexamer 1 (CH-1; prepore). (C) β-hairpin loops of CPE assemble to form a β-barrel that inserts into the cell membrane to create a cation-permeating pore. (D) Influx of Ca^{2+} ions disrupts the osmotic equilibrium and activates Ca^{2}-dependent proteases to lyse the cell that forms calpain. (E) Release of cytochrome C from the mitochondrion. (F) Activation of caspase 3/7 and formation of a large new CH-2 complex (approximately 600 kDa, consisting of CLDNs, occludins and the CPE hexamer. (G and H) Apoptosis, leading to DNA fragmentation. This illustration was constructed using BioRender (https://biorender.com/, accessed on 12 May 2022).

CPE, encoded by genes α, β, ϵ and ι located on the genome or on a plasmid, are transcriptionally regulated [190]. The *spo0A* gene, encoding the master sporulation regulator Spo0A, in conjunction with NanR, a transcriptional regulator, and three sporulation-associated sigma factors (SigE, SigK and SigF), are responsible for CPE production and gene regulation [191]. Transcriptional regulation of CPE expression has not been fully elucidated. However, a proposed mechanism of regulation has been compiled from various studies [188,192,193]. An Agr-like quorum-sensing (QS) system phosphorylates Spo0A, which activates the transcription of SigF, SigG, SigK and SigE [190]. SigK and SigE are required for CPE production, as they bind promotors to the *cpe* open reading frame (ORF) [190]. Three promoters (P1-P3) are located upstream of the ORF, with P1 being SigK-dependent, and P2 and P3 SigE-dependent [194].

The β-hairpin loops inserted in the membrane of the target cell create a pore through which cations are channelled. CPE binds to claudins (CLDNs) 3, 4, 5, 6, 7, 8, 9, 14 and 19 [187,188,195,196]. CLDN 4 is localized at tight junctions in normal human prostate epithelial cells (PrECs) but are distributed along the entire surface of cancer PrECs [197]. The size of cancerous tumours treated with CPE was reduced by 59%, suggesting that CLDN 4-targetted CPE treatment may be used to treat prostate cancer. Abedi et al. [198] constructed a recombinant plasmid containing the CPE and prostate stem cell antigen

(PSCA) and named it pBudCE4.1-CPE-PSCA. The expression of transgenes introduced into cancer cells, referred to as suicide gene therapy, may be the first step towards developing a vaccine against prostate cancer. The authors [198] reported a 62.6% death rate of PC3 prostate cancer cells. Genes encoding apoptosis were overexpressed, whereas genes encoding cell cycling were repressed. The influx of Ca^{2+} ions activates Ca^{2}-dependent proteases and causes cell lysis. Cytochrome C is released from the mitochondrion and caspase 3/7 is activated, leading to apoptosis [13,187,188,199–201].

Pahle et al. [202] used suicide gene therapy to treat mice with colorectal cancer. The authors amplified cDNA of CPE by PCR from plasmid pCpG-optCPE to construct a translation-optimized CPE vector (optCPE) and fused the amplicon to genes encoding green fluorescent protein (GFP), resulting in pcDNA3-optCPE-GFP (optCPE-GFP). This construct, and recombinant CPR (recCPE) were used to transfect different cell lines, including CaCo-2 and HT-29, and isogenic Sk-Mel5 and Sk-Mel5 Cldn-3-YFP melanoma cell lines. Colon carcinoma cell lines that overexpressed CLDN 3 and 4 were highly sensitive to recCPE and optCPE, but cells transfected with optCPE displayed rapid cytotoxic effects such as membrane disruption and necrosis. This suggested that suicide gene therapy may be used to suppress colon cancer in cells overexpressing CLDN 3 and 4. Gabig et al. [203] compared the cytotoxicity of CPE against the chemotherapeutics Dasatinib (Das) and Mitomycin C (MMC) used in the treatment of bladder cancer. The cells were killed within one hour when exposed to CPE, compared to 24 h when treated with Das or MMC. Furthermore, after one hour of treatment, 75% of primary bladder cancer cells died (in a 3D culture). Normal cells and cells derived from highly aggressive tumours survived all treatments.

Despite the success of CPE with experimental models, its use in treatment of cancer is limited due to the abundance of CLDNs in normal cells [204]. Shim et al. [200] tested DOX-C-SNP (doxorubicin-loaded C-CPE-polysialic acid) nanoparticles against pancreatic tumour cells in vitro and in vivo and have shown that DOX-C-SNPs accumulated only in tumour cells, without displaying significant cytotoxicity towards non-target cells [200]. A 5.9-fold increase in apoptosis was recorded in orthotopic murine models. Gao et al. [204] reported a decrease in CLDN 4 expression when epithelial ovarian cells (EOCs) were treated with C-CPE and ascribed this to the disruption of TJ proteins. CLDN 4^{+} EOC cell lines were also more sensitive to chemotherapeutic agents, as shown with 59% suppression of tumour growth when cells were treated with a combination of C-CPE and Taxol [204]. Treatment with C-CPE also resulted in the upregulation of genes in the ubiquitin-proteasome pathway that regulates apoptosis and angiogenesis, and downregulated genes involved in metabolic pathways. Becker et al. [205] linked gold nanoparticles (AuNPs) to C-CPE to form a C-CPE-AuNP complex that targets CLDN-overexpressing cancer cells. The Strep-Tag Strep-Tactin fusion system developed by Becker et al. [205] could also be used to conjugate C-CPE to chromophores, thereby allowing imaging and detection of cancer cells. Photonic activation of the AuNPs, referred to as AuNP-mediated laser perforation (GNOME-LP), used in combination with C-CPE is highly specific and targets only $CLDN^{+}$ cells. A 30% and 40% reduction in cell viability was recorded for MCF-7 and OE-33 cells, respectively [205]. C-CPE-targeted GNOME-LP had no significant effect on the survival of cells in the control group. Gabig et al. [203] have shown that C-CPE treatment of RT4 (non-invasive superficial) cancer cells enhanced the toxicity of Das and MMC. Moreover, a drastic decrease in CLDN 4 expression was recorded, without affecting normal cells [203]. Nanoparticles loaded with fluorescent rhodamine dye and superparamagnetic iron oxide, linked to C-CPE ($CPE_{290–319}$) were used to target CLDN 3 and CLDN 4 in cancer cells [206]. This technique may be used to determine the aggressiveness of cancer tumours.

3.3. Botulinum Toxins

Botulinum toxins (BoNT), produced by *C. botulinum*, *Clostridium butyrricum*, *Clostridium barati*, and *Clostridium argentinensis* are used in the treatment of muscle disorders [207], anismus [208], tremors [209], dystonia [210], cancer [211], and severe pain [212]. The inactive single-chain polypeptide is nicked by a protease to form a di-peptide of 100 kDa and

50 kDa [207]. The light chain (LC) is located at the N-terminal and contains the catalytic domain (C-domain), whereas the heavy chain (HC) is divided into the central translocation (T) domain and the C-terminal receptor-binding (R) domain [213]. The toxin associates with non-toxic neurotoxin-associated proteins (NAPs) to form a 300 to 900 kDa protoxin resistant to stomach acid and improved ability to be translocated across the intestinal epithelial barrier [207]. Eight types of botulinum toxins (A, B, C1, C2, D, E, F, and G) and a novel serotype (BoNT/H), isolated from an infant with botulism, have been described [207,214]. The mode of action of BoNT is illustrated in Figure 3.

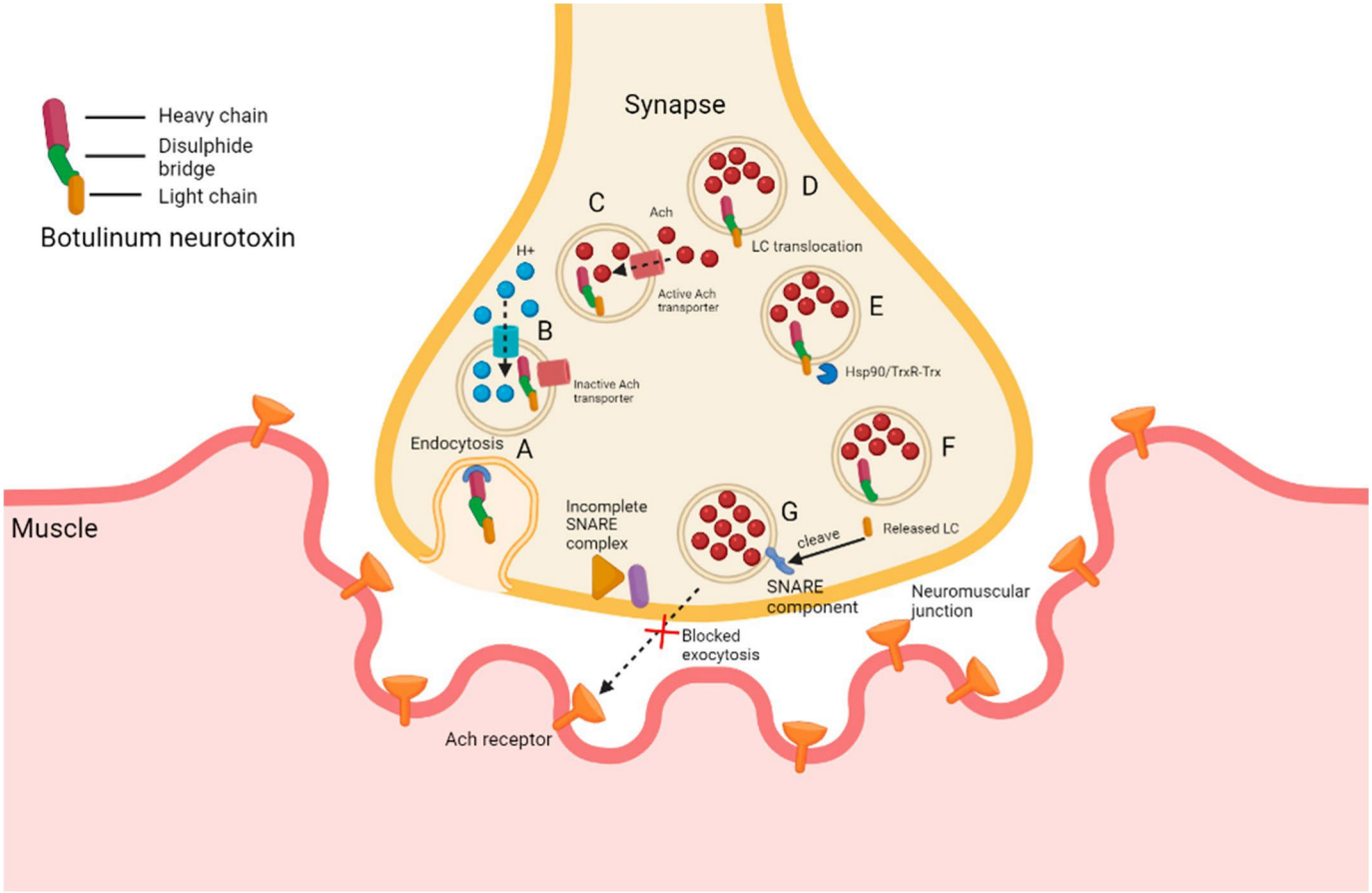

Figure 3. Botulinum neurotoxin (BoNT) mode of action. (A) BoNT binds to ecto-acceptors (polysialogangliosides) on the presynaptic cell surface of cholinergic neurons and is internalized via SV-2 or Syt-mediated endocytosis. (B) The synaptic vesicle is acidified with vesicular proton pumps, which in turn (C) activates Ach transporter proteins. The activated transporter proteins import acetylcholine (Ach) and the light chain (LC) of BoNT is translocated to the cytosol (D) with the heavy chain (HC) domain. (E) Heat shock protein 90 (Hsp90) and thioredoxin reductase-thioredoxin (TrxR-Trx) cleaves the LC and (F) liberates it into the cytosol. (G) The C-domain is a Zn^{2+}-dependent endopeptidase that cleaves proteins in the soluble N-ethylmaleimide-sensitive adaptor receptor (SNARE) protein complex. This complex is responsible for exocytosis and the fusing of acetylcholine-containing vesicles with the plasma membrane, allowing the release of acetylcholine. The cleaved SNARE component is non-functional, thereby blocking the release of acetylcholine from the presynaptic membrane to muscles. Blocking exocytosis of acetylcholine leads to failed skeletal muscle contracture. This representation was constructed using BioRender (https://biorender.com/, accessed on 12 May 2022).

BoNT binds to ecto-acceptors (polysialogangliosides) on the presynaptic cell surface of cholinergic neurons and is internalized via synaptic vesicles (SVs) or membrane-trafficking

proteins (synaptotagmins). Acidification of the SVs leads to the activation of acetylcholine (Ach) transporter proteins that import Ach, the LC and HC domains of BoNT. Heat shock protein 90 (Hsp90) and thioredoxin reductase-thioredoxin (TrxR-Trx) cleaves the LC and liberates it into the cytosol. The C-domain is a Zn^{2+}-dependent endopeptidase that cleaves proteins in the soluble SNARE protein complex. This complex is responsible for exocytosis and the fusing of Ach-containing vesicles with the plasma membrane, allowing the release of Ach. For more information BoNT mode of action, the reader is referred Nigam and Nigam [156], Gul et al. [213], Dolly et al. [215], Choudhury et al. [216] and Huang et al. [217].

Huang et al. [217] performed one of the earliest studies using BoNT/A to treat HIT-T15 insulinoma cells. Although the authors did not determine whether BoNT/A can be used to kill cancer cells, they showed that a transient transfection with the toxin can inhibit insulin expression. This paved the way for more in vivo and in vitro studies using BoNT. Treatment with BoNT/A render cancer cells radiosensitive [218] and may be used in the treatment breast and prostate cancer [211,219]. BoNT may also be used as an immunotoxin [220]. Toxin A induces apoptosis, inhibits the proliferation of LNCaP (infraclinical prostate cancer) cells, as shown with in vitro and in vivo studies [219]. In a separate study [221], toxin A showed cytotoxicity against cell lines LNCaP and PC-3 (prostate cancer), most probably due to the phosphorylation of phospholipase A2. Cell death of T47D breast cancer cells was attributed to the induction of caspase 3- and 7-dependent apoptosis [222]. Toxin C induced apoptosis and cell death in differentiated human neuroblastoma cells (SH-SY5Y and SiMa) [223]. Other cell-line anticancer studies performed with BoNT are listed in Table 2.

Table 2. Studies using BoNT as an anti-cancer agent.

Cancer or Cell Line	Study Type	Methodology	Results	Reference
VCap cells Cancerous human prostate	In vivo In vivo	OnaA injection into VCap cells transplanted into murine prostate OnaA injection into prostate before prostatectomy	Inhibited cancer progression and increased apoptosis Increased incidence of apoptosis	[224]
MIA PaCa-2 cells	In vivo	Co-injection of cancer cells and 20 U/kg BoNT, or BoNT injection followed by cancer cell injection (murine study)	Increase in apoptosis and a decrease in tumour size	[225]
SiMA and SH-SY5Y cell lines	In vitro	BoNT/C injection into retinoic acid-treated	Increase in apoptosis	[223]
3T3 fibroblast cells	In vitro	BoNT/A treatment	Cytoplasmic degradation and decreased cell viability	[211]
SCC-25 and HUVEC cells	In vitro	Cells grown in the presence of BoNT	No effect on cell growth	[226]
DBTRG glioblastoma cell line	In vitro	BoNT/A and BoNT/A + AMG	Increased apoptosis and decreased cell proliferation	[227]

Abbreviations: OnaA: OnabotulinumtoxinA (Botox); DBTRG: Denver Brain Tumour Research Group; AMG: transient receptor potential vanilloid 1 receptor antagonist.

BoNT inhibits the release of neurotransmitters [207] and may be used as a painkiller in cancer treatment. Van Daele et al. [6] were the first to report on the analgesic effect of BoNT/A. The authors treated patients with painful spams of the sternocleidomastoid muscle. Injection with BoNT/A relieved the pain in four of six patients. Wang et al. [228] administered BoNT/A to a lung cancer patient with Raynaud phenomenon and previously treated with chemotherapy. Conventional treatment of neoplasms is generally ineffective. However, after BoNT/A treatment patients reported relief in symptoms, with no adverse side effects. Incobotulinumtoxin A (INCO), a BoNT/A preparation used in clinical settings, has also been used in the treatment of cancer-related pain [12]. Twelve patients with head, neck and breast cancer were enrolled in the study. Two patients passed away due to

advanced cancer, one developed a skin rash, and another did not return due to poor general health. Three of the remaining eight patients reported an improvement in their quality of life. Pain amelioration was assessed using the Visual Analog Scale (VAS). All eight patients reported a significant improvement. A significant satisfaction of treatment was reported by seven of the eight patients by self-assessment using the Patients' Global Impression of Change Scale. Dana et al. [10] used Botox and Dysport to test the efficacy of BoNT/A in pain relief of neck and head cancer patients suffering from radiotherapy-induced trismus and masticator spasms. One month after BoNT/A injection, a significant improvement in pain and spams were recorded, but no improvement in trismus. No adverse side effects were observed and the authors concluded that BoNT/A may be prescribed to patients with radiotherapy-induced pain or muscle spasms. De Groef et al. [229] studied the effect of a single BoNT/A injection in conjunction with physical therapy on breast cancer patients that underwent a mastectomy. Of the 50 patients, 25 received the injection (intervention) and 25 a placebo. After three months of treatment, a significant change in pain was observed in the upper limb of patients from the intervention group. Other studies using BoNT/A in the treatment of cancer-related or cancer therapy-related pain that were successful included post-radiosurgical neck contracture [230], frontotemporal glioblastoma related pain [231], and postoperative pain in patients that underwent a mastectomy and tissue expander reconstruction [232].

3.4. Pseudomonas aeruginosa Toxin

Pseudomonas aeruginosa produces a potent 66-kDa A-B toxin (PE, also known as exotoxin A or ETA) that inhibits protein translation [233,234]. The A domain has enzymatic activity, and the B domain acts as a cell-binding moiety [235,236]. The first 25 amino acids form a highly hydrophobic signal sequence that is removed during secretion [237]. The remaining 613 amino acids is divided into three domains. Domain Ia is the receptor binding domain (first 252 amino acids) that attaches to target cells, domain II (amino acids 253–364) facilitates the translocation of PE across the cell membrane, and domain Ib (amino acids aa 365–404) together with domain III (amino acids 405–613), represents the catalytic part of toxin PE [238]. When secreted, the terminal amino acid residue of PE (lysine 613) is cleaved by a host plasma carboxypeptidase, which converts the REDLK (amino acids 609–613) motif into REDL (amino acids 609–612) [239]. PE interacts with the low-density lipoprotein receptor-related protein 1 (LRP-1) via the Ia domain and is then internalized by endocytosis. In the early endosome, which is acidic, PE dissociates from the receptor and changes conformation, exposing the furin-cleavable motif to be cleaved by furin into two fragments of approximately 28 kDa (279 amino acids) and 37 kDa (333 amino acids) [240]. The 28 kDa fragment consists of domain Ia and parts of domain II. The 37 kDa fragment contains parts of domain II, domains Ib, and domain III and has enzymatic activity. The 37-kDa fragment migrates from the late endosome to the trans-Golgi network (TGN) and from there to the ER via the retrograde pathway. The C-terminal REDL motif interact with KDEL receptors on the TGN [241]. The mode of action of PE is illustrated in Figure 4.

Moxetumomab pasudotox [241] is a recombinant anti-CD22 immunotoxin consisting of a single chain antibody fragment of a mouse anti-CD22 monoclonal antibody (scFv) fused to a *Pseudomonas* endotoxin A (PE38 domain). The immunotoxin binds to CD22 antigen expressed on B cells in various hematological malignancies. Upon internalization and intracellular proteolysis, the cytotoxic fragment (PE38) is released, which then induces cell death by apoptosis [241,242]. In September 2018, Moxetumomab pasudotox (LUMOXITI™; AstraZeneca, Cambridge, UK) was approved by the U.S. Food and Drug Administration (FDA) for the treatment of relapsed or refractory hairy cell leukemia [243]. Immunotoxins containing PE are highly immunogenic in patients with normal immune systems, but less so in patients with hematologic malignancies, whose immune systems are often compromised. SS1P, a first-generation, mesothelin-targeted immunotoxin, demonstrated little activity as a single agent [244]. In patients with solid tumors, neutralizing antidrug antibodies (ADAs) directed against PE developed after only three infusions of SS1P. To delay the

development of high-titer ADAs, SS1P was combined with a preconditioning regimen of lymphocyte-depleting chemotherapy [245]. LMB-100, a second-generation recombinant immunotoxin that targets the glycoprotein mesothelin on the surface of cancer cells is composed of a humanized antimesothelin antibody fragment fused to a truncated PE A [246]. The maximum tolerated dose (MTD) of LMB-100 was 140 µg/kg, administered every other day over 3 weeks [246]. Although LMB-100 was less immunogenic than SS1P, most patients developed antidrug antibodies after 2 cycles. Phase 2 clinical studies with LMB-100 plus pembrolizumab is conducted on patients with mesothelioma and lung cancer [246]. Several studies are devoted towards the developing of PE-based recombinant immunotoxins (RITs), especially against mesothelin and other proteins on solid tumors. For more information, the reader is referred to the review by Mazor and Pastan [247].

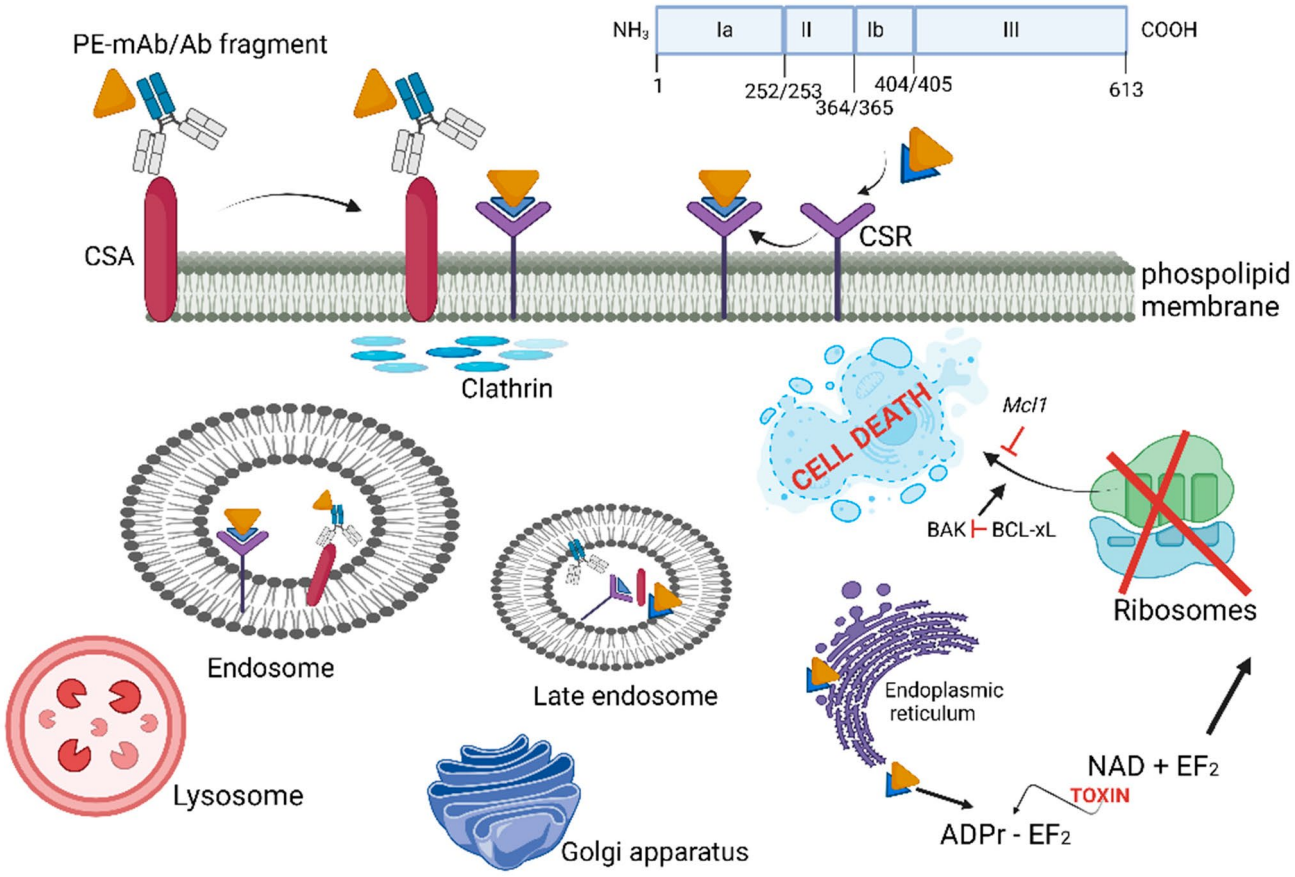

Figure 4. Top of presentation: The four domains (Ia, II, Ib and III) of *Pseudomonas* exotoxin (PE), with the sizes of each domain indicated in amino acid numbers. Interaction of PE-IT (immunotoxin) with a cancer-specific antigen (CSA) or cancer-specific receptor (CSR) on the surface of cancer cells. Intracellular events leading to cell death is illustrated below the phospholipid membrane. Abbreviations: PE-L = *Pseudomonas* exotoxin A, fused to a cancer-specific ligand; Ab = antibody; EF2 = eukaryotic elongation factor-2 on ribosomes; Mcl1 = gene encoding anti-apoptotic protein; BAK = Bak protein involved in mitochondrion outer membrane (MOM) permeabilization; BCL-xL = B-cell lymphoma-extra large that inhibits the activation of Bak, thereby preventing a loss of MOM integrity. This illustration was constructed using BioRender (https://biorender.com/, accessed on 15 August 2022).

4. Antibiotics

Although antibiotics are mainly used as bactericidal or bacteriostatic agents, some display anticancer properties and are classified as anthracyclines, of which Actinomycin D (Dactinomycin), Bleomycin, Doxorubicin (adriamycin and doxil), Epirubicin (ellence),

Cerubine and Daunorubicin (DaunoXome), Novantrone (mitoxantrone), Mitomycin C, Spergualin and Epothilone are the best known.

4.1. Actinomycin D

Actinomycin D, produced by *Streptomyces antibioticus* and *Streptomyces parvulus* has two pentapeptide lactone rings and a 2-aminophenoxazine-based chromophore [15]. Intercalation of the chromophore into DNA inhibits transcription and prevents the growth of tumour cells [248]. Interaction of actinomycin D with DNA is facilitated by a GpC (guanosine-cytosine) base pair. Increased binding was obtained with the formation of hydrogen bonds between L-threonine residues in the pentapeptide rings and the amino-terminal of guanosine [249]. Other amino acids of the two lactone rings, including proline, *N*-methylglycine and methylvaline, facilitates the binding of actinomycin D to the minor groove of DNA, thereby improving the stability of the interaction [15,249]. Despite the toxic effects of actinomycin D, such as tissue necrosis, dermatoxicity and gastrointestinal enterotoxicity, the drug has been approved for treatment of Wilms' tumour, gestational choriocarcinoma, neuroblastoma, germ cell cancers, trophoblastic tumours, Ewing sarcoma and rhabdomyosarcoma [250–253]. Actinomycin D has also been used in combination with antitumor agents to treat high-risk malignancies [249]. Actinomycin D increased the therapeutic efficacy of antitumor agents such as RG7787, a mesothelin-targeting immunotoxin. RG7787 (also referred to as LMB-100) is a recombinant immunotoxin formed through conjugation between exotoxin A of *P. aeruginosa* and anti-mesothelin Fab [246]. Synergistic cytotoxicity of actinomycin D and LMB-100 towards mouse xenografts of pancreatic and stomach cancer cells were illustrated with Phase I clinical trials [254]. This combination led to apoptosis via an extrinsic pathway and resulted in noteworthy regression of the tumours. Anticancer drugs containing Actinomycin D are available in the market under the trade names Cosmegen and Lyovac [8].

4.2. Bleomycin

Bleomycin is produced by *Streptomyces verticillus* [255,256]. A combination of Bleomycin A2 ($C_{55}H_{84}N_{17}O_{21}S_3$, Mw. 1415.56 Da) and Bleomycin B2 ($C_{55}H_{84}N_{20}O_{21}S_2$, Mw. 1425.52 Da) was approved by the FDA in July 1973 [207]. The mode of action is described as a two-step process. In the first step Bleomycin chelates metal ions (primarily iron) and produces a pseudoenzyme. The second step is the enzymatic conversion of oxygen to superoxide and hydroxyl free radicals damaging DNA [257,258]. Bleomycin is used to treat Hodgkin's lymphoma, non-Hodgkin's lymphoma, testicular cancer, ovarian cancer, and cervical cancer [259,260], and is commercially available as Bleomycin USP and Blenoxane [8].

4.3. Doxorubicin, Epirubicin, Daunorubicin and Novantrone

Doxorubicin (adriamycin and doxil; $C_{27}H_{29}NO_{11}$), produced by *Streptomyces peucetius* var. *caesius* [15,27,261], has amphipathic properties owing to a water-insoluble aglycone (adriamycinone, $C_{21}H_{18}O_9$) and water-soluble amino sugar group (daunosamine, $C_6H_{13}NO_3$). Its anti-cancer properties was first reported in 1969 [8] and has been approved by the FDA for treatment of malignant lymphoma, soft tissue sarcoma, and breast-, liver-, ovary-, neck-, head-, gastric- and childhood cancers [15,213]. Doxorubicin binds to DNA and RNA polymerases, which prevents DNA replication and transcription [261], intercalates with DNA and removes histones from chromatin during transcription [8]. The formation of covalent complexes between topoisomerase-II and DNA [15] leads to single- and double-strand DNA breaks and apoptosis [261]. Doxorubicin also binds to cardiolipin and mitochondrial creatine kinase [8]. Patients treated with doxorubicin displayed adverse side effects such as fatal cardiotoxicity and nonspecific cytotoxicity [15]. A nano-drug delivery system, based on liposome-encapsulation of doxorubicin (e.g., Doxil®), has been approved for treatment of ovarian, breast- and AIDS-related Kaposi's sarcoma. Doxorubicin and mitomycin C encapsulated in polymer-lipid hybrid nanoparticles (PLNs) were 20 to 30

times more active than the drugs in separate form and killed multidrug-resistant human breast cells MDA435/LCC6 more effectively [262]. Examples of drugs containing doxorubicin are Myocet, Doxorubicin-Ebewe, Adriblastine PFS, Caelyx, Doxorubicin medac, and Doxorubicinum Accord [8].

Epirubicin (ellence) is a semisynthetic derivative of doxorubicin, with a hydroxyl group in the 4^t position of the daunosamine ring [263]. It is mainly used in the treatment of early or metastatic breast cancer, but also other tumours such as lung, bladder, gastric, ovarian and hepatocellular carcinoma, and lymphatic cancers [264]. The mode of activity of Epirubicin is similar to that of Doxorubicin and also inhibits topoisomerase II activity [265].

Daunorubicin or Daunomycin, also referred to as Cerubine, is produced by *Streptomyces peucetius* and differs from doxorubicin by lacking a hydroxyl group at the the 14th position [266]. The name Daunomycin is derived from the pigment aglycone daunomycinone and the amino sugar daunosamine [267]. The liposome-encapsulated form of daunorubicin, referred to as DaunoXome, is more stable in an aqueous solution and is more toxic towards certain types of solid tumours [266].

Novantrone (mitoxantrone) is chemically related to doxorubicin and acts as a potent immunosuppressive agent for treatment of multiple sclerosis [268]. Novantrone inhibits the proliferation of B and T lymphocytes as well as macrophages, kills antigen-presenting cells, and suppresses the migration of activated leukocytes [269]. Other modes of action for mitoxantrone include lowering the secretion of IFN-γ, TNF-α, and IL-2 [270].

4.4. Mitomycin C, Duramycin and Epothilones

Mitomycin C ($C_{15}H_{18}N_4O_5$, Mw. 334 Da), produced by *Streptomyces caespitosus* [271], is an aziridine [$(CH_2)_2NH$]-containing antibiotic that cross-links DNA and inhibits alkylation [272]. The drug is used in the treatment of bladder, colorectal and pancreatic cancers, head and neck sarcoma, and lung-, hepatic and esophageal carcinoma [273]. Spergualin ($C_{17}H_{37}N_7O_4$, Mw. 403.53 Da), produced by *Brevibacillus laterosporus* BMG162-aF2, repressed fibrosarcoma cells (M5076) and cell lines of rat hepatomas (AH66, AH66F), as well as leukemia in mice models [274,275]. Duramycin induces apoptosis and reduces the proliferation in tumour cells [276,277], a phenomenon that may be ascribed to its high affinity for phosphatidylethanolamine [278]. The cytotoxicity of duramycin was reduced by fusion to IgG [279,280]. This did not influence binding to phosphatidylethanolamine. Fusion to IgG guide host immune cells to apoptotic cells, resulting in enhanced phagocytosis. Tumour growth in mice was inhibited after treatment with duramycin-IgG [280]. Since duramycin binds to PE and the Fc region (fused) IgG antibodies, it interacts with phagocytic cells to enhance phagocytosis. Duramycin is cleared from the site effectively soon after inducing apoptosis in cancer cells, via phagocytosis, which would explain its lower cytotoxicity to surrounding normal cells. Epothilone A and B, produced by *Sorangium cellulosum*, is classified as a macrolide polyketide [15]. Both variants inhibit mitosis and induce the formation of α/β-tubulin polypeptide heterodimers [186]. Low dosages of epothilone inhibits cell growth without blocking mitosis [186]. Ixabepilone (IXEMPRA), a synthetic analogue of epothilone B, represses the growth of a variety cancer cells. In 2007, the FDA approved IXEMPRA for treatment of breast cancer cells resistant to treatment with taxanes such as paclitaxel and docetaxel, anthracycline and capecitabine. IXEMPRA combined with capecitabine was more effective than capecitabine in the treatment of breast cancer cells resistant to taxane and anthracycline.

5. Bacteriocins

Bacteriocins of Gram-negative bacteria are divided into four main classes: colicins, colicin-like, phage-tail-like bacteriocins, and microcins [281]. Colicins are protease-sensitive, heat-sensitive and has a high-molecular weight (30–80 kDa). Most *E. coli* strains have genes encoding colicins. These proteins are expressed when cells experience stress and usually leads to self-destruction due to co-expression with lysis protein [282]. Depending on the mechanism of interaction with the target cell, colicins are divided into three main groups,

i.e., pore-forming, nuclease, and peptidoglycan degrading. Bacteriocins of Gram-positive bacteria are ribosomally-synthesized peptides with bacteriostatic or bacteriolytic activity and are usually below 10 kDa in size. These antimicrobial peptides are grouped into four classes. Class I are linear peptides, produced as pre-peptides, and are converted to active, mature, peptides after post-translational modification. They contain several disulphide bridges and unusual polycyclic thioether amino acids such as dehydrobutyrine, dehydroalanine and lanthionine. Class II bacteriocins do not contain lanthionine and do not undergo post-translation modification, except for the removal of the leader peptide [16]. Bacteriocins from both classes are thermostable. Class III bacteriocins are thermolabile and have a molecular mass exceeding 10 kDa. Class IV bacteriocins are circular [9,283].

The first report of a bacteriocin displaying anticancer properties was published in the late 1970s, but this was a crude extract [284]. Since then, several reports of bacteriocins with anticancer properties (also from our own group, unpublished), have been reported [285]. To the best of our knowledge only three bacteriocins have been patented for their anticancer properties, i.e., plantaricin A produced by *L. plantarum* C11 [8,286,287], microcin E492 produced by *Klebsiella pneumoniae* [288–291] and Pep27anal2, a 27-amino acid peptide produced by *Streptococcus pneumoniae* [291,292]. Anticancer properties have also been reported for bovicin HC5 produced by *Streptococcus bovus* HC5 [293,294], colicins A, E1, E3, and U isolated from *E. coli* [295–300], pyocins from *P. aeruginosa* [301], nisin from *Lactococus lactis* [302], and pediocins from *Pediococcus* and other lactic acid bacteria [303,304].

Pyocin S2, produced by *P. aeruginosa* M47 (PAO 3047), inhibited the growth of cancer cells XC, TSV-5, mKS-A TU-7, HeLa-S3, and AS-II, but did not affect normal cells such as mice cells BALB/3T3, rat kidney cells and human lung cells [305]. Abdi-Ali et al. [306] reported cytotoxic activity of pyocin S2 towards HepG2 cells and human immunoglobulin secreting (Im9) cells isolated from multiple myeloma [306]. Pep27anal2, an analog of signal peptide Pep27, produced by *S. pneumoniae*, causes caspase-dependent and cytochrome C-independent apoptosis in cancer cells, as shown with studies on OCI-AML-2 and HL60 (leukemia) cells, Jurkat cells, and MCF-7 and SNU-601 (adenocarcinoma) cell lines [292]. Bovicin (2.4 kDa), produced by *Streptococcus bovis*, showed cytotoxicity towards human breast cancer (MCF-7) and human liver hepatocellular carcinoma (HepG2) cell lines in a concentration-dependent manner [294]. Colicins, produced by *E. coli*, are larger than the average bacteriocins (>20 kDa). Colicin E3 showed cytotoxic and cytocidal effects against HeLa (human cervical cancer) cells and cleaves rRNA [297]. Colicin produced by *E. coli* HSC10 degraded DNA and is cytotoxic towards mammalian cells [295]. Colicin from the same strain, referred to as verotoxin 1, acted anticarcinogenic against human ovarian cell lines but protected cells in a murine metastatic fibrosarcoma model [296]. Colicins E1-E5 displayed cytotoxicity towards hamster V79 fibroblast cells [297]. Colicin A, E1, E3 and U caused cell cycle arrest in human fibroblast cell lines MRC5, MCF-7, MDA-MB-231 (mammary tumor), HOS (bone osteosarcoma), and HS913T (fibrosarcoma) [298]. In general, colicins depolarize the cytoplasmic membrane, prevents the synthesis of peptidoglycan, degrade DNA and RNA, seize cell cycles, and causes necrosis [299].

The interaction of bacteriocins with cancer cells is ill researched. Kaur and Kaur [16] hypothesized that cancer cells increase the expression of negatively charged cell-surface molecules when exposed to bacteriocins and, by doing so, promote cytotoxicity and apoptotic cell death. Azurin, an anticancer copper-containing bacteriocin of 14 kDa (Figure 4) produced by *P. aeruginosa* [307], provides some indication as to how bacteriocins may enter cancer cells. The peptide enters human cancer cells through receptor-mediated endocytosis [308] or, as reported with studies on breast cancer cells lines MCF-7, ZR-75-1 and T47D, via caveolin-mediated pathways [309]. The p28 domain of azurin (Figure 5) facilitates cell crossing and promotes apoptosis [310]. Sections within p28 have different adhesion and cell penetration properties, as summarized in the legend of Figure 5. Once inside a cancer cell, azurin attaches to the DNA-binding domain (DBD) of the tumor-suppressor protein p53 (Figure 6) and increases the intracellular level of the protein by inhibiting the binding of the E3 ubiquitin ligase COP1 to p53 [311]. This leads to repression of cell growth and

apoptosis [312]. Amino acids 88 to 113 in the C-terminal of azurin repressed the growth of MCF-7 breast- and DU145 prostate cancer cells [313]. More recent studies have shown that azurin also interferes with non-receptor tyrosine kinase (NRTK) signalling pathways [314]. Increased intracellular levels of p53 and Bax protein (a central cell death regulator) were detected in cells exposed to azurin [314]. This led to the release of cytochrome C in the cytoplasm, and activation of caspases 9 and 7 [8]. Azurin also reduces VEGFR-2 tyrosine kinase activity, thereby preventing the formation of new blood vessels and the expression of P-cadherin, a glycoprotein maintaining the structural integrity in epithelial tissue [314]. Phase I clinical trials were performed with p28 on recurrent, difficult to treat, and progressive solid tumours in adults, and on tumours from the central nervous system (CNS) of paediatric patients. No significant immune response or adverse side effects were reported [315]. Protein p21, a short amino acid fragment of azurin overexpressed in MCF-7 cells, inhibited cyclin-dependent kinases and prevented cell growth [316].

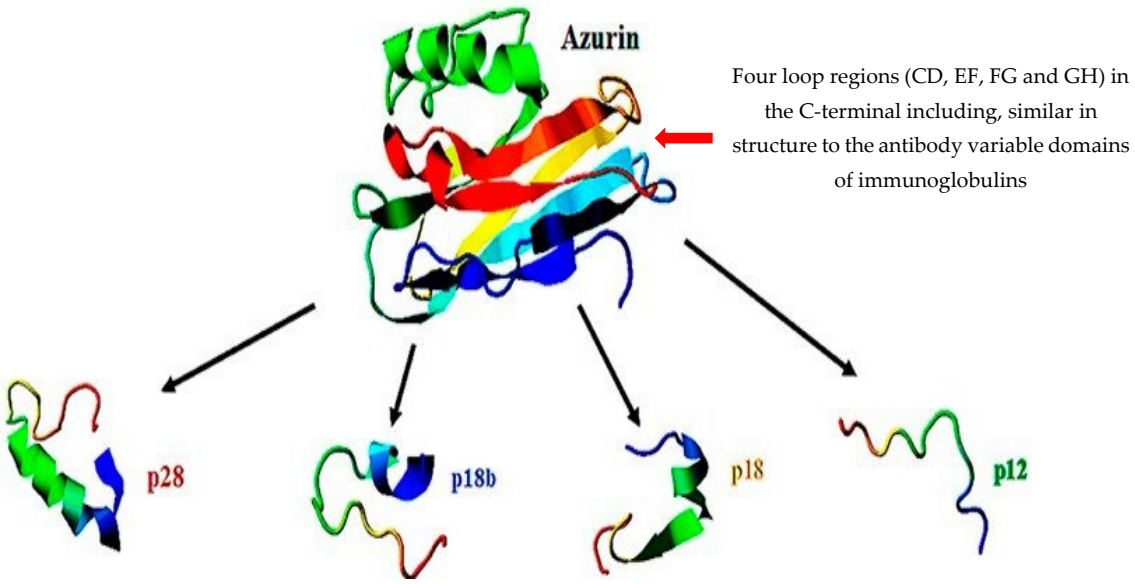

Figure 5. Azurin (128 amino acids), with an extended α-helix protein transduction domain, p28 (Leu50–Asp77), and four loop regions in its C-terminal (shown here in red, yellow, light blue and dark blue). The p28 peptide of 28 amino acids (amino acids 50 to 77) facilitates cell crossing and promotes apoptosis. Cell growth in tumours is repressed by 10 to 12 amino acids in the COOH terminal of p28. The α-helix, stretching over 18 amino acids (Leu^{50}–Gly^{67}), shown here as peptide p18, has a high affinity for cancer cells (less so for normal cells), excellent penetration abilities, and high binding to the tumour repressor protein p53. Peptide p18b also contains 18 amino acids (Val^{60}–Asp^{77}) but has a short α-helix and β-sheet and penetrates cancerous and normal cells. The p12 peptide of 12 amino acids (Gly^{66}–Asp^{77}) does not have an α-helical structure, binds poorly to p35, and penetrates cancer and normal cells. Adapted from Yaghoubi et al. [317].

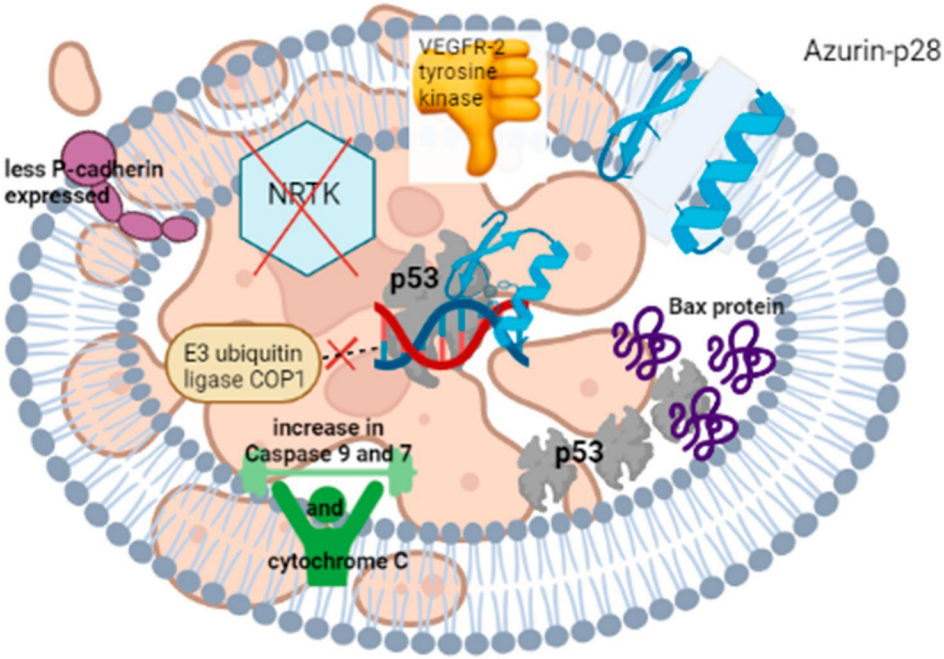

Figure 6. Mode of action of Azurin. Once inside a cancer cell, azurin attaches to the DNA-binding domain of the tumour-suppressor protein p53 (middle of presentation) and prevents binding of the latter to E3 ubiquitin ligase COP1, resulting in an increase in cytoplasmic p53 levels. Cell growth is repressed, and cells are destroyed by apoptosis. Azurin also interferes with non-receptor tyrosine kinase (NRTK) signalling pathways. Bax protein (a central cell death regulator) and cytochrome C levels increase, and caspases 9 and 7 are activated. VEGFR-2 tyrosine kinase activity is reduced, preventing the formation of new blood vessels and the expression of P-cadherin. Prepared using BioRender (https://biorender.com/, accessed on 12 May 2022).

A few bacteriocins produced by LAB are worth pointing out and are summarized here. Plantaricin A (2.98 kDa), a peptide pheromone produced by *L. plantarum* C11, binds to negatively charged phospholipids and glycosylated proteins in cell membranes of cancerous and normal cells, leading to the destabilization of cytoplasmic membranes [318]. The peptide also induces apoptosis and necrosis in Jurkat cells, accompanied by an increase in caspase 3 levels [286,287]. Microcin E492 (7.8 kDa) causes cell shrinkage, DNA fragmentation, release of phosphatidylserine and calcium ions, and apoptosis of cancerous cells such as HeLa, Jurkat. RJ2.25 and colorectal carcinoma cells [289]. Normal bone marrow cells, splenocytes, KG-1, human tonsil cells, and nontumor macrophage-derived cells were not affected [289].

An in vitro study showed that lacticin A164 and BH5, produced by *L. lactis* subsp. *lactis*, inhibited the growth of *Helicobacter pylori* [319], which is responsible for a sizeable number of gastrointestinal cancers. Probiotic preparations with these strains may thus be used to control of *H. pylori* infection and related intestinal cancers. Nisin, also produced by *L. lactis* subspecies *lactis,* has been used in the food industry for over 50 years [16,27].

Joo et al. [320] reported a decrease in the proliferation of head and neck squamous cell carcinoma (HNSCC) cells when exposed to nisin and illustrated decreased tumour growth in an oral cancer floor-of-mouth mouse model [320]. Athymic nude mice (NCr-nu/nu strain) were used. Gene array analyses performed on HSC-3 oral squamous cell carcinoma (SCC) cells treated with nisin revealed an increase in genes regulating calcium transport, membrane lipid functioning and apoptosis. The gene most upregulated encodes the cation transport regulator CHAC1 (ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1), which is known to be activated by oxidized phospholipids [320]. Since nisin interacts with phospholipids in cell membranes, especially phosphatidylcholine, it is possible that CHAC1 may be a "downstream" nisin target. In in vitro experiments conducted with nisin ZP on HNSCC cells and with NCr-nu/nu mice much higher levels of apoptosis were

reported compared to treatment with nisin [321]. Apoptosis of HNSCC cells increased with higher concentrations of nisin ZP [321]. This coincided with a decrease in cell proliferation, clonogenic capacity, and sphere formation [321]. Nisin ZP induced apoptosis through a calpain-dependent pathway in HNSCC cells but not in human oral keratinocytes [321]. Apoptosis of human umbilical vein endothelial cells (HUVEC) induced by nisin ZP coincided with a decrease in vascular sprout formation (in vitro) and lowering of intratumoral microvessel density (in vivo) [321]. No abnormalities in organ tissue, inflammation, fibrosis or signs of necrosis were observed in mice treated [321], suggesting that nisin ZP may be a promising alternative in cancer therapy. Reports of nisin showing potential in the treatment of colorectal cancers [321–323] and growth inhibition of blood, breast, brain, colon, gastrointestinal, liver and skin cancer cells [324] have been published.

Preet et al. [325] studied the synergistic effect of nisin and doxorubicin on dimethylbenzanthracene-induced skin cancer in murine models. Doxorubicin-alone-treatment and nisin-alone-treatment resulted in a mean tumour burden reduction of 51.3% and 14.18%, respectively. Mice treated with a combination of nisin and doxorubicin displayed a reduction of 66.82% in the mean tumour burden. Nisin combined with 5-FU killed skin cancer cells, induced with 7,12-dimethylbenz(a)anthracene, in vivo [326]. Anticarcinogenic properties was ascribed to modulation of apoptotic, angiogenic and cell proliferative pathways.

Pediocin PA-1, produced by *Pediococcus acidilactici* K2a2-3, is cytotoxic towards human colon adenocarcinoma (HT29) and HeLa cells [303]. Pediocin CP-2, produced by *P. acidilactici* MTCC 5101 and rec-pediocin CP-2 (recombinant) cells are cytotoxic towards MCF-7, HepG2, HeLa and mouse spleen lymphoblast (Sp2/O-Ag14) cells and induces apoptosis [304].

Bacteriocins may thus play a key role in prevention of intestinal and skin cancers. Only a few of such studies have been published and most were performed in vitro. Should these results be confirmed by in vivo studies, methods will have to be developed to protect these peptides from degradation by gastrointestinal enzymes and enhance their activity. Bacteriocin-producing strains with anticarcinogenic properties may be included in probiotic supplements. We are, however, still a long way from understanding the efficacy of bacteriocins in anticancer therapy and whether these peptides should be used alone or in combination with chemotherapeutic agents.

6. Non-Ribosomal Peptides and Polyketides

Non-ribosomal peptide synthetases (NRPS) were discovered in 1968 by Gevers and co-workers when they studied gramicidin S production by *Bacillus brevis* [327]. By adding RNAse and puromycin (a ribosome inhibitor) to *B. brevis* extracts containing gramicidin S, Gevers et al. [328] observed that aminoacyl-tRNA synthetases and tRNA were not used in the production of gramicidin S. Subsequent studies have shown that non-ribosomal peptides are produced by enzyme complexes in a nucleic acid-independent manner [329]. Further research is needed to determine if non-ribosomal peptides can be used in cancer therapy [8]. Polyketides are produced non-ribosomally by type 1 polyketide synthetases (PKSs) [330]. Almost one-third of all pharmaceuticals are polyketides which can be attributed to their structural and biological diversity. Type 1 PKSs are modular enzyme assemblies and act successively to elongate the polyketide chain. Different domains can be found in PKSs such as ketoreductase, dehydratase, and enoylreductase with conserved domains such as acyl carrier protein, ketosynthase and acyltransferase. Readers are referred to Baindara and Mandal [8] for further information non-ribosomal peptides and polyketides. Noteworthy is that no non-ribosomal peptides and polyketides with anticancer properties have been reported for lactic acid bacteria.

7. Phenylpropanoids

Phenylpropanoid-derived metabolites from plants inhibits the growth of several cancer cell types [331]. However, Bacteroides thetaiotaomicron, Bacteroides eggerthii, Bacteroides ovatus, Bacteroides fragilis, Parabacteroides distasonis, Eubacterium hallii and Clostridium

bartlettii ferment phenylalanine, tyrosine and tryptophan to phenylacetic acid (PAA) and 4-hydroxylphenylacetic acid (4-hydroxyPAA) in the colon [332]. An increase in cell numbers of Bacteroides fragilis was observed in patients with advanced stages III and IV) CRC [333].

The phenylpropanoid verbascoside protects the GIT from oxidative stress and represses the growth of MKN45 gastric epithelial cancer cells, but also stimulates appetite [334,335]. Further research on the stability of phenylpropanoids is required. Acteoside, a verbascoside, has anti-inflammatory properties [336] and prevents the onset of chronic diseases, including cancer [337,338], but is degraded by gut microbiota [339]. Further research is required to understand the degradation of phenylpropanoid by gut microbiota, and exactly how these compounds modulate inflammatory and microbial processes.

8. Phenylflavonoids

Xanthohumol (XN), a prenylated flavonoid found in hops, has promising anticancer properties [340]. Gut microbiota metabolites XN to 8-prenylnaringenin (8-PN), a very potent phytoestrogen with anticancer activity, as demonstrated with SK-MEL-28 and BLM human metastatic melanoma cells [341] and MCF7 breast cancer cells [342]. 8-PN also inhibited cell proliferation of the HT-115 and HT-116 colon cancer cells [342,343].

9. Natural Purine Nucleosides

Adenosine and inosine (formed by the catabolism of adenosine) bind to adenosine receptors, and initiates cAMP production and the phosphorylation of kinase-1 and -2 [344]. Inosine also enhances T cell antitumour activity in colorectal, bladder, and melanoma cancer cells [345]. Studies on bladder cancer cells indicated that inosine enhanced the function of anti-CTLA-4, causing to increase infiltration of IFN-γ+ CD4+ and IFN-γ+ CD8+ T-cells into the tumour, as well as reducing overall tumour weight [345]. CTLA-4, also known as CD152, is a protein receptor that functions as an immune checkpoint downregulating immune responses, a process referred to as immune checkpoint blockade (ICB). Inosine produced by *Bifidobacterium pseudolongum* increased the activation of a cDC-dependent TH 1 cell circuit and led to the enhancing of ICB in murine models with intestinal and epithelial tumours [345].

10. Short Chain Fatty Acids

Diet plays an important role in CRC and may promote the formation of tumours [346]. SCFAs such as butyrate, acetate, propionate and lactate are largely produced in the colon by *Bifodobacterium*, *Lactobacillus*, *Lachnospiraceae*, *Blautia*, *Coprococcus*, *Roseburia*, *Faecalibacterium*, *Clostridium*, *Eubacterium*, and species converting lactate and acetate, e.g., *Anaerostipes* spp. [347,348]. These SCFAs adhere to free fatty acid receptors (FFARs), e.g., GPR43 (FFAR2) and GPR41 (FFAR3) located on the surface of IECs [349]. Patients diagnosed with CRC had less *Bifidobacterium* spp. and lower levels of SCFAs [350]. Butyrate also protects intestinal barrier function by up-regulating the tight junction protein claudin-1 [351–353]. Other functions of butyrate include maintaining a balanced state of oxygen [301] and suppressing inflammatory responses [354,355]. The latter is achieved by downregulating histone deacetylase (also referred to as lysine deacetylase) inhibitors (HDACi) [354,355]. An increase in de-acetylated histones (due to the inhibition of HDACi), together with a decline in gene transcriptions, leads to autophagic cell death, the activation of extrinsic and/or intrinsic apoptotic pathways, an increase in production of reactive oxygen species (ROS), and a decrease in the expression of pattern recognition receptors, kinases, transcription regulators, cytokines, chemokines, and growth factors [356,357]. Molecules released from dying cells are recognised by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and form specific protein-protein interactions. These interactions, also prevalent in lymphocytes, macrophages and dendritic cells, play a key role in the regulation of cytokines, chemokines and the expression of genes coding for the production of antimicrobial compounds, collectively referred to as the innate immune response [358–360]. Downregulation of NLRs prevents the formation of multi-protein inflammasomes, the

signaling of caspase-independent nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK). These cascades of events counteract autoimmune and inflammatory disorders and as recently shown, represses the growth of cancerous cells [361].

Butyrate and propionate are more effective than acetate in inhibiting the growth of HT29 cells [362]. Butyrate significantly increased apoptosis in cancer cells [363] and activates ornithine decarboxylase, resulting in the inhibition of polyamine metabolism and an increase in alkaline phosphatase activity [364]. Butyrate also activates GPR41/GPR43 receptor signaling pathways [364], thus preventing the proliferation of cancer cells [365]. GPR43 is predominantly present in the large intestine of healthy cells, but less so in colon cancer cells [366]. Manipulation of HCT8 human colonic adenocarcinoma cells to express the GPR43 receptor led to an increase in apoptosis and G0/G1 cell cycle arrest [366]. Concluded from these studies, the GPR43 receptor serves as a tumour suppressor. SCFAs were ineffective against HCT-116 colon cancer cells with a deletion in the *p21* gene, suggesting that p21, a cell cycle inhibitor and anti-proliferative effector, is important in repressing tumour growth. Another study has shown that *p21* is regulated by the p53 transcription factor [367]. Diets rich in SCFAs suppresses T cell-mediated autoimmune responses, most probably through regulation of cytokine expression [364]. The ability of butyrate to derepress epigenetically silenced genes in cancer cells, such as cell cycle inhibitor p21 and the pro-apoptotic protein Bcl-2, has important implications for cancer prevention and therapy. Lightfoot et al. [368] tested possible epigenetic modifications induced by LTA-deficient *L. acidophilus* and found that oral NCK 2025 enhances the expression of tumor suppressor genes [368,369]. This indicates that differential epigenetic regulation of CRC-related genes by NCK2025 represents a potential therapy against CRC.

Overall, SCFAs are promising specifically in the context of colon cancer. Future studies should evaluate the effect of SCFAs on other cancer types, e.g., pancreatic and gastric cancer. Studies should also explore the impact of SCFAs on the efficacy and safety of standard chemotherapy and the prognosis of cancer.

11. Enzymes

Four enzymes with anticancer properties have been reported, i.e., arginine deiminase (ADI), produced by *Mycoplasma hominis* and *Mycoplasma arginine*, asparaginase (ASNase), produced by *E. coli* and *Erwinia chrysanthemi*, glutaminase [370] and methionase [371]. ADI converts arginine to citrulline and ammonia in vivo [372]. Pegilation of ADI (ADI-PEG20) significantly increased the half-life of ADI in serum and decreased its antigenicity [373], rendering ADI much more effective against cancerous cells. The mode of activity of ADI-PEG20 is ascribed to loss of argininosuccinate synthetase (ASS) activity and, thus, the inability to synthesize arginine from citrulline [374]. Due to this, growth of hepatocellular carcinoma cells (HCC), auxotrophic to arginine, were repressed, with evidence of apoptosis [375]. Treatment of metastatic hepatocellular carcinoma cells with ADI-PEG20 entered phase II clinical trials [376]. Autophagy was induced in prostate cancer cells (CWR22Rv1) treated with ADI-PEG20 [374]. ASNase degrades asparagine, which results in a severe reduction in protein synthesis and thus growth inhibition of cancer cells [377,378]. Studies performed with human cells lines (pediatric medulloblastoma, DAOY, and glioblastomas GBM-ES and U87) indicated that inhibition of cell growth by ASNase is dose-dependent [379]. ASNase has been used in the treatment of acute lymphoblastic leukemia (ALL), acute myeloid leukemia, ovarian carcinoma, myelosarcoma, Hodgkin lymphomas, and extranodal NK/T cell lymphoma [380–383]. Glutaminase, specifically glutaminase 1 (GLS1) expressed in mitochondria, converts glutamine to glutamate [384], but also stimulates the growth of tumour cells [385] and is involved in autophagy [386], signal transduction [387], and radioresistance [388]. Recent evidence emerged showing that GLS1 might be involved in tumorigenesis and progression of human cancers [370]. GLS1 is overexpressed in metastatic lymph nodes and colorectal cancer cells [370]. Methionase, also known as L-Methionine-γ-lyase (EC 4.4.1.11; MGL), methioninase, L-methionine-γ-demethiolase, and L-methionine methanethiol-lyase (deaminating), is produced by *Pseudomonas putida*,

Pseudomonas ovalis, *Micrococcus luteus*, *Arthrobacter* spp., *Corynebacterium glutamicum*, *Staphylococcus equorum*, *Citrobacter* spp., *Clostridium sporogenes*, *Trichomonas vaginalis* and *Entamoeba histolytica*, but has also been isolated from protozoans, fungal, archaeon, and plants [371]. Melignant tumours are highly dependent on methionine. Depletion of methionine through methionase-based therapy may be an important strategy to control the growth of cancer cells. One approach experimented with was linking L-methionase to human annexin-V to generate a fusion protein. The fusion protein catalyzed the conversion of nontoxic prodrug selenomethionine into toxic methylselenol and restricted the growth of tumour cells by depriving the cells from access to methionine [389–391]. The advantage of using the fusion protein is that it does not require to be delivered directly to the tumour cells but only to the bloodstream.

12. Conclusions and Future Directions

Cancer is a major health concern and treatment remains a challenge due to cells developing resistance. Despite numerous efforts to use viable bacteria in the treatment of cancer, the idea is still viewed with scepticism, as many strains experimented with are considered obsolete or opportunistic pathogens. To date, bacterial anticancer compounds studied were either toxins, antibiotics, bacteriocins, non-ribosomal peptides, polyketides, phenylpropanoids, phenylflavonoids, purine nucleosides, short chain fatty acids (SCFAs) or enzymes. Other natural antitumor compounds discovered are spliceostatins, such as spliceostatin B purified from cell-free extracts of *Pseudomonas* sp. 2663 and pladienolide B, a macrolide produced by *Streptomyces platensis* Mer-11107. More research on spliceostatins is required. It is interesting to note that many of the bacteria that produce anticancer compounds are either obligate or facultative anaerobes. Many anticancer compounds naturally produced by bacteria are specific in their mode of action, and some are selective in attacking only cancerous cells. Lactic acid bacteria form a major part of the gut microbiome, yet the role they play in cancer treatment is ill researched. The combined use of natural anticancer compounds with conventional anticancer therapy warrants further research. Progress in metagenomics, proteomics, heterologous gene expressions and nanotechnology, combined with the use of artificial intelligence software such as AlphaFold 2 (https://alphafold.ebi.ac.uk/) and Chemistry42 (https://arxiv.org/abs/2101.09050, accessed on 10 May 2022), may lead to the discovery and design of novel anticancer molecules.

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Chapter 7

Quorum Sensing between Gut Microbiota and Lactic Acid Bacteria, and its Effect on the Host

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Review

How Does Quorum Sensing of Intestinal Bacteria Affect Our Health and Mental Status?

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Abstract: The human gut is host to almost 3000 microbial species, of which 90% are bacteria. Quorum sensing (QS) molecules generated by intestinal bacteria are important in establishing species- and strain-level structures within the gut microbiome but are also used to communicate with the host. Although we do not know which QS molecules have the most direct interaction with intestinal and sensory neurons, it is clear they affect our physiological and mental health. Signals produced by bacteria are diverse and include autoinducers (AIs), homoserine lactones (HSLs), quinolines, peptides, toxins and proteases. These signaling molecules activate specific receptors in the bacterial cell wall and trigger sensors in the cytoplasm that regulate gene expressions. A better understanding of the gene structures encoding the production of QS molecules is of importance when selecting strains with neurogenerative and other probiotic properties. Furthermore, QS molecules may be used as biomarkers in the diagnosis of inflammable bowel disease (IBD), irritable bowel syndrome (IBS) and colorectal cancer (CRC). In the future, it should be possible to use QS biomarkers to diagnose neurological and psychiatric diseases such as anxiety and depression, major depressive disorder (MDD), schizophrenia, bipolar disorder, autism and obsessive-compulsive disorder (OCD).

Keywords: quorum sensing; intestinal bacteria; health

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1. Introduction

In a highly competitive and ever-changing environment such as the gastrointestinal tract (GIT), microbiota have developed unique methods to communicate with each other. Quorum sensing (QS) molecules produced by the gut microbiota regulate a variety of cell functions such as expression of virulence genes, formation of biofilms, competence and sporulation and usually only initiate these processes when cell numbers reach a certain density [1–3]. These signals include autoinducers (AIs) such as AI-1, AI-2 and AI-3; 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL); C4-homoserine lactone (C4-HSL); 2-heptyl-3-hydroxy-4(1H)-quinoline; 2-heptyl-4-hydroxyquinoline (HHQ); QS peptides (QSPs); peptide pheromone Agr; pore-forming toxins such as hemolysins, leucocidins and phenol-soluble modulins (psms) and proteases. Epinephrine (Epi), norepinephrine (NE), autoinducer 3 (AI-3), fucose, ethanolamine (EA) and vitamin B12 activate specific receptors in the bacterial cell wall and trigger sensors in the cytoplasm that regulate gene expressions. Conversation between microorganisms varies and ranges from interspecies communication, self-talk or intraspecies communication to cells from one genus responding to signals generated by another genus. Cells that are unable to produce their own communication signals are "listening" to signals generated by other cells, a phenomenon referred to as "eavesdropping" [4]. The gut microbiota uses certain metabolites as QS molecules to communicate with intestinal epithelial cells (IECs). *Staphylococcus aureus*, for instance, secretes a variety of virulence factors that manipulate the immune system of the host to safeguard its own survival [5]. The effect of these survival strategies on the host often manifests in

the form of immune misfunctioning, neurological disorders, diarrhea and vast changes in the gut microbiome [6].

Microbial QS could be seen as a partnership or agreement amongst microbiota and, in the GIT, between the gut microbiota and the host. This requires microbiota and the host to develop specific adaptation strategies. In a complex environment such as the GIT with close to 3000 bacterial species, strains are in constant survival mode and produce an arsenal of inorganic and organic molecules participating in inter-microbial and inter-host communications. This review addresses changes brought about by QS amongst intestinal bacteria and the gut wall. The impact these communications may have on the central nervous system (CNS) and mental health is summarized.

2. Interbacterial Communication

2.1. Gram-Negative Bacteria

Gram-negative bacteria use small molecules as autoinducers (AIs) that either target transcription factors or transmembrane two-component histidine sensor kinases [7]. Amongst these, N-acyl-homoserine lactone (AHL), a small neutral lipid molecule with a homoserine lactone (HSL) moiety linked to a 4 to 18 carbon acyl side chain [8], is the best studied. AHL is synthesized from S-adenosylmethionine (SAM), catalyzed by either LuxI or LuxM synthetases (Figure 1, left image) [9]. Not all AHLs are the same. The length of the acyl side chain and substituents at the third position of the acyl chain differ and allow a LuxR-type receptor to discriminate between signals [10]. Furthermore, some species have a single AHL synthase enzyme and produce predominantly one type of AHL, whereas others have multiple AHL synthases and produce several forms of AHL [11]. The level at which AHLs are produced depends on the availability of substrates and is tightly controlled [11]. Bacteria lacking the LuxI-type synthase have orphan or "solo" LuxR-type receptors that respond to AHLs produced by other bacterial species in the same environment. SdiA (a LuxR homolog) in *Escherichia coli* and QscR in *Pseudomonas aeruginosa* are examples of such orphan LuxR-type receptors (Figure 1). These receptors are highly conserved with a 67–84% sequence identity [12] and are also found in *Enterobacter*, *Citrobacter*, *Cronobacter*, *Klebsiella*, *Salmonella* and *Shigella* [13].

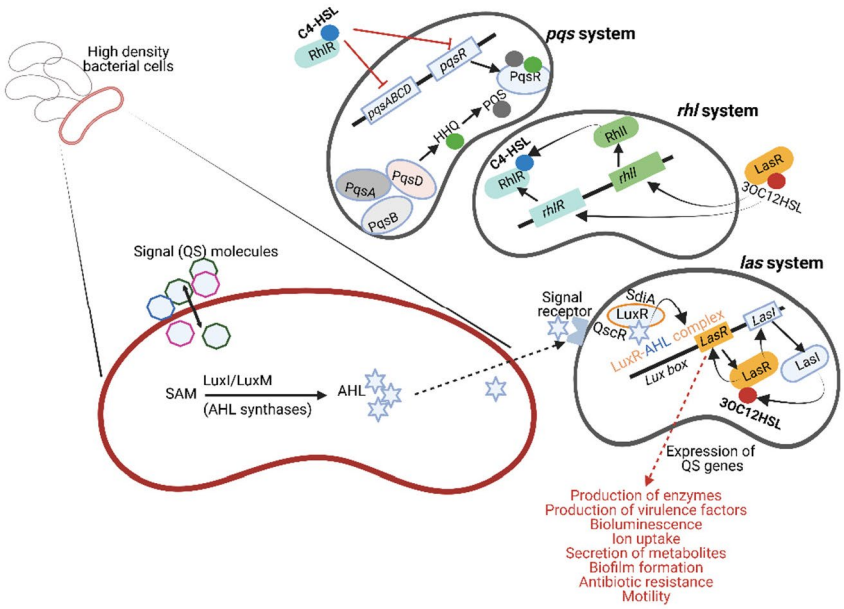

Figure 1. Quorum sensing (QS) molecules produced by Gram-negative bacteria. Acyl-homoserine lactone (AHL) is produced from S-adenosylmethionine (SAM) by AHL synthase (shown on the left). LuxI-type synthases are major producers of AHLs. LuxM synthase, described for *Vibrio harveyi*, is

important in intra-species communication. Signal receptors on the surface of the responding bacterial cell recognize AHL, form a LuxR–AHL complex and induce genes in the *Lux box* to produce 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL, abbreviated as 3OC12HSL in this figure to conserve space). This is referred to as the *las* system. In the absence of LuxI synthases, LuxR proteins detect different AHL molecules produced by other bacterial species. Examples of these receptors are SdiA (LuxR homolog) in *E. coli* and QscR in *P. aeruginosa*. *Pseudomonas aeruginosa* has three major QS systems, namely *las*, *rhl* and *pqs* (shown on the right), that mediate cell-to-cell communication and control the synthesis and secretion of virulence factors, bioluminescence, biofilm formation, etc. Additionally, 3-oxo-C12-HSL may also induce the *rhl* system to produce C4-homoserine lactone (C4-HSL). The *pqs* system uses two signal molecules, i.e., 2-heptyl-3-hydroxy-4(1H)-quinoline (also referred to as *Pseudomonas* quinolone signal or PQS) and its biosynthetic precursor 2-heptyl-4-hydroxyquinoline (HHQ). This illustration was constructed using BioRender (https://biorender.com/, assessed on 27 September 2022).

Five QS systems have been described for pathogenic *E. coli*, i.e., AI-2 signaling produced by the enzyme LuxS, SdiA signaling that suppresses cell division, AI-3/Epi/NE signaling involved in host–bacteria communication, indole signaling and extracellular death factor (EDF) signaling that triggers the activation of toxin–antitoxin systems [14]. SdiA of *E. coli* (SdiAEC) is activated by AHLs produced by *P. aeruginosa* [15]. The SdiAEC/AHL complex increases the transcription of genes in the gad operon (*gadW*, *gadE*, *yhiD* and *hdeA*) of *E. coli* [16] that encodes an acid resistance system critical to the survival of enterohemorrhagic *E. coli* (EHEC) in a low-pH environment [17].

E. coli uses QS to regulate virulence genes, biofilm formation, mobility, the type III secretion system (T3SS), toxicity and the production of curli [18]. QS systems in *Salmonella* regulate the pathogenicity island SPI-1 (invasion), the expression of genes encoding flagella formation and the pefI-srgC plasmid operon regulating the genes *rck* (resistance to complement killing) and *srgE* (sdiA-regulated gene E) involved in the Zipper invasion mechanism [19–21].

Pseudomonas aeruginosa has three major QS systems, namely *las*, *rhl* and *pqs* (Figure 1, right image), involved in cell-to-cell communication, control over synthesis and secretion of virulence factors, bioluminescence, biofilm formation, etc. (listed in Figure 1). The *las* system regulates both the *rhl* and *pqs* systems by initiating the expression of the AI receptors RhlR and PqsR. These receptors also act as transcriptional activators when bound to their respective AIs. Freely diffusible AHL communicates with other bacterial cells or binds to LuxR-type receptors in the cytoplasm of producing cells to form stable LuxR–AHL complexes [9], as depicted in Figure 1. These LuxR–AHL complexes bind to the *Lux box* (*las* system, Figure 1) and regulate the expression of QS genes [22–24]. Mutants without the *lasR* gene (Δ*lasR*) are more motile, survive stationary growth much better and produce higher levels of β-lactamase and pyocyanin [25,26]. However, Δ*lasR* mutants produce fewer exoenzymes and elicit a higher immune response in host cells, as evident from an increase in the secretion of pro-inflammatory cytokines and neutrophil recruitment [27]. Complexes similar to the LasR/LasI system, e.g., RhlR/RhlI (*rhl* system, Figure 1) described for *P. aeruginosa*, produce and detect C4-homoserine lactone (C4-HSL) [28,29]. In the absence of LuxI synthases, LuxR proteins may bind to AHLs produced by other bacterial species and initiate interspecies communication [30–32]. The *pqs* system (Figure 1) employs two signal molecules, i.e., 2-heptyl-3-hydroxy-4(1H)-quinoline (also referred to as *Pseudomonas* quinolone signal or PQS) and its biosynthetic precursor 2-heptyl-4-hydroxyquinoline (HHQ).

2.2. Gram-positive Bacteria

Gram-positive bacteria communicate using small linear or cyclized oligopeptides (QS peptides, QSPs) consisting of 5 to 17 amino acids [2,28,33,34]. The most studied QS systems are those produced by *Bacillus*, e.g., the competence sporulation factor (CSF), a pentapeptide, and the heptapeptide SDLPFEH (PapRIV). The heptapeptide forms after cleaving of the inactive 48-amino-acid pre-peptide by NprB protease [35,36].

QSPs are extracellularly secreted with the assistance of ATP-binding cassette transporters located in the cell membrane (Figure 2) and interact with either membrane-located receptors or cytoplasmic sensors such as the proteins Rap, NprR, PlcR and PrgX [33]. In the case of *Staphylococcus aureus*, the accessory gene regulator (*agr*), a four-gene operon encoding the peptide pheromone Agr, serves as the membrane-bound sensor. Agr regulates the expression of several genes, including virulence factors such as formylated peptides, proteases and pore-forming toxins (PFTs) such as hemolysins, leucocidins and phenol-soluble modulins (psms) [37]. Strains of *S. aureus* lacking the *agr* gene (Δ*agr*) form biofilms and are more prone to causing chronic infections and bacteremia [38–40]. *Enterococcus faecalis* uses the Fsr-QS system, which is controlled by the four-gene locus *fsrABDC* [41]. Once cleaved, the activated peptide is intracellularly transported using a transmembrane kinase (Figure 2). A cascade of phosphorylation reactions excites the peptide and induces the expression of target genes (Figure 2). For more information on the chemical characteristics and microbial background of QSPs, the reader is referred to the Quorumpeps® database [42].

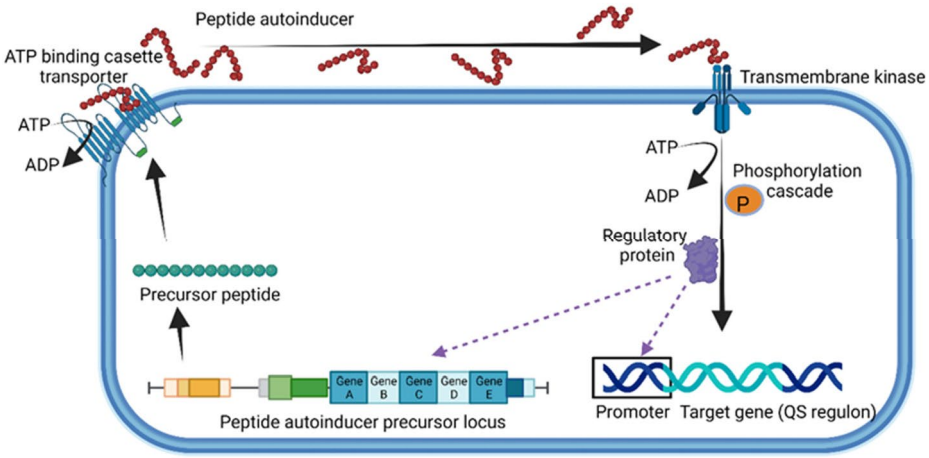

Figure 2. General representation of quorum sensing used by Gram-positive bacteria. This illustration was constructed using BioRender (https://biorender.com/, assessed on 1 September 2022).

3. Interspecies Communication

Autoinducer-2 (AI-2), a furanosyl borate diester produced by Gram-negative and Gram-positive bacteria [43], plays a key role in interspecies communication and the altering of specific behavior such as virulence, luminescence and biofilm formation [44–47]. The AI-2 system is also used by the gut microbiota to overcome stressful conditions in the GIT [48,49]. Production of AI-2 is regulated by the *luxS* gene (Figure 3). S-adenosylhomocysteine (SAH) is converted to homocysteine by SAH hydrolase (SahH) in a one-step reaction but may also be produced from the cleavage of the thioether linkage of S-ribosylhomocysteine (SRH). This is a two-step reaction that requires SAH nucleosidase (Pfs) and LuxS. The intermediate, 4,5 dihydroxy-2,3-pentanedione (DPD), is rearranged to form AI-2 (Figure 3) [50].

Genes encoding homologues of luxS have been detected in more than a third of bacterial genomes, including *Escherichia coli*, *Enterococcus faecalis*, *Campylobacter jejuni*, *S. aureus*, *Clostridium difficile*, *Bacillus* spp., *Streptococcus* spp., *Shigella flexneri*, *Helicobacter pylori*, *Salmonella enterica* serotype Typhimurium, *S. enterica* serotype Typhi [43,51,52], *Bifidobacterium* [53], *Lactobacillus* [54,55], *Eubacterium*, *Roseburia* and *Ruminococcus* [56]. Strains of *E. coli* [57], *Streptococcus pneumoniae* [58], *Streptococcus mutans* [59,60] and *Lactobacillus* [61,62] use the luxS system to regulate genes encoding bacteriocin production. The same group

of signaling factors is also used by *Bifidobacterium* to combat *Salmonella* infections [63]. An engineered strain of *E. coli* with increased production of AI-2 led to the reinstatement of streptomycin-repressed Firmicutes and suppressed the growth of Bacteroidetes [64]. It can be deduced from these findings that AI-2 may be used to restore balance in the gut microbiota after antibiotic treatment. Should this strategy be followed, it will have to be carefully controlled, as cytoplasmic levels of AI-2 are regulated by LsrK kinase (Figure 3). Mutants of *E. coli* with an inactive LsrK kinase were unable to phosphorylate AI-2 and lost their QS communication abilities [47,65]. However, when co-cultured with an AI-2-producing strain of *Vibrio harveyi*, the *E. coli* mutant responded to its own AI-2 and to that produced by *V. harveyi*. An increase in the adherence of *Actinobacillus pleuropneumoniae* to epithelial cells and an increase in the expression of motility genes in *E. coli* were observed in the presence of AI-2 [66,67]. In the case of *Helicobacter pylori*, AI-2 acted as a chemorepellent and prevented biofilm formation [68]. AI-2 may have the same anti-biofilm forming effect on Bacteroidetes, which would explain the decline in cell numbers as Firmicute numbers increase.

Changes in the populations of Firmicutes and Bacteroidetes alter the level and composition of SCFAs, which in turn affect gene expressions, cytokine secretion and regulatory T cell induction [69]. All these changes influence inflammatory responses. Increased levels of AI-2 could thus restore the balance between Firmicutes and Bacteroidetes and prevent, or revert, dysbiosis, IBD, obesity, autism and stress-related disorders. However positive this seems, the idea must be considered with care, as elevated levels of AI-2 may upregulate virulence, as shown with an increase in the release of bacteriophages from *Enterococcus faecalis* and an increase in gene transfer [70]. In mice, the administration of AI-2 had no effect on the expression of cytokines but aggravated *P. aeruginosa* lung infection by interfering with QS molecules produced by the pathogen [71]. These findings clearly show that implementing AI-2 aimed at maintaining gut homeostasis is far more complex and warrants further research.

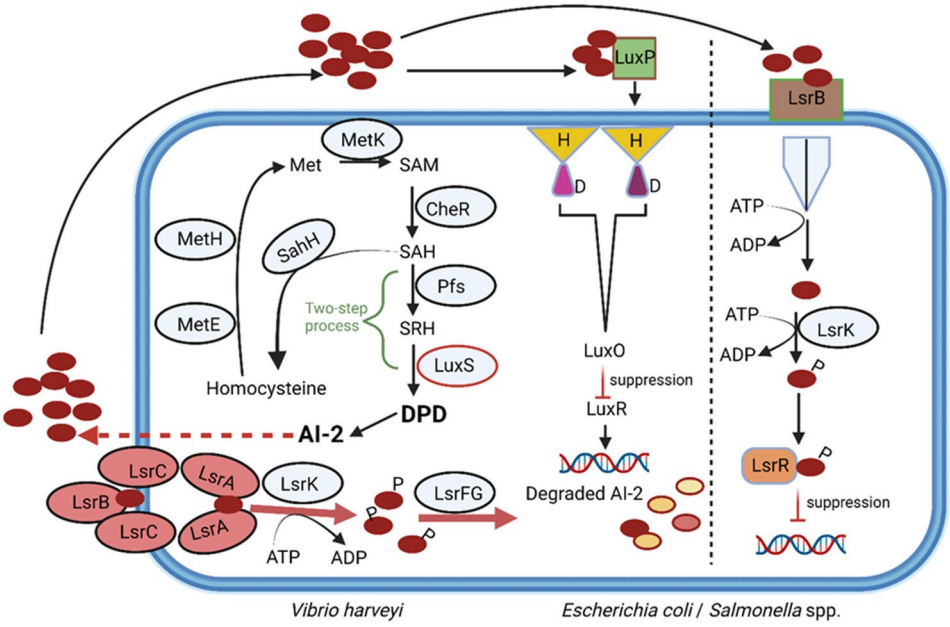

Figure 3. Production of AI-2, regulated by LuxS (an autoinducer synthase). S-adenosylhomocysteine (SAH) is converted to homocysteine by SAH hydrolase (SahH) in a one-step reaction but may also be produced from the cleavage of the thioether linkage of S-ribosylhomocysteine (SRH). This is a two-step reaction that requires SAH nucleosidase (Pfs) and LuxS. The intermediate 4,5 dihydroxy-2,3-pentanedione (DPD) is rearranged to form AI-2. Red circles = AI-2; LsrB-LsrC-LsrA = Lsr ABC-

type transporter; LsrK = Lsr kinase; LsrFG = genes F and G, part of the Lsr operon; MetE = cobalamin-independent methionine synthetase; MetH = cobalamin-dependent methionine synthetase; MetK = adenosylmethionine synthetase; CheR = methyltransferase; P = phosphate group; LuxO = a central regulator; LuxR = repressor. This illustration was constructed using BioRender (https://biorender.com/, assessed on 5 September 2022).

Two classes of AI-2 receptors have been identified, namely LuxP, common among members of Vibrionales, and LsrB (Figure 3), widely distributed across Proteobacteria, *Bacillus cereus* and *Bacillus anthracis* [72–74]. The two receptors are structurally different and share a sequence similarity of only 11% [75]. Other members of Firmicutes, including gut microbiota, may respond to AI-2 using receptors similar to LuxP and LsrB. *Streptococcus mutans* and *Staphylococcus epidermidis*, however, respond to AI-2 in the absence of these receptors [43]. The LuxS/AI-2 QS system regulates the expression of several genes, including drug resistance [50]. The effect of AI-2 on the host's immune system is less understood. Elevated levels of AI-2 were detected in tumors associated with colorectal cancer (CRC) [76]. This correlated with an increase in the expression of genes encoding TNFSF9 (tumor necrosis factor ligand superfamily member 9), as noted in tumor-associated macrophages [76]. AI-2 could thus be an important marker for CRC and warrants more research.

4. Interkingdom Communication

The autoinducer-3 (AI-3)/Epi/NE interkingdom signaling system [77] promotes the expression of virulence genes in pathogens such as *S. typhimurium, Citrobacter rodentium* and EHEC [78]. AI-3 controls the genes encoding the attachment of EHEC to epithelial cells in the colon (Figure 4), a process leading to the destruction of microvilli and the rearrangement of the cytoskeleton to form protective pedestal-like structures [79]. The direct effect of AI-3 on humans is unknown, apart from an increase in IL-8 production by THP-1 monocytes [80]. Epi and NE recognize the AI-3 receptor (Figure 4) [79] but do not activate or modulate adrenergic signaling [80]. Further research on microbial endocrinology is required to understand the effect of variations in AI-3 levels on the host.

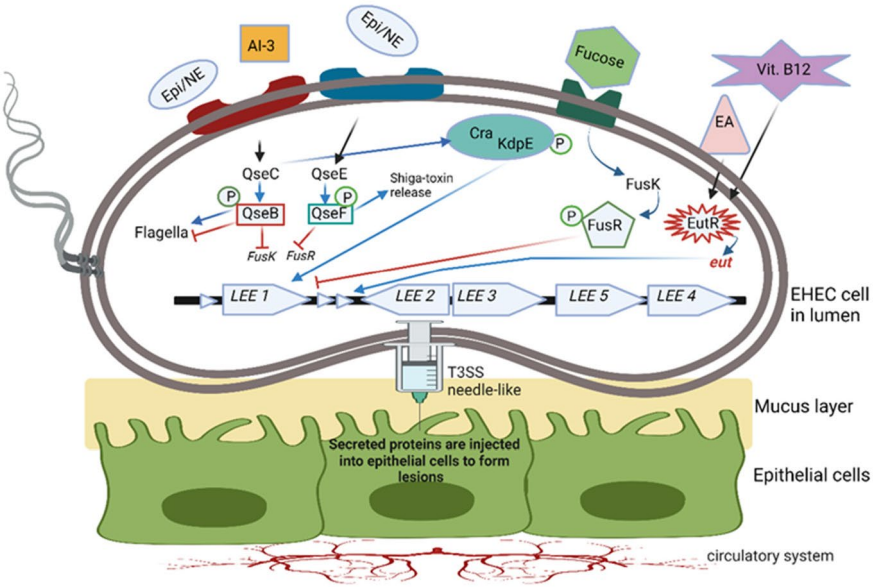

Figure 4. Two-component QS system (TCS) used by enterohemorrhagic *E. coli* (EHEC). Bacterial cells sensing environmental signaling compounds such as epinephrine (Epi), norepinephrine (NE), autoinducer 3 (AI-3), fucose, ethanolamine (EA) and vitamin B12 activate transmembrane histidine

kinase receptors (indicated here as blue and red rectangles). Response regulators either activate or repress the TCS. QseC histidine sensor activates QseB, and QseE activates QseF. QseB regulates the expression of flagella and, at the same time, represses the expression of *fusK/-R*, involved in fucose metabolism, and the expression of virulence genes. QseC controls the response regulator KdpE, which, together with Cra, stimulates genes in the LEE operon to form microscopic T3SS needle-like structures through which proteins are injected into the host cell. Microvilli on the surface of epithelial cells are eradicated, and lesions with actin-rich pedestals form, onto which EHEC cells attach. QseC also activates regulator QseF, which stimulates the production of Shiga toxin. FusK is activated by fucose and phosphorylates FusR to inhibit LEE expression. Genes in the *eut* operon encode the EutR transcription factor, which recognizes the presence of ethanolamine (EA) and vitamin B_{12}. Both compounds are required to promote the transcription of genes in the LEE operon. This illustration was constructed using BioRender (https://biorender.com/, assessed on 3 September 2022).

Intestinal pathogens such as EHEC, *Salmonella typhimurium* and *Citrobacter rodentium* regulate virulence by using a two-component QS system (TCS). In the case of EHEC, the TCS consists of the quorum-sensing *E. coli* regulators QseBC and QseEF (Figure 4). QseB is a response regulator with a receiver domain and a helix-turn-helix (HTH) DNA binding domain, QseC functions as a bacterial adrenergic receptor, QseE is a sensor kinase and QseF is a response regulator [81]. Cells sensing environmental signaling compounds such as Epi, NE, AI-3, fucose and ethanolamine (EA) respond by activating transmembrane histidine kinase receptors (shown as blue and red rectangles in Figure 4). Response regulators either activate or repress the TCS. The QseC histidine sensor activates QseB, which regulates the expression of flagella and, at the same time, represses the expression of *fusK/-R*, encoding fucose metabolism and the expression of virulence genes. QseC can also phosphorylate the response regulator KdpE, which, together with Cra, stimulates genes in the LEE operon to encode the formation of microscopic "needles" through which proteins are injected into the host cell. At the same time, microvilli on the surface of epithelial cells are eradicated, and lesions with actin-rich pedestals form, onto which EHEC cells attach (not shown in Figure 4). QseC may also activate the regulator QseF, which stimulates the production of Shiga toxin. FusK is activated by fucose and phosphorylates FusR to inhibit LEE expression.

The 3-oxo-C12-HSL produced by *P. aeruginosa* (Figure 5) is actively transported across epithelial and immune cells [2,82] and destroys the permeability of the gut wall by repressing the expression of genes encoding tight junction proteins (TJs). This leads to the re-arranging (misplacing) of occludin, tricellulin, ZO-1, ZO-3, JAM-A, E-cadherin and β-catenin and prevents mucin production [2,83,84]. This not only exposes epithelial cells to infection but also activates the mucosal immune system, leading to an increase in leucocytes and the accumulation of pro-inflammatory cytokines [85]. Furthermore, 3-oxo-C12-HSL also inhibits tumor necrosis factor (TNF)-α and IL-12 production, causes malfunctioning of the T helper cell-1 (Th1) response and stimulates Th2 to produce immunoglobulin G1 [86]. Inhibition of Th1 and Th2 T lymphocyte differentiation increases cytokine production [87], intensifies oxidative stress, stimulates apoptosis and inactivates mitochondria [2].

A structurally similar form of 3-oxo-C12-HSL, 3-oxo-C12:2-HSL, has the opposite effect on the gut wall. Instead of destabilizing epithelial cells, 3-oxo-C12:2-HSL protects the tight junction proteins occludin and tricellulin and cytoplasmic ZO-1 from pro-inflammatory cytokines such as interferon-gamma (IFN-γ), TNF-α and IL-8 [88–91]. Apart from a few pioneering studies, the impact of 3-oxo-C12:2-HSL on immune cells in the human intestinal tract remains largely unknown. Landman et al. [88] reported much lower concentrations of 3-oxo-C12:2-HSL in patients diagnosed with IBD. This suggests that 3-oxo-C12:2 HSL plays a major role in the protection of epithelial cells exposed to an immune onslaught. Further research is required to determine if 3-oxo-C12:2-HSL could be used in the treatment of IBD. This also requires a better understanding of the processes involved in 3-oxo-C12:2-HSL quorum quenching, the cleaving of AHL and the hydrolysis of the homoserine lactone (HSL) ring. Thus far, three paraoxonases (PON1, PON2 and PON3)

involved in the hydrolysis of the HSL ring have been identified in the GIT of humans and other mammals [92]. Of these, PON2 is the most active [93] and is predominantly expressed in the jejunum [94]. PON1 and PON3 are expressed at lower levels in patients diagnosed with Crohn's disease and ulcerative colitis [95]. It is thus possible that these gastrointestinal disorders may be reversed by reinstating PON1 and PON3 levels. An in-depth study on the role paraoxonases play in different areas of the GIT, and their possible application in the treatment of gastric disorders, is required.

Other examples of intestinal receptors interacting with bacterial cells or bacterial compounds are pregnane X receptors (PXR1 and PXR2) and peroxisome proliferator-activated receptors (PPARα, PPARβ/δ and PPARγ). Pregnane X receptors (PXRs) are primarily expressed by intestinal epithelial cells, but also to a lesser extent by kidney cells, T cells, macrophages and dendritic cells [96], and regulate the expression of proteins involved in detoxification and the metabolism of glucose, lipid, cholesterol and bile acid [97]. Peroxisome proliferator-activated receptors (PPARs) are widely expressed in human cells, including intestinal cells [98], and regulate energy production, lipid metabolism and inflammation. PPARα represses nuclear factor kappa B (NF-κB) signaling, which decreases the production of inflammatory cytokines [99,100]. PPARγ inhibits the activation of macrophages and the production of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-1β. These anti-inflammatory responses may restore gut dysbiosis and alleviate IBDs such as ulcerative colitis and Crohn's disease [101].

PQS and HHQ produced by *Pseudomonas* (Figure 1) interact with lymphoid cells, dendritic cells and macrophages, leading to the suppression of innate and adaptive immune responses [102,103]. In response, the aryl hydrocarbon receptor (AhR) senses the PQS signals and alerts the immune system to activate the most beneficial immune response [104,105]. This involves the expression of IL-22 and IL-17 [106]. Activation of AhR also stimulates the p38 pathway, which initiates the apoptosis of epithelial cells [107]. This is an excellent example of "eavesdropping" on an interkingdom level.

CSF, produced by *Bacillus subtilis*, binds to the cation transporter OCTN2 (Figure 5). This activates HSP-27, mediates the uptake of organic cations and carnitine and promotes intestinal barrier integrity [108]. Once in the cell, CSF acts as a reporter monitoring changes in the behavior or composition of the gut microbiota [109]. HSP-27 acts as a protein chaperone and an antioxidant but also facilitates the refolding of damaged proteins, thus preventing apoptosis and actin cytoskeletal remodeling [2,110]. The autoinducers AI-2 and AI-3 promote the expression of the immune mediators TNSF9 and IL-8 (Figure 5), and 3-oxo-C12:2-HSL reduces the production of IL-8 by epithelial cells. Host cells retaliate against QS by the binding of Epi and NE to AI-3 receptors (Figure 5). Epithelial cells then secrete AI-2 and PON that degrade HSLs (Figure 5).

PapRIV, produced by *Bacillus*, crosses the gastrointestinal tract (GIT), albeit slowly, and enters the circulatory system from where most peptides (87%) cross the BBB in a one-directional way. It can be deduced from in vitro studies that PapRIV activates microglia and may thus play a role in gut–brain interactions [111]. According to Yorick Janssens et al. [111], the second (aspartic acid) and the fourth (proline) amino acids play a key role in the activation of microglia. PapRIV also induces the production of the pro-inflammatory cytokines IL-6 and TNFα, increases intracellular ROS and stimulates an increase in ameboid cells [112]. Autoinducer peptides (AIPs) produced by *Clostridium acetobutylicum* cross the BBB much more easily than AIPs produced by *Streptococcus pneumonia* [113]. AIPs produced by Gram-positive bacteria crossing the gut wall have been shown with in vivo studies on Caco-2 cells [102,103]. De Spiegeleer et al. [114] have shown that AIPs produced by *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* in the GIT have pro- and anti-inflammatory effects on muscle cells. The crossing of these barriers seems to depend on the structure and size of the peptide. Small diffusible molecules produced during the degradation of signal peptides, referred to as diffusible signal factors (DSFs), may also act as autoinducers [115,116].

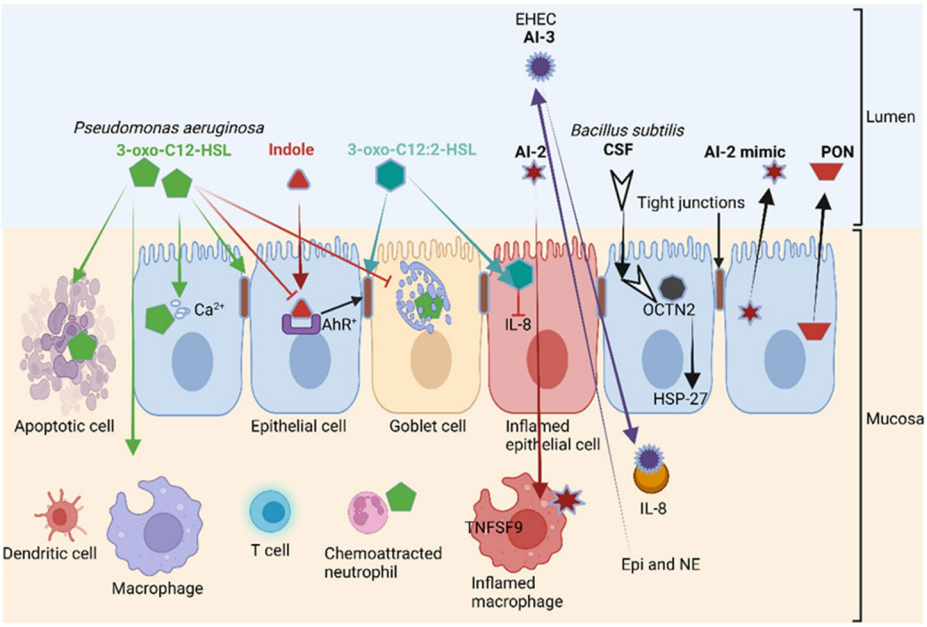

Figure 5. The 3-oxo-C12-HSL produced by *P. aeruginosa* (also shown in Figure 1) induces apoptosis in various cell types, including epithelial cells; disrupts tight junctions and decreases mucin production. Intestinal acyl-homoserine lactone (3-oxo-C12:2-HSL) and indole (produced from tryptophan) protect tight junctions. The competence and sporulation factor (CSF), a pentapeptide produced by *B. subtilis*, binds to the cation transporter OCTN2 and activates heat shock protein 27 (HSP-27), which increases the strength of intestinal barriers. Meanwhile, 3-oxo-C12-HSL stimulates chemoattraction and phagocytosis in neutrophils and induces cell death. The autoinducers AI-2 and AI-3 induce the expression of the immune mediators TNSF9 and interleukin (IL)-8, respectively, leading to the inflammation of macrophages, while 3-oxo-C12:2-HSL reduces IL-8 production. Epinephrine (Epi) and norepinephrine (NE) bind to the AI-3 receptor in enterohemorrhagic *E. coli* (EHEC), thereby interfering with QS. Intestinal epithelial cells secrete a mimic form of AI-2 and paraoxonase (PON) to degrade HSLs. AhR: Aryl hydrocarbon receptor. This illustration was constructed using BioRender (https://biorender.com/, assessed on 6 September 2022).

Signals generated by gut bacteria are recorded by specialized cells in the gut wall (Figure 6), resulting in temporary or long-lasting changes in physical or mental health. These cells differentiate between signals produced by autochthonous (endemic) and foreign, potentially pathogenic, microbiota by using pattern recognition receptors (PRRs). The pro-inflammatory properties of AHLs, associated with an increase in neutrophil activity and the differentiation of fibroblasts into myofibroblasts, are crucial for tissue regeneration [117,118]. These onslaughts on the immune system are driven by systems independent of pathogen-associated molecular pattern (PAMP) recognition pathways, TLRs and the nucleotide-binding oligomerization domain proteins Nod1 and Nod2 [99]. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) specialize in the recognition of microbial cell wall components, and G protein-coupled receptors (GPRs) activate G proteins involved in hormonal regulation [119,120] (Figure 6). GPRs are localized on the surface of intestinal immune cells, leukocytes, regulatory T cells, monocytes, macrophages and colonic epithelial and mesenchymal cells, thus playing a role in anti-inflammatory and pro-inflammatory processes and barrier control [121] (Figure 6).

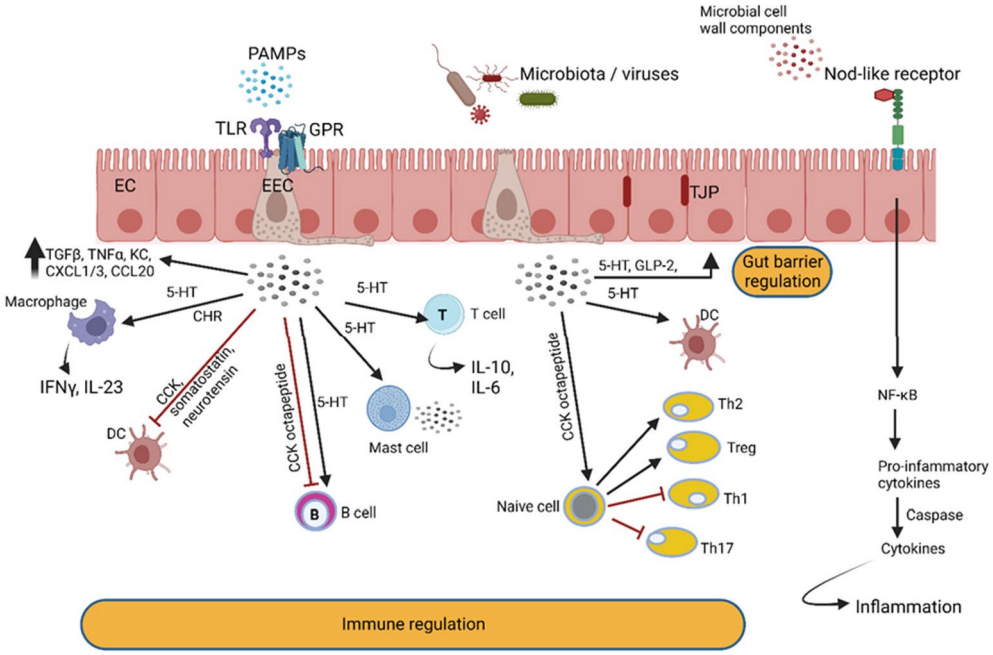

Figure 6. Enteroendocrine cells (EECs) in the gut wall detect intestinal bacteria and microbial metabolites and react by secreting peptide hormones and cytokines that react with immune cells. Hormones produced by EECs modulate intestinal barrier functions and react with enteric nerves. The latter communicate with the central nervous system via the vagus nerve. PAMPs = pathogen-associated molecular patterns; TLR = Toll-like receptor; GPR = G protein-coupled receptor; TGF = tumor growth factor; TNF = tumor necrosis factor; KC = neutrophil chemokine; CXLC = chemokine (C-X-C motif) ligand; CCL = chemokine ligand; IFN = interferon; IL = interleukin; CCK = cholecystokinin; DC = dendritic cell; 5-HT = serotonin; Th = T helper cell; Treg = regulatory T cell. This illustration was constructed using BioRender (https://biorender.com/, assessed on 6 September 2022).

AhRs regulate immune responses and pathogenesis (Figure 7) [122]. Large quantities of AhR are expressed by intestinal epithelial cells and immune cells such as innate lymphoid cells, intraepithelial lymphocytes, TH17 cells and Treg cells but are also present in the liver, lung, bladder and placenta [123–125]. One of the key functions of AhR is restoration of barrier homeostasis, a phenomenon eminent in IBD [7].

AhR is activated by metabolic compounds produced from the bacterial degradation of tryptophan [126] but also by 2,4-dihydroxyquinoline (2,4-DHQ) and quinolone derivatives [127], pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxylic acid and phenazine-1-carboxamide [128]. Tryptophan degradation products such as indole, indolo [3,2-b]carbazole, indole acetic acid (IAA), 3-methylindole and tryptamine react with AhR and stimulate the production of interleukin-22 [126,129,130]. Individuals with inflammatory bowel disease (IBD) [131], metabolic syndrome [132] or celiac disease [133] have decreased fecal concentrations of AhR ligands and reduced AhR activity. Higher indole concentrations were detected in patients suffering from *Clostridioides difficile* (*Clostridium difficile*) infection (CDI) compared to healthy individuals [134]. Elevated levels of indole produced by enterotoxigenic *E. coli* stimulated the colonization of *C. difficile* whilst other intestinal bacteria were repressed [134]. When indole was administered to GF mice, the expression of genes encoding tight junction proteins increased and improved the resistance of epithelial cells to colitis [135]. Since Trp is not synthesized by gut microbiota or the host, indole levels are directly linked to diet. Roasted cashew nuts, sunflower seeds, cheddar cheese, chicken breast and boiled eggs are rich in Trp [136].

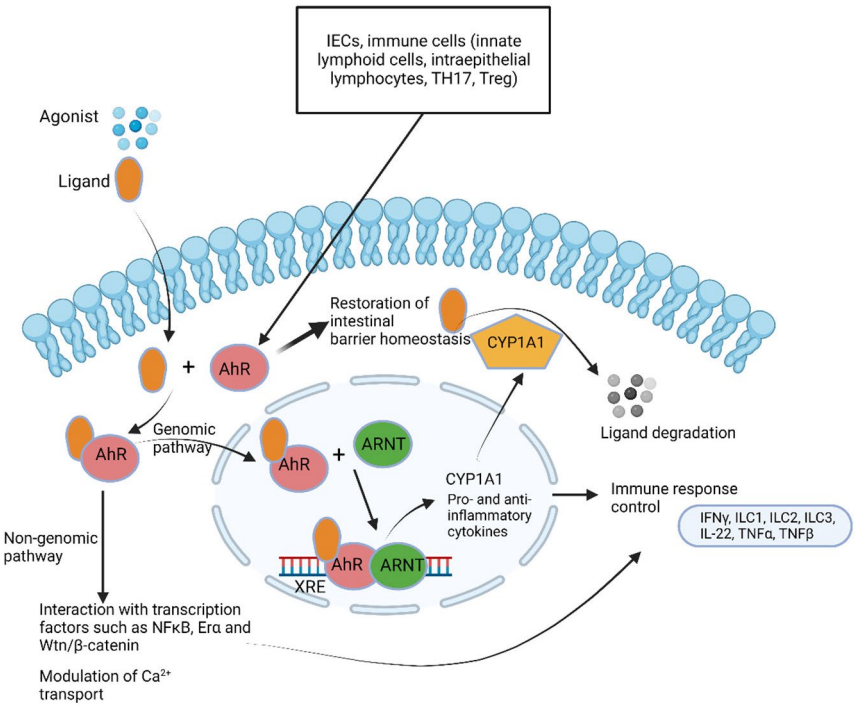

Figure 7. Summary of aryl hydrocarbon receptor (AhR) pathways. AhR ligands such as 6-formylindolo [3,2-b]carbazole; 2,3,7,8-tetrachlorodibenzo-p-dioxin and polycyclic aromatic hydrocarbons cross the cytoplasmic membrane and bind to AhR. The ligand–AhR complex crosses into the nucleus and forms a heterodimer with the AhR nuclear translocator (ARNT). Binding of the ligand–AhR–ARNT complex with xenobiotic response elements (XREs) leads to the expression of cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1). CYP1A1 degrades AhR ligands. This leads to the induction of several genes encoding pro-inflammatory and anti-inflammatory cytokines. The non-genomic pathway involves interaction between AhR and other transcription factors and modulates Ca^{2+} transport. This illustration was constructed using BioRender (https://biorender.com/, assessed on 6 September 2022).

The importance of AhR cannot be overemphasized as it plays a crucial role in barrier integrity; intestinal and immune homeostasis, mostly due to the regulation of tight junction proteins; the generation and survival of intraepithelial lymphocytes (IELs); the production of IL-22 and IL-10; the regulation of peristalsis and microbiota density; the regulation of goblet cell differentiation in the colon, specifically preventing goblet cell depletion in the elderly [2,122,127,137]; and the stimulation of antimicrobial peptide production via IL-22 [138–142]. Individuals with IBD and celiac disease have low levels of AhR in their feces [133]. Similarly, patients with Crohn's disease have decreased levels of AhR, especially in the ileum, and have the tendency to convert ILC3 to type 1 innate lymphoid cells (ILC1) [143,144]. ILC1 elicits the production of IFNγ and TNFα, which are both known to induce apoptosis of epithelial cells in vitro, and most likely also in vivo, disrupting the intestinal barrier and increasing microbial-driven inflammation [145]. LC1 also induces TGFβ, which is associated with tumor formation and colorectal cancer (CRC) [145]. Further research is required to have a better understanding of the cross-talk between host AhR and bacterial QS molecules.

5. Can QS Be Used to Control Microbial Infections?

Five years ago, the World Health Organization (WHO) published a list of pathogenic bacteria most resistant to currently used antimicrobials. *Acinetobacter baumannii*, *P. aeruginosa* and enterobacteria resistant to carbapenems and species producing extended spectrum beta-lactamases (ESBLs) were amongst the top ones on the list [146]. This urged

many scientists to investigate the possibility of using anti-QS therapy, referred to as quorum quenching (QQ), to prevent or control bacterial infections [147]. In recent years, many published articles have reported promising results indicating the possibility of reducing the pathogenicity of microorganisms and easier eradication when co-treated with antibiotics. Wu et al. [148] suggested that AHL-based QS may be used to control infections caused by Gram-negative bacteria. This approach is only possible where disrupting QS has a major effect on the expression of virulence genes [149]. Limited successes were reported using QQ in the treatment of infections caused by *P. aeruginosa* and *S. aureus* [150], notably concerning biofilm-associated infections [151]. The rationale behind this concept is to interrupt receptor proteins, degrade autoinducing signals or inhibit the synthesis of QS signaling molecules [152–155]. Another approach is using synthetic compounds analogous to QS signaling molecules [156].

Van den Abbeele et al. [157] reported a decline of approximately 60% of mucosa-associated pathogens, mostly *Clostridium* spp., when QQ was applied. Although promising from a perspective of infection management, such drastic changes may lead to the development of pro-inflammatory diseases such as cystic fibrosis [158], sclerosis [159] and IBD [160,161] and an increase in *Enterococcus* and *C. difficile* cell numbers [162]. Perhaps the most alarming of all is evidence of increased cell aggregation and biofilm formation in bacteria with a dysfunctional or absent luxS QS system, as reported for *Helicobacter pylori* [163], *Vibrio cholerae* [164], *Aggregatibacter actinomycetemcomitans* [165], *Actinobacillus pleuropneumoniae* [166], *Haemophilus parasuis* [167], *S. aureus* [168], *S. epidermidis* [169], *Streptococcus mutans* [170], *Enterococcus faecalis* [171] and *Bacillus cereus* [172]. Meropenem and levofloxacin stimulated the expression of an efflux pump in *A. baumannii* that promoted the release of an AHL, resulting in an increase in QS-mediated biofilm formation [173]. Ciprofloxacin, ceftazidime and azithromycin prevented QS when used at sub-inhibitory concentrations [174]. Despite these challenges and limitations, QQ may still be the answer to controlling bacterial infections [147,153–155,175–177], especially those caused by multi-drug-resistant (MDR) and extensively resistant microorganisms (XDR) [178]. However, with the variable reactions of pathogens to QQ therapy combined with antibiotics, the treatment of infections will have to be evaluated on a case-to-case basis

Strains may develop resistance to QS inhibitors, as shown for *P. aeruginosa* exposed to brominated furanones. Resistant cells had mutations in genes encoding efflux pumps [179]. Strains of *P. aeruginosa* resistant to carbapenems and azithromycin lost antibiotic-associated QS inhibition [180]. *Streptococcus pyogenes* and *S. aureus* mutants without LuxS (Δ*luxS*) were more resistant to macrophages [181,182]. This may lead to the development of persistent pathogens difficult to eradicate. For further information on resistance to QQ, the reader is referred to Defoirdt et al. [183], García-Contreras et al. [179], Kalia et al. [184] and Liu et al. [185].

In the case of *S. aureus,* the accessory gene regulator (agr) regulates the expression of several genes, including virulence factors such as formylated peptides, proteases and pore-forming toxins (PFTs) such as hemolysins, leucocidins and phenol-soluble modulins (psms) [38]. Strains of *S. aureus* lacking the *agr* gene (Δ*agr*) are more prone to causing chronic infections and bacteremia [39,38]. These findings suggest that treatment of *S. aureus* infections with QQ is not an option.

Pseudomonas aeruginosa uses psms to alter cell membrane properties [186] and activate the immune system [187]. These amphipathic peptides lyse neutrophils, erythrocytes and T cells [186]. By using QS inhibitors, the number of microbial enzymes degrading mediators of inflammatory responses may be limited. Since oxidative stress selects for cells with an active QS system [188], success in treatment using QQ techniques depends on the exposure of the infected area to oxygen and the overall immune status of the individual [189].

An interesting advance in QS research in the last decade is the discovery that QSPs may promote tumor cell invasion and angiogenesis (at least in vitro), suggesting that these peptides may stimulate stem cell differentiation and the migration of cancer stem cells

[190,191]. The influence microbiota have over colon cancer stem cells, "instructing" them to become treatable or non-treatable, was raised by Trosko and Lenz [192].

6. Effect of QS on the CNS and Mental Health

The effect of QS molecules on the CNS is ill-researched. Several QSPs can diffuse through the intestinal mucosa and enter the circulatory system, from where they may penetrate the blood–brain barrier (BBB) [113]. Based on these findings, QSPs may play a key role in communication between the gut microbiome and the brain. If this is the case, QSPs may affect neurodevelopment and initiate neurodegenerative diseases. Further research is needed to confirm these findings.

Exotoxins produced by *S. aureus* activate the transcription factor accessory gene regulator (Agr)A, which regulates the expression of several genes, including virulence factors, pore-forming toxins (PFTs) and bacterial proteases [37]. These toxins increase intracellular calcium levels, leading to the activation of sensory neurons [193]. This is especially true for psms attached to formyl peptide receptor-like proteins (FPRs) [194]. The structural similarity of FPRs to b-defensins and ligands of mas-related G protein-coupled receptor (MRGPR) X2 [195] suggests that MRGPRs are involved in psm-mediated effects such as skin allergies [196]. The expression of FPRs in sensory and dorsal root ganglia of the colon has been well documented and linked with QS-dependent pathways involved in the gut–brain axis (GBA) [197,198]. The pore-forming toxin alpha-hemolysin (Hla) produced by *S. aureus* excites neurons by increasing the transfer of calcium [199]. According to Uhlig et al. [198], Hla produces smaller, less disruptive pores in cell membranes compared to psms [200]. The authors have also observed the expression of Adam10, a membrane-bound metalloprotease produced in sensory neurons, to which Hla binds [201]. The importance of exotoxins in GBA communication is unknown. However, since *S. aureus* is associated with irritable bowel syndrome and food [202,203], these QS molecules have the potential to directly modulate gut–brain communication and intestinal reflex.

Janssens et al. [204] screened 85 quorum sensing peptides on six different neuronal cell lines and found 22 peptides with a possible effect on the GBA. Of these, four peptides induced neurite outgrowth, two peptides inhibited nerve growth factor (NGF)-induced neurite outgrowth and eight peptides induced neurite outgrowth in human SH-SY5Y neuroblastoma cells. Two peptides killed SH-SY5Y cells and six peptides induced either IL-6 expression or nitric oxide (NO) production.

Several reports have been published on the role that cell wall components such as lipopolysaccharides, polysaccharides and peptidoglycans play in neuron activation and the GBA [37,205–208]. Cell wall components also induce the release of neuropeptides, ATP and cytokines [209]. Short-chain fatty acids, tryptophan, trace amines [142,210] and exotoxins [182] also have neuromodulator properties. Serotonin and histamine excite mast cells in the proximity of nerve endings [210,211].

Neuronal conditions such as Alzheimer's disease (AD), autism spectrum disorder (ASD), multiple sclerosis (MS), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are associated with dysfunctional microglia [212]. Fecal transplants from humans with attention deficit hyperactivity disorder (ADHD), AD and PD to mice activated microglia in the brain and aggravated cognitive and physical impairments [213–215]. These findings along with more evidence of a clear link between microbial dysbiosis and neurodevelopmental, neurodegenerative and psychiatric disorders such as ASD, schizophrenia, AD, major depressive disorder (MDD) and PD [216–219] prompted researchers to have a closer look at the GBA. For more information on gut bacteria and neurotransmitters, the reader is referred to a recent review by Dicks [220]. The role that gut bacteria play in neuropsychiatric disorders has recently been reviewed by Dicks et al. [221].

7. Conclusions

With bacteria representing the largest fraction of almost 3000 microbial species in the GIT, it is no surprise that they have developed mechanisms to communicate with host

cells. Some QS molecules are genus-specific, but a few are used by Gram-negative and Gram-positive bacteria. Hormones such as Epi and NE and certain carbohydrates (e.g., fucose and EA) activate specific receptors in bacteria that, in turn, trigger sensors in the cytoplasm to regulate gene expressions. In a healthy GIT, these signaling molecules are important in maintaining a homeostatic status. Some QS molecules, such as 3-oxo-C12:2-HSL, protect tight junction proteins and may be important in the treatment of leaky gut syndrome. Some QS molecules stimulate tumor growth and are closely associated with the development of specific cancers, whilst others are linked to neurological disorders. QSPs that penetrate the blood–brain barrier (BBB) constitutes an area that warrants more research, especially since the gut microbiome is increasingly recognized as a key player in neuropsychiatry. This warrants a better understanding of the underlying mechanisms involved in the regulation of gut bacterial QS systems. We also need to have a better understanding of bacterially produced QS molecules in adrenal pathways. With more in-depth knowledge of the different QS systems produced by intestinal bacteria, we may be able to develop biomarkers that can be used to diagnose neurological and psychiatric diseases such as anxiety and depression, MDD, schizophrenia, bipolar disorder, autism and OCD. More research is required to understand the integral communications between autoinducer-based signaling molecules and neurons in the human CNS. Research on using QS inhibitors (QQ therapy) to control microbial infections is gaining interest. Although a microbial infection may not be fully controlled using this strategy, it may provide the host's immune system a better chance to overcome the infection. Before this approach can be implemented, we need to investigate the effect that QS inhibitors may have on non-pathogenic, beneficial, commensal gut microbiota.

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Chapter 8

Gut Bacteria, Neurotransmitters and Neuropsychiatric Disorders

Do bacteria use neurotransmitters solely to establish communication with the CNS to release molecules in the bloodstream that regulate physiological functions in the gut wall, or are there other more direct benefits?

What role does gut bacteria play in the maintenance of neuronal pathways and how are signals, generated by gut bacteria, orchestrated to communicate with the CNS?

To answer these questions, we need to have a better understanding of the receptors on neurons, neuro-signaling pathways, and other physiological functions of signaling molecules.

Could SCFAs produced by gut bacteria play a central role in neurotransmission?

Would it be possible to develop novel therapeutics and probiotics to treat gastro-intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)?

How far away are we in the design of psychobiotics to improve cognitive functions and prevent or treat mental disorders?

This chapter is presented in two sections:

Gut Bacteria and Neuropsychiatric Disorders, published by Dicks, L.M.T., Hurn, D. and Hermanus, D., **2021**, *Microorganisms* 9, 2583. https://doi.org/10.3390/microorganisms9122583

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microorganisms

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Review

Gut Bacteria and Neuropsychiatric Disorders

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Abstract: Bacteria in the gut microbiome plays an intrinsic part in immune activation, intestinal permeability, enteric reflex, and entero-endocrine signaling. Apart from physiological and structural changes brought about by gut bacteria on entero-epithelial cells and mucus layers, a vast number of signals generated in the gastro-intestinal tract (GIT) reaches the brain via the vagus nerve. Research on the gut–brain axis (GBA) has mostly been devoted to digestive functions and satiety. Less papers have been published on the role gut microbiota play in mood, cognitive behavior and neuropsychiatric disorders such as autism, depression and schizophrenia. Whether we will be able to fully decipher the connection between gut microbiota and mental health is debatable, especially since the gut microbiome is diverse, everchanging and highly responsive to external stimuli. Nevertheless, the more we discover about the gut microbiome and the more we learn about the GBA, the greater the chance of developing novel therapeutics, probiotics and psychobiotics to treat gastro-intestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), but also improve cognitive functions and prevent or treat mental disorders. In this review we focus on the influence gut bacteria and their metabolites have on neuropsychiatric disorders.

Keywords: gut microbiota; mental health

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1. Introduction

The human gut hosts close to 4 trillion microorganisms and represents between 400 and 500 species [1,2]. Slightly outnumbered by our gut microbiota (1.3:1), it is no surprise that the genetic material they carry represents 99% of our total genetic makeup [2–4]. At natural birth, the gastro-intestinal tract (GIT) of an infant is largely colonized with microorganisms from the mother's uterus and vagina [5,6]. However, bacteria from the placenta, amniotic fluid and circulatory system of the mother may reach the fetus before birth [7–13]. Mechanisms involved in the translocation of bacteria from the mother to the fetus have not been well documented. It may be that bacteria from the mother's GIT are captured by dendritic cells (DCs) penetrating the gut epithelium and are then translocated to lymphoid tissue and the placenta [14,15]. This hypothesis was proven with the transfer of a genetically labelled strain of *Enterococcus faecium* from pregnant mice to off-spring [8,9]. Another study conducted on rats [16] have shown that bacteria can be transferred to off-spring during pregnancy and lactation. The presence of *Escherichia*, *Enterococcus*, *Staphylococcus* and *Propionibacterium* in murine blood isolated from the umbilical cord indicated that bacteria may reach the fetus via the placenta and bloodstream [9,13]. Dasanayake et al. [17] reported that *Actinomyces naselundii*, normally present in the oral cavity, may reach the uterus via the circulatory system. This was supported by high cell numbers of oral microbiota in the placenta of healthy mothers [11].

Drastic changes in maternal gut bacteria have been recorded throughout pregnancy. In 57% of pregnant women studied, cell numbers of proteobacteria and actinobacteria increased drastically [18]. The first three months of pregnancy is characterized by an increase in butyrate-producing *Faecalibacterium* and *Eubacterium* spp. During the last three months higher cell numbers of *Enterobacteriaceae, Streptococcus* spp. and proteobacteria

have been reported. The latter is known to promote inflammatory responses, but is kept under control with elevated cytokine levels at the placental interface [19,20].

Infants are generally not affected by later changes in the mother's gut microbiome and tend to maintain a bacterial population characteristic to that of the mother during the first three months of pregnancy. However, should the placenta of mothers be infected with *Prevotella* and *Gardnerella*, newborns may develop distinctive inflammatory responses [21,22]. Transfer of microorganisms to the fetus and colonization of the GIT is not only influenced by the mother's health and changes in physiological conditions, but also by stress, alcohol, nicotine, and medication prescribed during pregnancy [21,22]. Detailed studies performed on the meconium of healthy fetuses, and the first stool of newborns revealed that *Streptococcus mitis*, *Lactobacillus plantarum*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, staphylococci and enterococci are amongst the first bacteria to colonize the GIT [23–25].

Further development of the gut microbiome is highly dependent on the infant's health. Unusually high cell numbers of *Bacteroidetes* have been isolated from the GIT of diabetic infants [26]. In another study [27], high cell numbers of lactic acid bacteria and enteric bacteria present in the meconium were associated with maternal eczema and respiratory problems later in life. Most researchers are of the opinion that vast changes in composition of gut microbiota occur during the first two years of an infant's life [28]. A metagenomic study conducted on 98 infants and their mothers have shown that one-year-old infants delivered via Caesarean (C)-section hosted *Enterobacter hormaechei*, *Enterobacter cancerogenus, Haemophilus parainfluenzae*, *Haemophilus aegyptius*, *Haemophilus influenza*, *Haemophilus haemolyticus, Staphylococcus saprophyticus*, *Staphylococcus lugdunensis*, *Staphylococcus aureus*, *Streptococcus australis*, *Veillonella dispar*, *Veillonella parvula* and a few *Bacteroides* spp. [29]. In contrast, the GIT of same age infants vaginally delivered contained fewer species, with *Bacteroides*, *Bifidobacterium*, *Parabacteroides*, *Escherichia* and *Shigella* the core bacteria [29]. During the first 4 months of the screening program 52 MetaOTUs (metagenomic operational taxonomic units) identified in a group of mothers could not be in the GIT of their infants. The species were thus either not transmitted to the infants or did not colonize the GIT of infants during the first few months [29]. On the other hand, *Propionibacterium acnes*, *Streptococcus agalactiae* and *Veillonella* spp., identified in more than 10 newborns, were not detected in any of the mothers [29]. The developing of a gut microbiome is thus clearly far more complex than originally understood and the first 5 years seem to be the critical phase in developing a core group of microorganisms [30]. During these years, changes in gut microbiota are influenced by altering physiological conditions and diet. *Bacteroides* spp., for example, are associated with high-fat or high-protein diets and *Prevotella* spp. with high-carbohydrate diets [31].

Accumulating evidence concurs that abnormal or disturbed gut microbiota is a contributing factor to the pathophysiology of various neurological and psychiatric diseases, including anxiety and depression, major depressive disorder (MDD), schizophrenia, bipolar disorder, autism and obsessive-compulsive disorder (OCD). It is thus important to learn more about the effect a healthy, balanced, gut microbiome has on the CNS, but also understand the effect an imbalanced microbiome (GIT in dysbiosis) has on gut–brain communication.

Exploration of the intimate cross-talk between the gut and brain may further unveil novel approaches towards combatting various disorders associated with the GBA. This cross-talk extends across a multitude of pathways, involving endocrine, immune and neural mechanisms which depend on extensive interactions between gut microbes and host. It is thus important to explore signals produced by gut microbiota and study the influence these pathways have on neuropsychiatric disorders. This review addresses the influence of gut bacteria and their metabolites have on a select few neurological and psychiatric diseases.

2. Gut Microbiota Alters Neural Signals

The bidirectional communication between gut microbiota and the brain is illustrated in Figure 1. The first bacteria that colonize the GIT of a new-born are aerobic and convert

lactose in breast or formula milk to organic acids and short chain fatty acids (SCFAs) [32]. The glucose component in milk is critical in the shaping of an infant's gut microbiome [32], but also plays an important role in brain development [33,34]. This is especially true for vaginally born infants. As *Lactobacillus* spp. represents the largest component of vaginal bacteria [11], they may have a profound influence on the manifestation of the initial gut microbiome and may play a distinctive role in the development of the central nervous system (CNS).

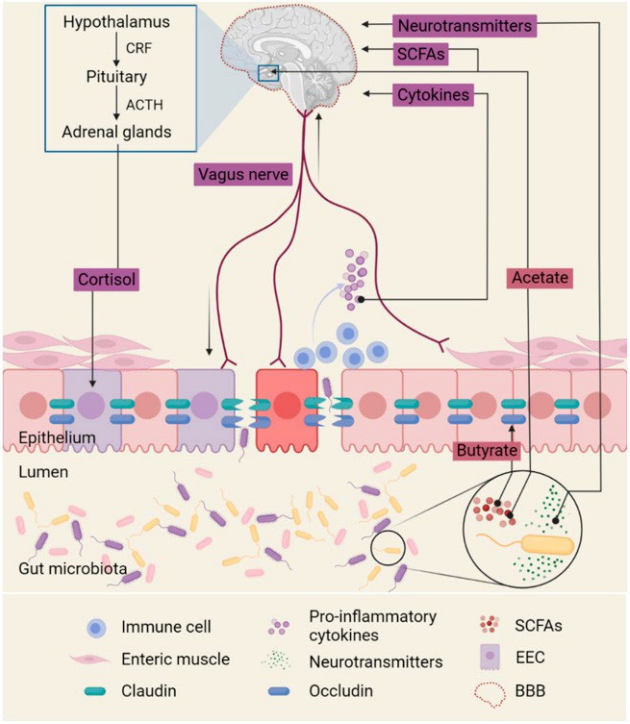

Figure 1. Mechanisms of bidirectional communication between gut microbiota and the brain. A network of entero-epithelial cells (EECs) along the gut wall mediates the bidirectional communication. In response to various stimuli and external cues, the central nervous system (CNS) modulate EECs via vagal efferents and the hypothalamic pituitary adrenal (HPA) axis. Gut microbiota return signals to the brain through different afferent pathways. Microbial metabolites, cytokine induction and neurotransmitters function via endocrine pathways; vagal afferents form part of the neurocrine pathway. Short chain fatty acids (SCFAs) produced by bacteria in the gut include acetate, lactate, butyrate and propionate. SCFAs modulate the integrity of the blood–brain barrier (BBB). Butyrate induces the expression of tight junction proteins, including claudins and occludins, and is therefore important for maintaining gut epithelial barrier integrity. A disrupted barrier encourages translocation of gut microbiota and their metabolites from the lumen to the circulatory system, resulting in the production of pro-inflammatory cytokines by immune cells, which can lead to changes in cognition and mood. Acetate crosses the BBB and accumulates in the hypothalamus, thereby controlling appetite. The bidirectional flow of information via the gut–brain axis can modify the gut microbiota and modulate behavior, mood and mental health.

Anaerobic bacteria in the large intestine produce acetate, lactate, butyrate and propionate. Butyrate acts as an inhibitor of histone deacetylase (HDAC) [12,35]. This is an important observation, as studies conducted on animals with HDAC inhibitors have shown promising results in the treatment of brain trauma and dementia [35]. Overproduction of HDAC has been implicated in neurological disorders such as Parkinson's disease, schizophrenia, and depression [35]. On the other hand, an increase in acetylated histones (ACHs) elevates the expression of the *bdnf* (brain-derived neurotrophic factor) gene in the frontal cortex and hippocampus, stimulating brain development [36,37]. Decreased levels of BDNF are linked to mood changes, depression, and anxiety [38–41]. Studies on

germ-free mice have shown lower levels of BDNF expression in the hippocampus [36,39]. Similar findings have been reported in mice treated with antibiotics and antimicrobial supplements [36,38]. Treatment of neurological disorders may thus vest in the control of SCFAs and HDAC levels. This emphasizes the importance of a well-balanced gut microbiome.

Fluctuation in butyrate levels may be due to inadequate numbers of intestinal butyrate-producing bacteria, or abnormal high binding of butyrate to free fatty acid receptors (FFARs) located on entero-epithelial cells (EECs) [35]. Butyrate also activates certain G-protein-coupled receptors (GPCRs) and is associated with multiple neurodegenerative disorders [12,35]. Butyrate is also known to promote regulatory T cells and subsequently produce inflammatory cytokines [42]. The increased anti-inflammatory response keeps *Proteobacteria* numbers in the GIT under control and, by doing so, also prevents the production of inflammatory cytokines [43]. Controlling butyrate levels in the GIT is important, as a decrease inhibits GPCRs and interrupts immune or endocrine responses [44]. Apart from this, butyrate and other SCFAs also modify the integrity of the blood–brain barrier (BBB), thus affecting the CNS and maturation of microglia [44,45]. In germ-free (GF) mice, the malfunctioning of microglia could be reversed by administering high levels of a combination of butyrate, propionate and acetate [46]. The function of acetate is different in that it crosses the blood–brain barrier and accumulates in the hypothalamus from where it controls appetite [47]. Activation of the hypothalamic-pituitary-adrenal (HPA) axis also affects the enteric nervous system (ENS) which, in turn, sends signals to EECs [12].

Butyrate induces the expression of the tight junction proteins claudin-2, occludin and cingulin [48]. This minimizes the translocation of microorganisms and their antigens across the gut wall and is described as an anti-inflammatory response [35]. Propionic acid displays properties similar to butyrate [35]. However, propionate may act as a neurotoxin and is associated with autism [49]. Translocation of bacteria and their antigens from the lumen to the circulatory system stimulate the secretion of pro-inflammatory cytokines such as interleukins (IL-6, IL-1b), tumor necrosis factor-alpha (TNF-α), and C-reactive protein [35,50,51]. Other studies have shown that an increase in these cytokines lead to changes in cognitive behavior and mood [48,52]. Immunologically induced GI barrier defects in rodents caused neurodevelopmental-related behavioral disorders [53]. Rodents exposed to specific pathogens showed anxiety-like behavior and impaired cognitive functions [13]. Obese mice on a high-fat diet produced offspring that were more prone to social and behavioral dysfunctions [45], confirming that gut microorganisms play a critical role in neural signaling and mental health. The role gut microorganisms play in control of behavior, mood, and stress-related brain disorders is a relatively young, but fast evolving, research field [36].

Given the substantial influence of the gut microbiota on neurodevelopment and sequential neurological health, a balanced gut microbiome is imperative for favorable brain development and a healthy mental status. This is especially important in neonates, as the brain is then most vulnerable to internal and external changes [13]. However, the brain is also susceptible to environmental and pathological adversities during adolescence and is thus sensitive to signals leading to neurodevelopmental and brain disorders. Growing up is associated with drastic changes in hormones. Although the composition of gut microbiota remains relatively stable during adulthood, changes in populations may still influence behavior [13]. The GIT secretes more than 20 hormones that bind to specific receptors that communicates with the CNS. Production of hormones is regulated by gut nutrient content, and the interaction between gut microbiota and intestinal epithelial cells [54,55]. Chemical signals generated by EECs, either directly or in response to microbial metabolites, travel through the ENS and regulates digestion, salivation, lacrimation, urination, defecation and sexual arousal [56]. A clear association exists between chronic stress and gut inflammation disorders, such as IBD and IBS [57]. Signals from the CNS are sent back to EECs and gut microbiota via the ENS and peripheric nervous system (PNS) [58]. In a healthy person, the bi-directional flow of information through the GBA helps to keep the gut microbiota in a homeostatic state.

3. Gut Microbiota Regulates Serotonin Levels

Serotonin (5-hydroxytryptamine or 5-HT) plays a vital role in neuronal and endocrine signaling pathways [53] and is involved in the regulation of appetite, sleeping patterns, mood, and cognition [40,48]. Although serotonin is synthesized by enterochromaffin cells (EC) and neurons of the ENS (Figure 2), more than 80% is produced in the GIT by *E. coli*, and species of *Lactococcus, Lactobacillus, Streptococcus, Morganella, Klebsiella, Hafnia, Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Eubacterium*, *Roseburia* and *Prevotella* [48,53]. Enteric serotonin levels are regulated by tryptophan hydroxylase TPH1 and serotonin from the ENS by tryptophan hydroxylase TPH2 [59]. Furthermore, the expression of *Tph1* (one of two tryptophan hydroxylases), is induced by SCFAs [53], whereafter TPH1 modulates EC-cell derived serotonin [59]. This confirms the association of elevated levels of SCFAs with a decrease in anxiety and depression-like behaviors [48]. At physiological concentrations, SCFAs have been noted to cause an eight- to ten-fold increase in serotonin production, at least in an in vitro colonic mucosal system [12]. Excess serotonin is transported across the cell membrane by a serotonin reuptake transporter (SERT) and intracellularly inactivated by monoamine oxidase (MAO) [59]. Homologs of eukaryotic monoamine transporters produced by bacteria therefore play an important role in the distribution of serotonin in the gut mucosa. The precursor of serotonin tryptophan (Trp), present in the mucosal layer, modulates intestinal permeability. Elevated levels of serotonin cause a decrease in the permeability of the gut wall [48]. Additionally, low levels of serotonin lead to a decrease in the expression of occludin, thus increasing gut wall permeability. The latter was reported in patients diagnosed with IBS [48].

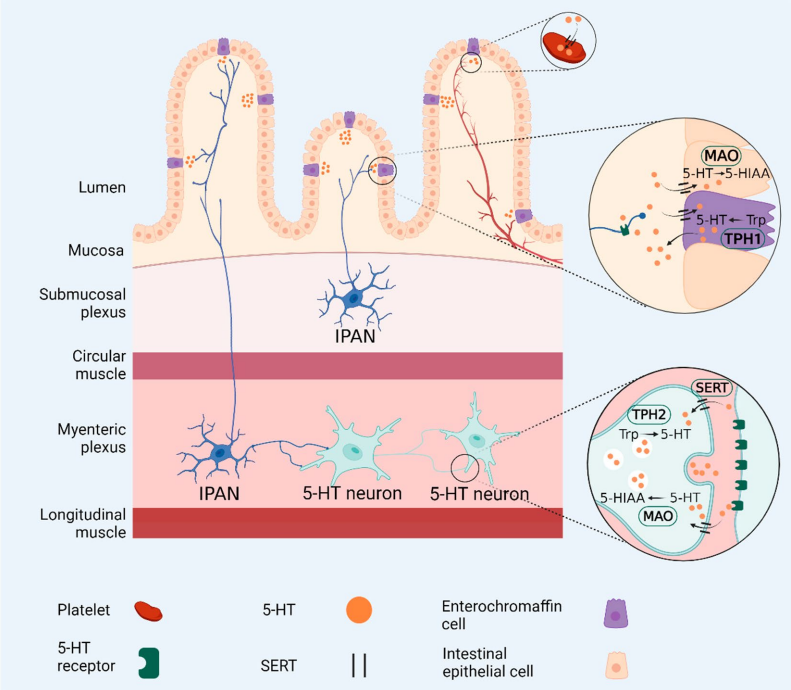

Figure 2. Synthesis, metabolism, and degradation of serotonin in the intestinal epithelium. Serotonin (5-hydroxytryptamine or 5-HT) is synthesized in the intestinal epithelium by enterochromaffin cells (ECs), serotonin-synthesizing neurons of the enteric nervous system (ENS) (5-HT neurons) and bacterial inhabitants of the gastro-intestinal tract (GIT). ECs convert tryptophan (Trp) to 5-HT with tryptophan hydroxylase 1 (TPH1). Enteric neurons use tryptophan hydroxylase 2 (TPH2) to convert Trp to 5-HT. Secreted 5-HT activates postsynaptic 5-HT receptors and is subsequently inactivated through pre-synaptic serotonin reuptake transporter (SERT) reuptake, where it can either be packaged into vesicles for release or degraded by monoamine oxidase (MAO). Release of 5-HT into the mucosal layer activates 5-HT receptors on intrinsic primary afferent neurons (IPANs) in both the submucosal and myenteric plexuses. SERT facilitates 5-HT inactivation. 5-HT is converted into 5-hydroxyindoleacetic acid (5-HIAA) by MAO. Platelets express SERT and are hypothesized to collect and store intestinal 5-HT as they move through enteric circulation.

4. Role of Gut Microbiota in Psychiatric Disorders

The link between gut microbiota and disorders such as anxiety, depression, schizophrenia, bipolar behavior, autism, and obsessive-compulsive disorder (OCD) has been clearly demonstrated (summarized in Figure 3). Changes in gut microbiota with each of these disorders are listed in Table 1.

Table 1. Changes in gut microbiota associated with mental disorders.

Anxiety/Depression	
Reference	**Findings**
[60,61]	↑ *Alistipes*, *Oscillibacter* ↓ Bacteroidales
[61]	↑ *Clostridium*, *Roseburia* ↓ *Bacteroides*, *Prevotella*, *Ruminococcus*
[62]	↓ *Bifidobacterium*, *Lactobacillus*
[63]	↓ *Coprococcus, Dialister*
Schizophrenia	
Reference	**Findings**
[64]	↑ *Anaerococcus* ↓ *Proteobacteria*, *Haemophilus*, *Sutterella*, *Clostridium*
[65]	↑ Firmicutes ↓ Proteobacteria
[66]	↑ Actinobacteria, Fusobacteria, Acidobacteria, *Staphylococcus, Megasphaera* ↑ Proteobacteria, *Succinivibrio*, *Collinsella*, *Clostridium*, *Klebsiella* ↓ *Blautia*, *Coprococcus*, *Roseburia*
[67]	↑ Firmicutes, *Lactobacillus gasseri* ↓ Bacteriodetes, Acinetobacteria
[68]	↑ *Lactobacillus* phage phi adh, *Lactobacillus gasseri*
[64]	↓ Proteobacteria, *Haemophilus, Sutterella, Clostridium*
[66]	↑ *Proteobacteria, Succinivibrio*, *Collinsella*, *Clostridium, Klebsiella* ↓ *Blautia*, *Coprococcus*, *Roseburia*
[69,70]	↑ *Anaerococcus, Collinsella*
Bipolar disorder	
Reference	**Findings**
[71]	↑ *Flavonifractor*
[72]	↑ *Actinobacteria*, Coriobacteriaceae ↓ *Faecalibacterium*
[73]	↓ *Faecalibacterium*, Ruminococcaceae
[74,75]	↓ *Bifidobacterium*
Autism	
Reference	**Findings**
[76]	↑ *Clostridium*
[77]	↑ Bacteroidetes, *Actinobacterium*, Proteobacteria, *Clostridium defense*, *Clostridium hathewayi, Clostridium orbiscindens* ↓ Firmicutes
[77,78]	↓ *Faecalibacterium, Ruminococcus*
[77]	↑ *Roseburia*
OCD	
Reference	**Findings**
[79]	↑ Systemic inflammation markers ↓ *Oscillospira*, *Odoribacter*, *Anaerostipes*

Arrows facing upwards (↑) denotes an increase in cell numbers and arrows facing downwards (↓) a decrease in cell numbers.

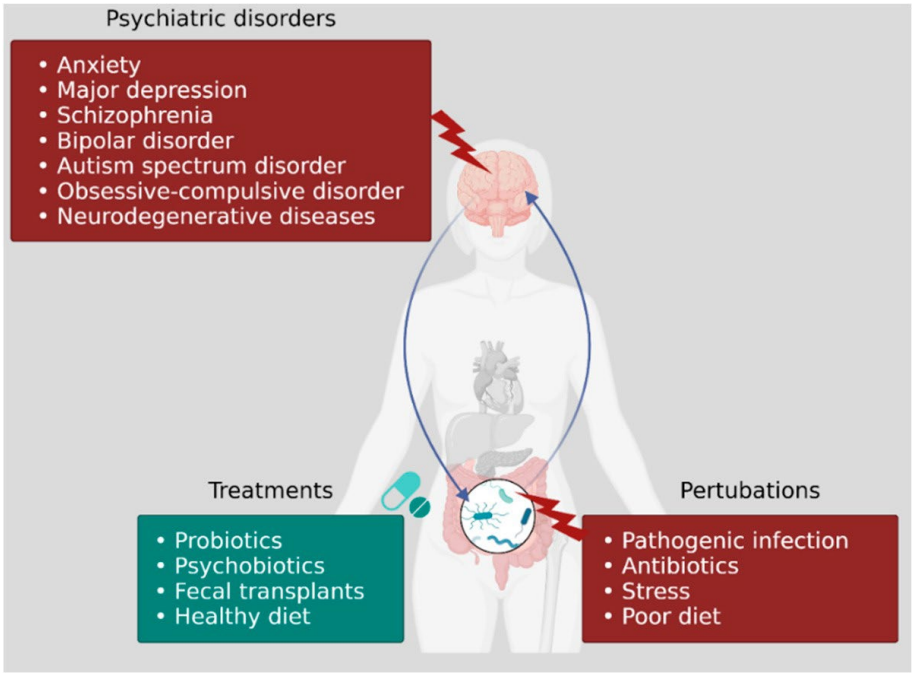

Figure 3. Link between perturbations in the gut microbiota and cognition, mood, and neuropsychiatric disorders. Disturbance of the gut microbiota may occur upon infection or administration of antibiotics, stress or with poor diet. There is evidence of a link between imbalances in gut microbiota and consequent psychiatric disorders, including anxiety and major depression, schizophrenia and autism spectrum disorder. Potential treatments include administration of probiotics to restore balance to the gut microbiota, fecal transplants from healthy individuals and maintaining a healthy, balanced diet.

4.1. Anxiety and Depression

Stress, anxiety, mental illnesses, and methods used in treatment have a profound effect on the gut microbiome; reviewed by Cryan et al. [80]. Anxiety and depression may have a more profound effect on an infant. Animals administered specific strains of bacteria displayed changes in behavior [38,81–83]. The human GIT may be host to 3000 bacterial species, as recently reported by the Human Microbiome and MetaHIT studies [84–86]. A chronic inflammatory state of the GIT may lead to increased responsiveness to stress and to development of major depressive disorder, MDD [87]. Treatment with antibiotics not only change the microbiome, but may have a lasting effect on the brain, spinal cord and the ENS [88,89]. This may occur without changes in immune response, as animal studies have shown changes in behavior with low levels of microbial infections had little effect on immune activation [90]. On the other hand, individuals suffering from autoimmune disorders and chronic inflammation often develop comorbid depression [91]. Treatment of these individuals with proinflammatory agents such as interferon-alpha (IFN-α) led to an increase in depression [92]. This coincides with studies that linked an increase in the secretion of proinflammatory cytokines with changes in depression [93–95]. In some cases, chronic inflammation led to the disruption of the blood–brain barrier (BBB), caused cellular and structural changes in the central nervous system, and induced the release of glutamate from microglia [96]. Although studies done on animals and macrophages have shown that some antidepressants have anti-inflammatory properties [96–101], this is not supported by all studies [102,103]. There is, however, evidence that levels of IL-1β in the hippocampus, and its effect on hippocampal neurogenesis, is reduced by certain antidepressants [104–106].

Anxiety often develops from a young age and can lead to other mental disorders such as depression [6,107]. Major depressive disorder (MDD) is world-wide the leading cause of disability and is characterized by irritability, loss of concentration, loss of appetite and sleep,

and depressed moods [108,109]. Since depression is often associated with a deficiency in the functioning of serotonin and/or norepinephrine at specific synapses in the brain, most of the currently available antidepressants prevents the reuptake of these biogenic amines into nerve terminals [87]. However, many patients treated for MDD developed resistance to antidepressants, which led to studies investigating the relationship between gut microbiota and depression [110,111].

Naseribafrouei et al. [60] studied the microbiota of 37 depressed patients by comparing 16S rRNA sequences of fecal bacteria with those isolated from non-depressed patients. Based on this study, *Bacteroides* spp. were present at low cell numbers in depressed patients, although high cell numbers of *Alistipes* and *Oscillibacter* spp. were recorded. Similar findings were reported by Jiang et al. [61] when 46 depressed and 30 non-depressed patients between ages 18 and 40 were studied. In addition, high cell numbers of *Clostridium* and *Roseburia*, but lower numbers of *Prevotella*, and *Ruminococcus* were reported in depressed individuals. Aizawa et al. [62] reported an underrepresentation of *Bifidobacterium* and *Lactobacillus* spp. in depressed patients and Valles-Colomer et al. [63] linked a reduction of *Coprococcus* and *Dialister* to depression. Dysbiosis observed in the GIT of depressed individuals may cause IBD and in some cases accentuate depression [111]. Zanoli et al. [112] reported an association between depression, Crohn's disease, and cardiovascular complications. Common symptoms associated with IBD is diarrhea, rectal bleeding, intermittent nausea and abdominal pain or tenderness [57]. Although the authors [112] did not associate changes in gut microbiota with any of these symptoms, it is likely that an imbalanced microbiome did play a role.

Oscillobacter spp. are known to produce valeric acid, a compound that closely resembles gamma-amino butyric acid (GABA) and binds to GABA(a) receptors [113]. Binding of GABA to GABA(a) and GABA(b) receptors block CNS signals, which alleviates anxiety and depression [113]. With the binding of valeric acid to these receptors, GABA binding is inhibited, and the CNS signals are no longer blocked, resulting in anxiety. Of interest is the lowering in *Lactobacillus* cell numbers in patients that suffer from anxiety and depression. Certain species of *Lactobacillus* are responsible for GABA secretion as well as the neurotransmitter acetylcholine [114,115]. It may thus well be that low cell numbers of these *Lactobacillus* spp. contribute to anxiety and depression.

Alistipes spp. is associated with chronic fatigue and IBD [116,117]. Inflammatory factors produced by *Alistipes* spp. could play a role in depression and anxiety. Of interest was the low numbers of *Prevotella* spp. reported in patients with anxiety and depression. This is a conflicting finding, as *Prevotella* spp. are often associated with pro-inflammatory characteristics [118].

Studies on rats have shown that oral administration of *Faecalibacterium prausnitzii* (ATCC 27766) relieved anxiety and depression, suggesting that the strain may have psychobiotic properties [119]. The increase in SCFA levels in the cecum, and elevated plasma IL-10 levels, accompanied with a reduction in corticosterone and IL-6 levels, may explain the anxiolytic and antidepressant properties observed [104]. Lukic et al. [120] have shown that *Ruminococcus flavefaciens* upregulated genes involved in mitochondrial oxidative phosphorylation, whilst downregulating genes involved in synaptic signaling and neurogenesis. The authors [120] also reported a reduction in serotonin and norepinephrine in the prefrontal cortex. Studies such as these and reports on probiotic bacteria that influence neurotransmission, neurogenesis, expression of neuropeptides and neuroinflammation [58], opens a new research field in psychobiotics [121], especially probiotics affecting the CNS. For more information on probiotics and the effect on the nervous system, the reader is referred to the review published by Cryan et al. [80].

4.2. Schizophrenia

Schizophrenia is defined as a mental disorder characterized by abnormal thinking, perceptual disturbances, impaired memory, slow mental processing, and sporadic emotional expression [121,122]. This disorder affects at least 20 million people throughout the world

and is amongst the top 10 global causes of disability [123]. Symptoms differ vastly and reasons for developing schizophrenia is not fully understood [122,124], apart that it manifests at adolescence and remains with the individual throughout life [125]. At least one study reported a connection between schizophrenia and early childhood development [122]. As the gut microbiome is drastically altered during the first few years of life, certain microbiota may play a role in the developing of schizophrenia. A recent study [65] established a link between the salivary microbiome and gut microbiota associated with schizophrenia. Although their findings confirmed previous reports of the association between salivary microbiota and anxiety, depression, and autism spectrum disorder, ASD [126–129], much more detailed observations were made. The study involved 208 individuals diagnosed with symptoms of first-phase schizophrenia, psychosis (high risk schizophrenia) and no symptoms (classified as healthy). Concluded from this study [65], Firmicutes had a competitive advantage over Proteobacteria and may live in synergy with actinobacteria, fusobacteria, and Acidobacteria during early stages of schizophrenia. The dominance of Firmicutes over Proteobacteria has also been observed in the salivary microbiome of patients with primary Sjögren's syndrome [130], an autoimmune disease involving chronic inflammation of the salivary and lacrimal glands. Findings of Qing et al. [65] also suggest a switch towards microbiota that produce branched-chain amino acids (BCAA) and lysine in individuals with early-phase schizophrenia. This may indicate an increase in *Staphylococcus* and *Megasphaera*, as both genera has been associated with increased BCAA and lysine production [131,132].

The microbiome of schizophrenic patients, deduced from oropharyngeal studies, is largely represented by *Firmicutes*, especially lactic acid bacteria and in particular *Lactobacillus gasseri.* whereas Bacteriodetes and *Acinetobacteria* were in the minority [67]. In contrast to previous studies, the presence of *Proteobacteria* did not differ significantly between schizophrenic and non-schizophrenic patients. Yolken et al. [68] reported on the presence of bacteriophage *Lactobacillus* phage phi adh in schizophrenic patients. This phage prevails in the lysogenic state within *L. gasseri*, confirming that this species may have strong links to schizophrenia.

Nguyen et al. [64] were the first to report the effect an altered gut microbiome may have on schizophrenic individuals. Significantly lower levels of *Proteobacteria*, *Haemophilus*, *Sutterella*, and *Clostridium* spp. were reported in patients 30 to 76 years old. Cell numbers of *Anaerococcus* spp. remained unchanged compared to healthy individuals. In a separate study conducted on patients between 18 and 65 years of age [66], high levels of *Proteobacteria*, *Succinivibrio*, *Collinsella*, *Clostridium* and *Klebsiella* spp., but low levels of *Blautia*, *Coprococcus*, and *Roseburia* spp. were reported. Contradictory findings reported in these two studies suggests that age plays a major role in the extent to which gut microbiota may change in patients with schizophrenia. *Proteobacteria* within the gut is the most unstable over time compared to the other three main phyla, especially when in a non-healthy state [133]. Lipopolysaccharides produced by *Proteobacteria* elicits the production of proinflammatory cytokines such as interferon-γ (IFN-γ), TNF-α, and interleukin-1β (IL-1β) [134]. This may cause intestinal inflammation and modification of tight junctions in the gut wall, leading to several intestinal diseases [135]. Given that a healthy human gut microbiome is seen to be relatively stable over time, Shin et al. [136] has proposed that fluctuations of *Proteobacteria* in the GIT could indicate microbial dysbiosis and could potentially be used as a diagnostic criterion [133,137].

The observation of a higher abundance of *Anaerococcus* and *Collinsella* in schizophrenic individuals is of interest, as species from these genera produce butyrate [69,70]. *Coprococcus* and *Rosburia* were less prevalent in schizophrenic individuals. Although these bacteria are also known butyrate producers, they are underrepresented in the gut compared to *Anaerococcus* or *Collinsella*. A similar observation was made with studies on bipolar and autistic patients. In these individuals, butyrate-producing *Faecalibacterium* spp. were present in low numbers [138]. Reasons for different reports on populations of butyrate-producing bacteria is unclear. It may be that population differences amongst these bacteria

play an important role in the regulation of pro-inflammatory cytokines, which in turn influences certain psychiatric disorders.

Most *Haemophilus* spp. are regarded commensal, but some may cause meningitis. As psychiatric disorders are associated with inflammation, *Haemophilus* spp. was expected to be present at high cell numbers. However, the genus was less prominent in schizophrenic patients [139]. *Sutterella* spp., associated with reduced inflammation and low blood glucose levels [140], are also less prominent in schizophrenic patients. *Clostridium* and *Oscillospira* were in a lower abundance in schizophrenic and OCD patients, respectively [64,76,141,142]. This suggests that *Clostridium* may play a different role in schizophrenic and OCD patients than in patients suffering from anxiety, depression and autism.

Bacteroides fragilis, often isolated from schizophrenic patients, plays an important role in $CD4^+$ T cell activation by producing zwitterionic polysaccharides (ZPS) that bind to peptide-binding sites on class II molecules of antigen-presenting cells. This stimulates T cells to produce anti-inflammatory IL-10, IL-2 and IL-12, thus playing a key role in host immune response [143]. Since *B. fragilis* is Gram-negative and contains a LPS capsule, the species may promote inflammation and be considered a pathogen [144,145]. Low numbers of *B. fragilis* observed in schizophrenic individuals suggests that they are most likely not pathogenic.

4.3. Bipolar Disorder

Bipolar disorder is similar to schizophrenia and depression, and is characterized as recurrent episodes of depression, along with cognitive, physical, and behavioral changes that, if severe enough, can lead to mania [146]. According to the World Health Organization (WHO), bipolar disorder affects approximately 60 million people worldwide and presents a high risk of suicide. Lithium is the choice of treatment, due to the drug's anticonvulsant and antipsychotic characteristics. However, up to 50% of patients undergoing treatment still experience severe bipolar episodes [147].

Individuals suffering from bipolar disorder may experience an increase in gut wall permeability [51]. Coello et al. [71] reported a significantly higher abundance of *Flavonifractor* in bipolar individuals. The genus is known for its ability to cleave quercetin [148], a flavonoid with anti-oxidative and anti-inflammatory properties [149]. Changes in flavonoid levels could thus play a role in bipolar disorder.

A decrease in *Faecalibacterium* and an increase in *Actinobacteria* and Coriobacteriaceae was reported in bipolar patients [72]. In another study by Evans et al. [73], a decrease in *Faecalibacterium* and Ruminococcaceae was recorded in bipolar patients. The decrease in *Faecalibacterium* suggests a decline in anti-inflammatory reactions [150].

Actinobacteria consists of many different genera, some of which are pathogens. *Bifidobacterium* spp. with probiotic properties have been associated with the alleviation of IBD [74,75]. Cell numbers of *Bifidobacterium* spp. were, however, lower in schizophrenic patients and individuals suffering from anxiety, suggesting that the GIT could be inflamed. The role other actinobacteria play in bipolar disorder has been less researched.

Coriobacteriaceae play an important role in bile salt and steroid conversion, and the activation of polyphenols [151]. Species from this family may, however, become opportunistic pathogens, but this must be confirmed.

4.4. Autism

Autism is a disorder characterized by restricted or repetitive behavior as well as difficulties with communication and social interactions [152]. Symptoms may manifest in infants as young as one year [153,154]. Although autism is considered to have a genetic origin, environmental factors may lead to the development of a series of co-occurring medical conditions, including anxiety [155,156].

Reports that as many as 90% of individuals diagnosed with autism suffer from dysbiosis led researchers to study the role gut microbiota play in such cases [157–159]. Mccartney et al. [76] reported a significant increase of *Clostridium* spp. in autistic individuals, support-

ing previous findings [141,160]. In a more detailed study on the complete microbiome of autistic patients, Finegold et al. [77] indicated a significant increase in Bacteroidetes, *Acintobacterium* and *Proteobacterium* spp., but a decline in *Firmicutes* in autistic patients. High cell numbers of *Clostridium defense*, *Clostridium hathewayi* and *Clostridium orbiscindens* were recorded in autistic patients. *Faecalibacterium* and *Ruminococcus* spp. were less abundant, which is an important observation given the anti-inflammatory properties of these species. Most of the *Clostridium* spp. are, however, considered commensal with a key role in maintaining gut homeostasis [161]. They also induce colonic T regulatory cells [162]. *Roseburia* spp., well represented in autistic patients, produce butyrate that has anti-inflammatory properties. Compared to controls, individuals diagnosed with autism had lower cell numbers of *Ruminococcus*, a genus within *Clostridium* cluster XIVa. *Ruminococcus albus* degrades cellulose and produces acetate [78].

4.5. Obsessive-Compulsive Disorder (OCD)

OCD is a psychiatric disorder characterized by recurrent, intrusive thoughts or obsessions, and ritualistic compulsions [79]. This condition can have a lifetime prevalence in 2.3% of the population, with a predominance in men [163]. OCD was originally classified as an anxiety disorder, similar to autism, but has now been classified as an obsessive-compulsive spectrum disorder.

Few studies have been conducted on the microbiome of individuals with OCD. Experiments on mice showed a decrease in OCD when treated with *Lactobacillus rhamnosus.* In humans, similar findings were reported with the administration of *Lactobacillus helveticus* [164,165]. These observations led Turna et al. [79] to conduct a detailed study on the microbial diversity of the GIT of OCD patients. The authors reported low numbers of *Oscillospira*, *Odoribacter* and *Anaerostipes* spp. in OCD patients. In addition to this, an increase in systemic inflammation markers were noted. *Odoribacter* produces butyrate and is considered an anti-inflammatory species [166]. A decrease in *Odoribacter* in OCD patients could thus lead to an increase in inflammation, which may be the onset of OCD.

5. Trace Amines Influence Cognitive Functions, Anxiety and Depression

Trace amines are endogenous compounds comprising of β-phenylethyalmine, *p*-tyramine, tryptamine, *p*-octopamine, and some of their metabolites [167]. They are also abundant in food and are produced, and degraded, by intestinal microorganisms. Six functional isoforms of trace amine-associated receptors (TAARs) have been identified in humans, i.e., TAAR1, TAAR2, TAAR5, TAAR6, TAAR8, and TAAR9. Of these, TAAR1 is the most thoroughly studied and has both central and peripheral roles. In the CNS, TAAR1 acts as a regulator of dopaminergic, glutamatergic, and serotonergic neurotransmission and is a novel therapeutic target for schizophrenia, depression, and addiction. TAAR1 also regulates nutrient-induced hormone secretion and may be a therapeutic target for diabetes and obesity. TAAR1 may also regulate immune responses by regulating leukocyte differentiation and activation [167].

Decarboxylation of L-phenylalanine, L-tyrosine, and L-tryptophan by aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28) leads to formation of the trace amines β-phenylethylamine (PEA), *p*-tyramine (TYR) and tryptamine (TRP) [168]. *p*-octopamine (OCT) and p-synephrine are formed in the presence of dopamine-b-hydroxylase (EC 1.14.17.1) and phenylethanolamine-N-methyl transferase (PNMT; EC 2.1.1.28), respectively [169,170]. It is, however, noteworthy to mention that the K_m value of AADC is within the solubility of many precursor amino acids [171,172], which suggests that the synthesis of PEA, TYR and TRP may depend on the regulation of AADC [173,174], or specific variants of AADC [175]. An example of this is an exon 3-depleted variant of AADC expressed in neuronal and non-neuronal cells that lacks the ability to decarboxylate L-DOPA and L-5-hydroxytryptophan [176]. An AADC variant without exons 11–15 is expressed in non-neuronal tissue [175]. The enzymatic activity of this variant is not known.

AADC variants with no clear enzymatic activity has also been detected in pancreatic b cells [177].

Production of PEA, TYR, and TRP by commensal gut microbiota is well documented [178–180]. Decarboxylation of precursor amino acids in the stomach [181] and entero-epithelial cells [182] play an important role in host-microbiota interactions. Decarboxylation of precursor amino acids also takes place in the glia, blood vessels [183], kidneys [184], liver [185], lungs [186] and pancreas [177]. In the brain AADC activity is regulated by dopamine, serotonin and glutamate. The activity of AADC may, however, also be affected by systemic lupus erythematosus [187,188]. Unlike dopamine, norepinephrine, epinephrine and serotonin, PEA, TYR and TRP are not stored and rapidly diffuse across membranes [78,79,189,190]. PEA diffuses across the blood–brain barrier [191] and TYR across intestinal epithelial cells [192]. Tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of the catecholamines dopamine, norepinephrine (noradrenaline) and epinephrine (adrenaline). A deficiency in L-tyrosine may thus lead to anxiety and low mood [192]. Treatments that increase monoamine neurotransmitter receptor activation leads to a decrease in PEA and TYR synthesis. Likewise, treatments that decrease receptor activation results in an increase in PEA and TYR synthesis. Reports on changes in AADC activity are almost exclusively based on L-DOPA as substrate. Binding of PEA, TYR, TRP and OCT to TAAR1 in the brain regulates the release of neurotransmitters dopamine and serotonin [167].

Inhibition of the reuptake of monoamine neurotransmitters occurs when PEA and TYR concentrations exceed 10 mM [193,194], which is 100-fold higher than physiologic concentrations [195]. Similar indirect sympathomimetic responses to OCT have been reported [194,196]. N-methylated metabolites of PEA, TYR, N-methylphenylethylamine, N-methyltyramine and N-methyl metabolite of TRP N,N-dimethyltryptamine (DMT) are TAAR agonists [170]. Under- or over-expression of TAAR1 may lead to schizophrenia, depression and addiction [197]. TAAR1 is expressed in key areas in the brain where dopaminergic, serotonergic, and glutamatergic neurotransmission is modulated. These reactions also occur in the amygdala, hypothalamus, rhinal cortices, and subiculum [197]. TAAR1 may thus be a novel target for the developing of antipsychotic, mood-stabilizing, and antidepressant drugs. Some TAAR1 agonists exert incretin-like activity that leads to an increase in insulin secretion. Since TAAR1 releases the hormones peptide tyrosine-tyrosine (PYY) and glucono-like peptide 1 (GLP-1), TAAR1 antagonists may regulate obesity.

Anxiety and depression are controlled by "blocking" neurotransmission. During early life, gamma-amino butyric acid (GABA), produced by GABAergic neurons, serves as a neurotransmitter [198,199]. Later in life, when GABAergic neurons mature, glutamate is transferred between synaptic cells. Adhesion of GABA to GABA receptors (GABARs) on the postsynaptic surface de-activates ion channels involved in the transfer of Na^+, K^+, Ca^{2+} and Cl^- [200]. The inflow of positively charged ions into a cell excites GABA. Outflow of these ions leads to the inhibition of GABA formation. Three classes of GABARs have been described, i.e., $GABAR_A$, $GABAR_B$ and $GABAR_C$. $GABAR_B$ is a G protein–linked receptor (GPLR) that directs signals received from pheromones, hormones and neurotransmitters to signal transduction pathways [200–202]. Glycoproteins, 80-kDa in size and containing multiple transmembrane regions, act as transporters of GABA. At least six different GABA transporters are known. The levels of unbound GABA in the cleft are tightly regulated by reuptake into presynaptic nerve terminals and surrounding glial cells [203]. Under normal physiological conditions, the intracellular level of GABA exceeds extracellular levels by approximately 200. The uptake of GABA by nerve cells occurs when Na^+ levels decrease. In the glia GABA is converted to glutamine, which is transferred back to the neuron [203]. Glutamine is then converted by glutaminase to glutamate, which re-enters the GABA shunt. *Lactobacillus rhamnosus* JB-1 altered the expression of GABARs in the brain, which resulted in the reduction of anxiety-like and depressive behavior [81].

Acetate, propionate, and butyrate interact with G-protein-coupled receptors 41 (GPR41) and 43 (GPR43) on the surface of EECs [204]. This, in turn, leads to the expression of the *pyy* gene encoding PYY. Most of PYY is released from L cells in the mucosa of the ileum

and colon [39]. At elevated PYY levels a loss in appetite is experienced, which leads to a decrease in the rate of gastric emptying and the sensation of fullness [205]. Since PYY is present in the ileum at high levels, it is often referred to as an "ileal brake" [206]. At a state of satiety, water uptake increases, and electrolytes accumulate in the colon, leading to an increase in nutrient uptake. Smaller quantities of PYY (1–10%) is released in the esophagus, stomach, duodenum and jejunum [207]. Cleavage of the Tyr-Pro amino terminal residues of PYY_{1-36} by dipeptidyl peptidase IV (DPP-IV) produces more PYY_{3-36}. [208]. During fasting, PYY_{1-36} levels are much higher compared to $PYY_{3-36.}$ The latter is released within 15 min of food intake, thus before the ingesta reaches the lower part of the small intestine and colon [207]. This suggests that the initial post-prandial release of PYY_{3-36} is controlled by the CNS. Highest PYY_{3-36} levels have been recorded in the colon after approximately 90 min of food intake [209]. Secretion of PYY, GLP-1 and cholecystokinin (CCK) send signals to the vagus nerve. The levels remain high for up to 6 h. A diet rich in lipids increases PYY_{3-36} production, whereas a diet rich in proteins delays the release of PYY_{3-36} by as much as 2 h after a meal. Bile acids interact with the G protein-coupled bile acid receptor (GPCR) TGR5 (also known as GPBAR 1) and farnesoid X receptors (FXR) on EECs. Binding of SCFAs and bile to these receptors stimulate the secretion of gut hormones such as PYY, GLP-1 and CCK.

A protein-rich diet stimulates the production of CCK. The hormone interacts with CCK-A receptors on acinar cells in the pancreas, CCK-B receptors in the brain and stomach and other CCK receptors distributed throughout the CNS [54]. This sends a signal to the small intestine to stop gastric emptying, thus mediating satiety. CCK also stimulates the pancreas to release enzymes involved in the digestion of lipids, proteins and carbohydrates [54]. CCK also interacts with calcineurin in the pancreas, which in turn activates the transcription factors NFAT 1–3 [210]. The latter stimulates hypertrophy and growth of the pancreas. The release of CCK is inhibited by somatostatin and pancreatic peptide. Trypsin, released by the pancreas, hydrolyses the CCK-releasing peptide and shuts down further secretion of CCK. The presence of CCK stimulates the contraction of the gall bladder to increase the secretion of bile into the duodenum [211]. CCK cannot cross the blood–brain barrier, but certain parts of the hypothalamus and brainstem are not protected by the barrier. Gastrin, a gastrointestinal hormone, binds to CCK_B receptors, which stimulates the release of gastric acid and the production of mucosa. Studies conducted on humans and rodents have shown that elevated CCK levels increases anxiety [54].

6. Conclusions

Gut microbiota has an adverse impact on our GBA and overall mental health. Chemicals secreted by these bacteria, such as GABA, in addition to other metabolites, play an important role in anti-inflammatory responses and help alleviate psychiatric symptoms stemming from inflammation. Treatment of schizophrenic and bipolar patients with probiotics alleviated symptoms associated with IBD, autistic children benefitted from probiotic treatment and OCD-like behavior could be controlled. The effect IBD has on depression, stress and anxiety requires in-depth studies. Our understanding of exactly how gut microorganisms control cognitive behavior, mood, and neuropsychiatric disorders remains limited. The deciphering of this complex, everchanging network between cells and neurons requires in-depth research by scientists from diverse disciplines. Although preclinical and clinical investigations have shown that treatment with probiotics may improve mood, extensive and carefully controlled clinical trials need to be performed to evaluate the effectiveness in treating mental disorders. Biomarkers need to be developed to identify differences in the gut microbiome of individuals suffering from psychological disorders. Interactions between drugs used in treatment and gut microbiota need to be studied in greater depth. Studies should include multi-omics of gut and oral microbiota to have a better understanding of the mutual interplay between phyla. The identification of changes in the gut microbiome associated with psychological disorders may provide valuable information in the choice of treatment.

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microorganisms

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Review

Gut Bacteria and Neurotransmitters

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Abstract: Gut bacteria play an important role in the digestion of food, immune activation, and regulation of entero-endocrine signaling pathways, but also communicate with the central nervous system (CNS) through the production of specific metabolic compounds, e.g., bile acids, short-chain fatty acids (SCFAs), glutamate (Glu), γ-aminobutyric acid (GABA), dopamine (DA), norepinephrine (NE), serotonin (5-HT) and histamine. Afferent vagus nerve (VN) fibers that transport signals from the gastro-intestinal tract (GIT) and gut microbiota to the brain are also linked to receptors in the esophagus, liver, and pancreas. In response to these stimuli, the brain sends signals back to entero-epithelial cells via efferent VN fibers. Fibers of the VN are not in direct contact with the gut wall or intestinal microbiota. Instead, signals reach the gut microbiota via 100 to 500 million neurons from the enteric nervous system (ENS) in the submucosa and myenteric plexus of the gut wall. The modulation, development, and renewal of ENS neurons are controlled by gut microbiota, especially those with the ability to produce and metabolize hormones. Signals generated by the hypothalamus reach the pituitary and adrenal glands and communicate with entero-epithelial cells via the hypothalamic pituitary adrenal axis (HPA). SCFAs produced by gut bacteria adhere to free fatty acid receptors (FFARs) on the surface of intestinal epithelial cells (IECs) and interact with neurons or enter the circulatory system. Gut bacteria alter the synthesis and degradation of neurotransmitters. This review focuses on the effect that gut bacteria have on the production of neurotransmitters and vice versa.

Keywords: gut bacteria; neurotransmitters

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1. Introduction

Most studies on the human gut focus on the composition of the gut microbiome, variations in gut microbiota with changing diets or medication, the role bacteria play in digestion and immune responses, and regulation of entero-endocrine signaling pathways. The more we discover about the gut microbiome, gut–brain axis (GBA), hypothalamic pituitary adrenal axis (HPA), cognitive behavior and neuropsychiatric disorders, such as autism, depression, and schizophrenia, as reviewed by Dicks et al. [1], the more questions arise concerning the influence that gut bacteria have on the production of prominent neurotransmitters, such as γ-aminobutyric acid (GABA), dopamine (DA), norepinephrine (NE, also called noradrenaline, NAd), serotonin (5-HT), and histamine.

Neurotransmitters are divided into four main categories, i.e., excitatory neurotransmitters (glutamate (Glu), acetylcholine (Ach), histamine, DA, NE, and epinephrine (Epi), also known as adrenaline (Ad)), inhibitory neurotransmitters (GABA, 5-HT and DA), neuromodulators (DA, 5-HT, Ach, histamine and NE), and neurohormones released from the hypothalamus (oxytocin (Oxt) and vasopressin, also known as antidiuretic hormone (ADH)) [2]. Neurotransmitters released into the synaptic cleft between the presynaptic- and postsynaptic membranes are either destroyed by enzymes or reabsorbed into the terminal button of the presynaptic neuron by reuptake mechanisms and then recycled. Examples of fast and short-lived neurotransmitters with an excitatory action are Ach, NE and Epi. GABA is the major inhibitory neurotransmitter. As soon as a neurotransmitter binds to the receptor on the postsynaptic membrane, ligand-gated ion channels either open or close, regulating the flow of Ca^{2+}, Na^{+}, K^{+} and Cl^{-}. Opening of the ion channels leads to a

stimulatory response and closing leads to an inhibitory response [2]. Neuromodulators remain for a longer period in the synaptic cleft, modulating the activity of neurons [2]. Neurohormones are secreted into the bloodstream and transported to tissue [2].

Gut bacteria use primarily GABA, DA, NE, 5-HT and histamine to communicate with the central nervous system (CNS) [3], but also intermediate compounds, notably short-chain fatty acids (SCFAs) [4], tryptophan [5], and secondary bile acids [6]. Signals generated by these neurotransmitters and molecules are transported to the brain via afferent vagus nerve (VN) fibers. In response, the brain sends signals back to enterochromaffin cells (ECCs) and enteroendocrine cells (EECs) in the gut wall, and the mucosal immune system via efferent VN fibers [7]. Activation of the VN improves the integrity of the gut wall, reduces peripheral inflammation, and inhibits the release of pro-inflammatory cytokines [8]. Signals generated by the hypothalamus reach the pituitary and adrenal glands and communicate with EECs via the hypothalamic pituitary adrenal axis (HPA) [9]. The intricate control of entero-endocrine signaling and immune-responses keeps the gut microbiome in a balanced state. If unbalanced, the gut enters a state of dysbiosis, characterized by a drastic increase in *Enterobacteriaceae*, especially *Escherichia*, *Shigella*, *Proteus* and *Klebsiella*, and an increase in enterotoxin levels [10]. If left untreated, major gastrointestinal disorders, such as diarrhoea, ulcerative colitis (UC), Crohn's disease, and other inflammable bowel diseases (IBDs) may develop [11–13]. In severe cases, elevated toxin levels may alter the functioning of the intestinal– and blood–brain barrier (BBB) that may lead to neurodegeneration [10,14–16].

Since gut microbiota co-evolved with humans and animals to perform neuronal functions, it is safe to assume that certain species, notably those that colonize the gastro-intestinal tract (GIT) first, play the largest role in the synthesis and degradation of neurotransmitters. From a philosophical point of view, one may ask what intestinal bacteria gain from the synthesis of neurotransmitters. Do bacteria use neurotransmitters solely to establish communication with the CNS to release molecules in the bloodstream that regulate physiological functions in the gut wall, or are there other more direct benefits? What role does gut bacteria play in the maintenance of neuronal pathways and how are signals, generated by gut bacteria, orchestrated to communicate with the CNS? To answer these questions, we need to have a better understanding of the receptors on neurons, neuro-signaling pathways, and other physiological functions of signaling molecules. Could SCFAs produced by gut bacteria play a central role in neurotransmission?

The purpose of the review is not to discuss metabolic pathways and the physiology of gut bacteria, but rather focus on the synthesis and degradation of neurotransmitters and identify prominent gut bacteria that influence neurotransmission.

2. Bacteria Dominates the Human Gut Microbiome

According to reports from the Human Microbiome Project (HMP) [17,18] and the METAgenomics of the Human Intestinal Tract (MetaHIT) consortium [19,20], the human gut is host to 2766 microbial species. More than 90% of the gut microbiome is represented by bacteria from the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes [20–22]. The majority of the gut bacteria are Firmicutes, dominated by Gram-positive *Lactobacillus* spp. and Gram-negative bacteroidetes [23]. Fusobacteria and Verrucomicrobia make up the remaining 10% of the gut microbiome [24].

The gut microbiome changes with fluctuations in hormone levels, variations in diet [25,26], stress and physiological changes brought about by the intake of drugs, especially antibiotics [27,28]. Most species in the GIT are represented by strains with unique phenotypic and genotypic characteristics [29–31]. Strains within species adapt as their environment changes. Strains within the same species isolated from different individuals have at least one variation in every hundred base pairs [32–35]. Species that cannot adapt or compete are replaced by novices that are able to regulate their own gene expressions, or alter their genetic composition [32,36]. Once adapted to the GIT, strains are not easily replaced [33]. *Bacteroides fragilis* adapted so well to humans that the species is represented by a single strain [37]. *Helicobacter pylori, Mycobacterium tuberculosis* [38–40], *Eubacterium*

rectale [41] and *Prevotella copri* are host-specific and contain strains that are associated with individuals from specific geographic regions [42].

A healthy GIT is characterized by a balanced gut microbiome with a core population of beneficial microbiota. Strain-level structures are maintained by keeping genetic changes under control, as illustrated in the studies on *Escherichia coli*. Genome sequences of 24 intestinal isolates of *E. coli* ED1a, studied over a year, showed a mutation rate of only 6.9×10^{-7} per base [43]. This was, however, complemented by a reduction in population size [43], suggesting that external stress factors have a profound influence on the survival of bacteria in the GIT. Roodgar et al. [44] have shown that strains exposed to stress, such as antibiotics, develop resistance rapidly and change the overall composition of the gut microbiome. Although the outcome of the reports by Ghalayini et al. [43] and Roodgar et al. [44] were different, both illustrated that the frequency at which strains adapt to an ever-changing and stressful environment, such as the GIT, is unpredictable. Whatever changes takes place in the microbial population, these will affect the synthesis and degradation of neurotransmitters and, ultimately, communication with the CNS.

3. Wiring of the Gut Wall to the CNS

Most signals to and from the gut run through a bi-directional VN that exits the brain at the medulla oblongata and leaves the skull at the jugular foramen (Figure 1). Vagus nerves in the neck communicate with muscles of the pharynx and larynx that control swallowing and speech. Vagus nerves in the thorax downregulates heart rate. Branches of the VN leading to the GIT relax and contract smooth muscles and control secretion from glandular tissue. The celiac branch of the VN connects with the duodenum and the rest of the intestine to the distal part of the descending colon [45]. Preganglionic neurons of the VN in the medulla communicate with muscular and mucosal layers in the lamina propria and muscularis externa [46]. Sensory cells in the nodose ganglia send signals to the nucleus tractus solitarii (NTS), from where messages are sent to the locus coeruleus (LC), amygdala, thalamus, and rostral ventrolateral medulla [46].

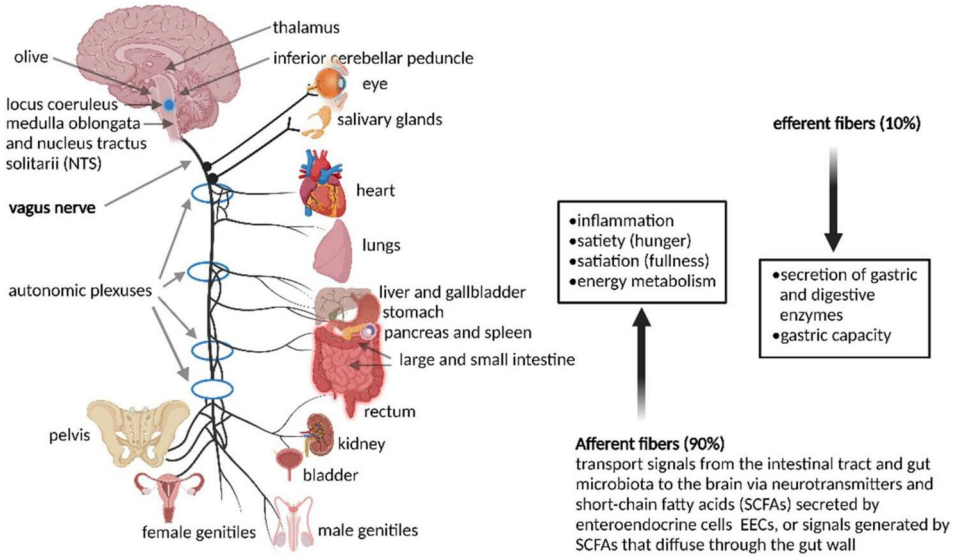

Figure 1. The vagus nerve (VN) connects the gastro-intestinal tract (GIT) with the central nervous system (CNS), but is also connected to various other organs. This illustration was constructed using BioRender (https://biorender.com/, assessed on 15 July 2022).

The importance of signaling from the GIT to the brain is emphasized by the overwhelming presence of afferent fibers that outnumber efferent fibers by 9:1. Afferent fibers are also linked to receptors in the esophagus, liver, and pancreas (Figure 1). Although the VN is in contact with all layers of the gut wall, fibers do not cross the gut wall and are, thus, not in direct contact with gut microbiota [47]. Signals reach the gut microbiota via 100 to

500 million neurons in the enteric nervous system (ENS) in the submucosa and myenteric plexi of the intestinal wall (Figure 2), stretching from the esophagus to the anus [3,48,49]. Thus, although associated with the VN, the ENS in the small and large intestinal tract functions independently from the VN. This is possible due to independent sensory and motor neurons, capable of regulating muscle activity, gut wall motility, secretion of fluids, blood flow in mucosal layers and mucosal barrier functions. A decline in functions associated with the ENS often manifests as constipation, incontinence, and evacuation ailments. This is often observed in the elderly and is referred to as Hirschsprung's disease or intestinal pseudo-obstruction [49]. Recent studies have shown that the ENS is dynamic and ever changing, maintained by a network of apoptotic and neurogenetic processes [50].

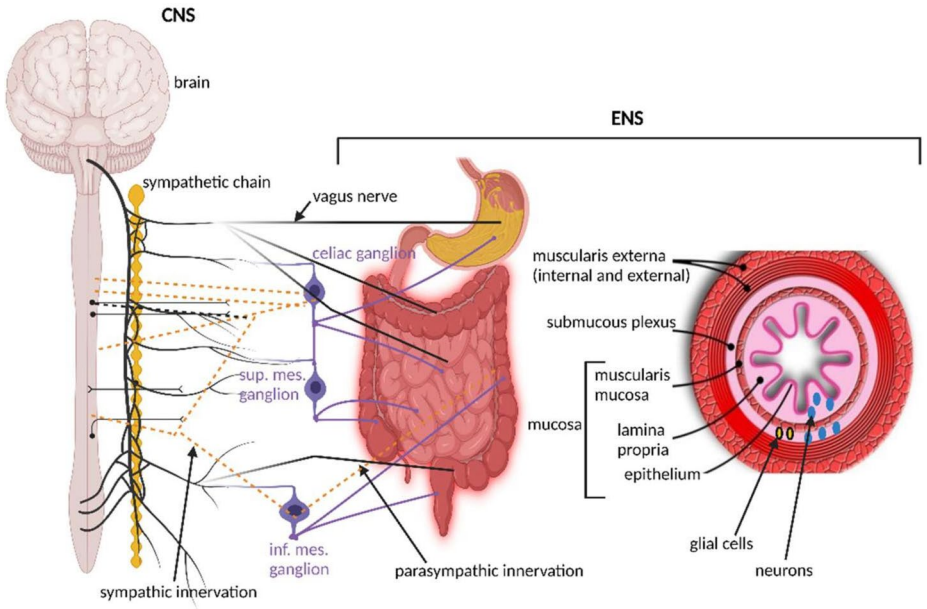

Figure 2. Although the vagus nerve (VN) is in contact with all layers of the gut wall, fibers do not cross the gut wall and are, thus, not in direct contact with gut microbiota. Signals reach the gut microbiota via 100 to 500 million neurons in the enteric nervous system (ENS) in the submucosa and myenteric plexi of the intestinal wall. The ENS in the small and large intestinal tract functions independently from the VN. This illustration was constructed using BioRender (https://biorender.com/, assessed on 15 July 2022).

Neurons of the ENS stem from enteric neural crest cells (ENCCs) and are, by far, the largest of all nervous systems in the human body. Proliferation and migration of ENCCs are highly dependent on the glial cell line-derived nerve growth factor (GDNF) protein that is predominantly expressed by neurons in the septum, striatum, and thalamus. Expression of GDNF and other neurotrophic factors, such as neurturin (NTN), artemin (ART) and persephin (PSP), are regulated by Toll-like receptors TLR2, TLR4, TLR5 and TLR9 [51]. GDNF, NTN, ART and PSP bind to growth factor receptors (GFR)α-1, GFRα-2, GFRα-3 and GFRα-4, respectively, all tethered to the cell membrane with a glycosyl phosphatidylinositol anchor and to the transmembrane receptor tyrosine kinase (RET). The dimeric GFRα1–GDNF complex changes to the active tyrosine-phosphorylated form and sends a signal to ENCCs to express the ENS precursors and neurons required to alter the ENS during prenatal development and ensures further development of the ENS [52].

Neurons connected to the GIT have several chemical and mechanosensitive receptors that interact with hormones and regulatory peptides released from EECs, also known as Kulchitsky cells. Although these cells constitute only 1% of the epithelial cells in the GIT, they play an important role in maintaining gut homeostasis [53]. To date, 10 different types of EECs have been characterized. They act as sensory cells that coordinate changes in the secretion of chromogranin/secretogranin, 5-HT, neuropeptide Y (NPY), vasoactive

intestinal peptide (VIP), cholecystokinin (CCK), somatostatin, glucagon-like peptide (GLP)-1/2, ghrelin, and substance P (SP) [54,55]. Receptors on these sensory cells are expressed by gut enteric neurons, but also vagal afferents, the brainstem and hypothalamus [56,57].

Levels of CCK, GLP-1 and PYY remain high for up to six hours after a meal. Production of CCK is stimulated by a protein-rich diet. CCK binds to specific receptors in the pancreas (CCK-A receptors), receptors in the brain (CCK-B receptors), and other receptors in the CNS. This sends a massage of fullness to the small intestine [58]. At the same time, lipolytic, proteolytic and carbolic enzymes are released from the pancreas [58]. When CCK interacts with calcineurin in the pancreas, transcription factors NFAT 1–3 are activated, which stimulate hypertrophy and the proliferation of pancreatic cells [59]. Somatostatin and pancreatic peptides prevent the release of CCK [60]. Gastrin, a hormone produced in the GIT, binds to CCK-B receptors, resulting in the release of gastric acid and mucosa production. High CCK levels increases anxiety [58]. A diet high in fats increases $PYY_{3–36}$ production, whereas a protein-rich diet slows down the release of $PYY_{3–36}$ for up to two hours. Bile acids interact with the G protein-coupled bile acid receptor TGR5 (GPBAR 1) and farnesoid X receptors (FXRs) on the surface of EECs [58,60].

Ghrelin, released by the stomach during fasting, enters the circulatory system and crosses the BBB. Once in the brain, ghrelin activates receptors on neurons in the arcuate nucleus, leading to increased production of NPY and agouti-related protein (AgRP) [61]. Receptors for ghrelin are located on the NPY and AgRP neurons in the hypothalamus [62]. High levels of NPY in the brain and spinal cord are secreted together with other neurotransmitters, such as GABA and glutamate [63]. NPY stimulates appetite and regulates the storage of energy in the form of fat, but also reduces anxiety, stress, and pain. NPY also regulates sleeping patterns and keeps blood pressure low [64]. AgPR stimulates the hypothalamic–pituitary–adrenocortical axis to release ACTH (adrenocorticotropin, also known as corticotropin), cortisol and prolactin (lactotropoin) [65]. Ghrelin also stimulates appetite through interaction with ghrelin receptors (GHSRs) located on neurons attached to the nodose ganglia of the VN [66].

Higher ghrelin levels are associated with elevated DA levels [67] that, in turn, send signals of satiety to the CNS [68]. A diet rich in oligofructose and inulin represses ghrelin production and increases GLP-1 production by endocrine L-cells of the intestinal epithelium [69]. GLP-1 activates GLP-1 receptors on cells in the pancreas, kidneys, and the GIT [70]. In mice with dysbiosis, activation of an enteric NO-dependent pathway resulted in the development of resistance to GLP-1 and insulin, and an increase in body mass [71–73]. GLP-1 is rapidly metabolized and inactivated by dipeptidyl peptidase IV and does not enter the circulatory system [74]. It would, thus, seem as if GLP-1 adheres to receptors in the GIT from where signals are transmitted to the brain via sensory neurons. The secretion of insulin stimulated by GLP-1 and inhibition of glucagon secretion keep glucose levels low during or immediately after a meal. GLP-1 also acts as an "ileal brake" by slowing down gastrointestinal motility [74]. Compounds structurally similar to GLP-1 (agonists) may, thus, be used to control type 2 diabetes. On the other hand, a decrease in the secretion of GLP-1 may lead to obesity. GLP-1 levels must be carefully controlled, as over secretion may lead to hypoglycemia. The effect of gut microbiota on ghrelin production is summarized in a study published by Schalla and Stengel [75]. According to this study, ghrelin production is stimulated by *Bacteroides* (certain species), Coriobacteriaceae, Veillonellaceae, *Prevotella*, *Bifidobacterium* (certain species), *Lactobacillus* (certain species), *Coprococcus* and *Ruminococcus*, but inhibited by some species of *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *Faecalibacterium*, *Bacteroides*, *Escherichia*, *Shigella* and *Streptococcus*, and members of Prevotellaceae. This clearly indicates that the regulation of ghrelin levels is species-specific, and more research is required to determine the specific factors involved.

Bacteroides produce molecules homologous to insulin, NPY and melanocyte-stimulation hormone (α-MSH) [76]. These molecules induce cross-reactions with immunoglobulins in the circulatory system that act directly against ghrelin, leptin, insulin, PYY and NPY [77]. Some strains of *Rikenellaceae* and *Clostridiaceae* produce caseinolytic protease B (ClpB) that

mimics satiety experienced with increased levels of α-MSH [78]. Immunoglobulins produced by interaction with ClpB act against α-MSH and reduces its anorexigenic effects, leading to a decrease in satiety [78].

Enterochromaffin cells (ECs) controls reflexes and the secretion of gastric acid, but also produce 5-HT. Type D, G, I, K and L cells control enzymatic secretions, Mo cells initiate myoelectric migration, N cells regulate contractions, S cells (located in the small intestine) regulate acidity levels, A cells secrete ghrelin and nesfatin-1, and P cells secrete leptin. Goblet cells secrete glycosylated mucins into the lumen to form the mucus layer, which is important in maintaining intestinal barrier homeostasis. Mucin 2 (Muc2) binds to glycan receptors on dendritic cells (DCs) in the lamina propria to induce anti-inflammatory signals. Mucins also regulate the adhesion of microbial cells to the gut wall [79]. Secretion of Muc2 is regulated by intestinal microbiota and SCFAs. Intestinal trefoil factor (ITF) and resistin-like molecule-β (RELM-β), also produced by goblet cells, assist in the formation of mucosal barriers [80]. ITF regulates the formation of the tight junction proteins claudin and occludin, and controls cell apoptosis and the repair of epithelial cells. Claudin and occludin are supported by zonula occludins and actins produced by, and positioned between, epithelial cells [81]. Adheren proteins link with actin to form a cytoskeleton but are also involved in cell signaling and gene transcription regulation. Cadherin proteins bind to α-catenin to form a P120–catenin–cadherin complex that regulates cadherin formation in the plasma membrane [82]. RELM-β is more involved in altering T-helper 2 (Th2)-mediated responses. Cup cells account for 6% of epithelial cells in the ileum. The function of these cells is unknown. Tuft cells (taste chemosensory epithelial cells) secrete cytokines [80].

4. The Smaller Brain in Our Gut

The ENS (Figure 2), referred to as the "brain within the gut" or "second brain" [83], composed of an outer myenteric plexus and inner submucosal plexus, is structurally similar to the brain and operates on a similar "chemical platform" [84]. The modulation, development, and renewal of ENS neurons are controlled by gut microbiota, especially those with the ability to produce and metabolize hormones. Studies with germ-free (GF) mice [85,86] have shown that most enteric neurons are formed during embryogenesis and early postnatal life. A small population of neural crest cells that express Sox10 (a protein that acts as a transcriptional activator) colonizes the foregut, multiplies and then colonizes the entire bowel to form neurons and glial cells [87]. Development of enteric neurons relies on the presence of microbial cells, as shown in a study conducted on mice [88]. The authors have shown that neuroepithelial stem cells produce the protein Nestin and expresse the nuclear protein Ki67 3 to 15 days after the GIT of GF mice has been recolonized with microbiota. Nestin maintains the balance between neuronal apoptosis and neurogenesis [88,89] and protein Ki67 is associated with rRNA transcription [90]. A deficiency in any of these proteins is, thus, an indication of a decline in the health of neural stem cells (NSCs), and the inability to renew damaged cells [91]. Nestin is also expressed in the placenta [92] and is used as a marker to determine neuron health in the CNS of the unborn.

Several enzymes are involved in the maintenance of the ENS. Phosphatidylinositol 3-kinase (EC 2.7.1.137) and Akt (protein kinase B) in the PI3K/AKT signaling pathway promote neural survival and transmission, phospholipase C gamma 1 (PLC-γ1; EC 3.1.4.3) is involved in cell growth, apoptosis and transmission of neural cells, and a ras/mitogen activated protein kinase (RAS/MAPK) controls cell survival and neurogenesis [50,89]. ENS cells destroyed by apoptosis are replaced by newly formed cells [89]. However, little is known about the mechanisms that control ENS cell replenishment. A study published by Vicentini et al. [93] shed some light on this. The authors noted that the small intestinal tracts of antibiotic (Abx)-induced mice (thus without gut microbiota) were longer than normal mice, which led to slower transit of gut contents, increased carbachol (carbamylcholine)-stimulated ion secretion, and increased gut wall permeability. Since carbachol binds to, and activates, acetylcholine (Ach) receptors [94], increased levels would stimulate muscarinic and nicotinic receptors. Muscarinic Ach receptors form G protein-coupled

receptor complexes in the cell membranes of, for instance, the parasympathetic nervous system [94]. Nicotinic acetylcholine receptors are polypeptides that are present in the central and peripheral nervous system, muscle, and many other tissues [95]. The authors also noted a decrease in neurons of the submucosal and myenteric plexuses of the ileum and proximal colon, and thus the neuronal network of the ENS. In addition, the myenteric plexus of the ileum contained fewer glial cells (neuroglia), which is an indication that neurons of the ENS had been deprived of nutrient and oxygen supply and neuronal cells were not insulated from each other, lost the ability to destroy pathogens and were unable to remove dead neurons; all of which are functions of neuroglia [96].

The role gut microbiota plays in association with neurotransmitters such as Ach and regulatory neuropeptides has become more apparent in research conducted on experimental animals. Ach, the main neurotransmitter of the parasympathetic nervous system involved in the contraction of smooth muscles, dilation of blood vessels, secretion of bodily fluids and the downregulation of heart rate, is stored in vesicles at the terminal of Ach-producing neurons. Recent findings [97,98] have shown that the secretion of Ach may be stimulated by certain species of *Lactobacillus*. Studies with animal models have shown that a stroke, created by restricting blood flow at the proximal cerebral artery, altered the composition of gut microbiota, which led to a decrease in the production of Ach, changes in peristalsis, increased gut permeability and dysbiosis [99,100]. A decline in Ach also led to an increase in adrenergic signaling and inflammation of the GIT. This, in turn, restricted the middle cerebral artery that led to a decline in goblet cells in the cecum and lowering of mucin production [101]. This indirect effect on gut health by a neurotransmitter of the parasympathetic nervous system, under microbial control, illustrates the complexity of the mechanisms involved in controlling ENS functions.

When GF mice were reconstituted with intestinal microbiota and administered lipopolysaccharides (LPS) and SCFAs, intestinal functions were restored, and enteric neurons and neuroglia recovered. Treatment with LPS assisted in the recovery of damaged neurons and gut microbiota but did not stimulate the formation of new neurons. Treatment with SCFAs, on the other hand, restored neuronal loss [93]. Concluded from the data presented in this study, a lack in SCFAs, as would be expected in patients with dysbiosis, may lead to a loss of enteric neurons, and thus a weakened ENS. This emphasizes the importance of SCFA-producing gut microbiota.

5. Neurotransmitters and Neuropeptides

Neurotransmitters are divided into amino acids (e.g., Glu, aspartate, D-serine, GABA and glycine), monoamines (DA, NE, Epi or Ad, histamine and 5-HT), trace amines (e.g., phenethylamine, *N*-methylphenethylamine, tyramine, 3-iodothyronamine, octopamine, tryptamine), peptides (oxytocin, somatostatin, substance P, cocaine and opioid peptides), gasotransmitters (nitric oxide, carbon monoxide and hydrogen sulfide), purines (adenosine triphosphate and adenosine), and smaller compounds, such as Ach and anandamide. Of these, Glu, GABA, glycine, DA, NE, 5-HT and histamine are considered the key neurotransmitters and will be discussed in more depth.

5.1. Glutamate (Glu)

Glu is the principal excitatory neurotransmitter in the brain and plays a key role in memory storage [102]. Glu is released from presynaptic nerve terminals and adheres to ionotropic glutamate receptors (iGluRs) located on postsynaptic terminals. This increases the transfer of Ca^{2+} through voltage-activated calcium channels (VACCs) located in the terminals [103] and activates calcium calmodulin-dependent kinase (CaMK; EC 2.7.11.17), extracellular signal-regulated kinase (ERK, EC 2.7.11.24) and cyclic AMP response element binding (CREB) protein, all crucial for protein synthesis and maintenance of synaptic density. This process is carefully controlled. Excess Glu is taken up by glial cells via transporters EAAT1 and EAAT2. Glu is then converted to glutamine, which is transported back to presynaptic nerve terminals and converted to Glu by glutaminase (EC 3.5.1.2). Vesicular

glutamate transporters VGLUT1 and VGLUT2 then transport the newly formed glutamate to vesicles in the presynaptic neuron. The over-excitement of Glu (glutamate excitotoxicity) accelerates the progression of Alzheimer's disease [104]. For more information on glutamate excitotoxicity, the reader is referred to the review by Esposito et al. [105].

D-Glu is part of the peptidoglycan structure in the cell walls of bacteria and is produced via Glu racemase (EC 5.1.1.3) Mur1 [106]. *Corynebacterium glutamicum, Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus paracasei, Brevibacterium avium*, *Mycobacterium smegmatis, Bacillus subtilis* and *Brevibacterium lactofermentum* convert L-Glu to D-Glu [20,107,108]. The latter is converted to GABA by Glu decarboxylase (GAD; EC 4.1.1.15) [109]. The metabolism of D-amino acids in the brain is regulated by gut bacteria, as shown in the studies on specific pathogen-free (SPF) mice [110]. The authors have shown that L-arginine, L-glutamine, L-isoleucine, and L-leucine were significantly higher in these animals. D-Aspartate, D-serine, and L-serine were, however, higher in certain areas of the brain. Low plasma D-Glu levels are associated with cognitive impairment in Alzheimer's disease [111]. *Lactobacillus rhamnosus* JB-1 changed the expression of GABA receptors (GABARs) in the brain, which led to lower anxiety and less depressive behavior [111].

5.2. Gamma-Aminobutyric Acid (GABA)

GABA is found in high (millimolar) concentrations in many brain regions and is released into the synaptic cleft upon depolarization of presynaptic neurons [112]. GABA is also produced in glia (astrocytes) connected with pre- and post-synaptic neurons [113] but different metabolic pathways [114]. In GABA-expressing neurons, α-ketoglutarate, synthesized in the Krebs cycle, is converted to L-glutamic acid by GABA α-oxoglutarate transaminase (GABA-T, EC 2.6.1.19) and decarboxylated to GABA by GAD. In glial cells, glutamate is not decarboxylated to GABA, as these cells do not express GAD. Instead, GABA is synthesized from N-acetylputrescine with monoamine oxidase B (MAOB, EC 1.4.3.4) as a key enzyme [114]. GABA in glia is converted to succinic semialdehyde by GABA-T but also to glutamine, which is deaminated to glutamate before it re-enters the GABA shunt [112]. Several reports of GABA produced by gut microbiota have been published, with special reference to *Lactobacillus, Bifidobacterium* and *Bacteroides,* specifically *B. fragilis* [115]. *Escherichia coli* K12 uses GABA as its sole carbon and nitrogen source [116]. More recently, a new "GABA-eating" species, *Evtepia gabavorous* within the family *Ruminococcaceae*, has been described [115,117]. The dependency of *E. gabavorous* on GABA is evident in that strains only grow in the presence of GABA-producing *Bacteroides fragilis* [118].

During early life, GABA serve as neurotransmitters [119,120]. With further development, GABAergic neurons transfer glutamate between synaptic cells. The adhesion of GABA to GABARs to postsynaptic neurons prevents the transfer of Na^+, K^+, Ca^{2+} and Cl^- [121]. Three classes of GABARs have been described, i.e., $GABAR_A$, $GABAR_B$ and $GABAR_C$. $GABAR_B$ transfers signals received from hormones, neurotransmitters and pheromones to signal-transferring pathways [112,120,121]. A select few microorganisms have been known to alter the function of GABARs. *Lactobacillus rhamnosus* JB-1 changed GABARs expression in the brain, which led to a decrease in anxiety and depression [122]. It may, thus, well be that certain *Lactobacillus* spp. play a key role in the regulation of anxiety and depression. Treatment with *Lactobacillus* elevated GABA levels in hippocampal and prefrontal cortex [123]. Acetate produced by microbiota in the colon is transferred across the BBB to the hypothalamus and enters GABA neuroglial cycling pathways [124]. Gut-derived GABA, unlike catecholamines, reaches the CNS via specific GABA transporters expressed in the BBB [125]. At least six different GABA transporters regulate uptake into presynaptic nerve terminals and surrounding glial cells [126]. Re-uptake of GABA by neurons coincides with a decrease in Na^+ levels. Under normal physiological conditions, intracellular levels of GABA are approximately 200 times higher than extracellular levels. In glia, GABA is transformed to glutamine. The latter is then transferred back to neurons [126]. Glutaminase converts glutamine to glutamate, which re-enters the GABA shunt. Other microbiota, e.g.,

Akkermansia muciniphila, *Parabacteroides merdae* and *Parabacteroides distasonis*, may also play a role in the regulation of GABA by altering GABA/glutamate ratios and increase glutamate levels in the brain [127].

5.3. Glycine

Glycine is an excitatory and inhibitory neurotransmitter [128]. As an excitatory neurotransmitter, it serves as a co-agonist with Glu at the N-methyl-D-aspartate receptor (NMDAR) transmitter, allowing the removal of magnesium from the passage of Na^+ and Ca^{2+}, which is critical in the enhancement of learning and neuronal flexibility [129]. As an inhibitory neurotransmitter, glycine plays a role in the processing of motor and sensory information that permits movement, vision, and audition [129]. Glycine is often co-released with GABA and moderates excitatory neurotransmission by enhancing the action of glutamate at NMDARs. Excess glycine is taken up by the sodium-and-chloride-coupled transporters GLYT1 (located in the plasma membrane of glial cells) and GLYT2 (found in pre-synaptic terminals) [130].

5.4. Dopamine (DA)

DA is produced in the substantia nigra, ventral tegmental area, and hypothalamus and is released into the nucleus accumbent and prefrontal cortex of the brain. It is generally referred to as the reward neurotransmitter, but also has a role in the modulation of behavior and cognition, voluntary movement, motivation, inhibition of prolactin production, sleep, dreaming, mood, attention, working memory and learning [131]. Tyrosine is hydroxylated to L-dihydroxyphenylalanine (L-DOPA) and then decarboxylated to DA. In the presence of DA, β-hydroxylase DA is converted to NE and Epi (Ad) [132]. DA is also produced by some *Bacillus* and *Serratia* species in the GIT [133].

Gut microbiota may alter the function of a neurotransmitter, as shown by studies conducted on individuals with Parkinson's disease. Levodopa, a natural precursor of DA, crosses the blood–brain barrier when administered peripherally and increases DA levels in the brain. However, levodopa metabolized by gut microbiota lowers its availability and DA produced peripherally causes unwanted side effects. *Enterococcus faecalis* decarboxylates L-DOPA to DA, but the latter is immediately dehydroxylated to m-tyramine by *Eggerthella lenta* [134]. The conversion of L-DOPA to DA by *E. lenta* is encoded by a single-nucleotide polymorphism in the gene encoding DA dehydroxylase. In future, screening of gut microbiota or mapping of the gut microbiome may become important in selecting a drug used for psychiatric treatment. This is possible with recent advances in sequencing, cloning, genetic manipulation, viral (including bacteriophage) targeting, imaging techniques and knowledge gained through research on GF animals. This may lead to the discovery of new pathways in the GBA that regulate brain functions and behavior. With recent progress in research on sensory neurons, intestinal epithelial cells and compounds affecting the activity of neurotransmitters, future psychiatric treatment may lean towards the production of specific metabolites in fermented foods.

5.5. Norepinephrine (NE) or Noradrenaline (NAd)

NE is structurally similar to EPi (Ad) and is produced during excitement, but is also involved in behavior and cognition, such as memory, learning, and attention [135], and is involved in inflammation and modulates responses of the autonomic nervous system [136]. NE shares characteristics with autoinducer-3, a quorum sensing molecule that stimulates enterohemorrhagic *E. coli* motility and virulence [137,138]. Cell growth of *Klebsiella pneumoniae*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Staphylococcus aureus* was also stimulated by NE, most likely due to iron acquisition [139]. *Bacillus mycoides*, *Bacillus subtilis*, *E. coli K12*, *Proteus vulgaris* and *Serratia marcescens* produce between 0.45 and 2.13 mM NE [140] and there is now evidence that these species may use the hormone in quorum sensing [3,141]. GF mice had reduced levels of NE in the

cecum and in tissue cells, but these could be restored by colonization with a combination of *Clostridium* spp. [133].

5.6. Serotonin (5-HT)

5-HT regulates appetite, gut motility, mood, cognition and sleeping patterns [142–144]. Enteric 5-HT levels are regulated by tryptophan hydroxylase TPH1 and TPH2 [145]. Although 5-HT is synthesized by neurons of the ENS, more than 90% of 5-HT is produced in the gut by ECCs [146]. Some authors claim that as much as 80% 5-HT is produced in the GIT by *E. coli*, *Hafnia, Bacteroides*, *Streptococcus, Bifidobacterium*, *Lactococcus, Lactobacillus, Morganella*, *Klebsiella, Propionibacterium*, *Eubacterium*, *Roseburia* and *Prevotella* [142,143]. *Candida* and *Escherichia* convert tryptophan in food to 5-HT [58]. Serotonin production by gut microbiota may have a greater effect on the CNS than originally anticipated, as ECs interact with 5-HT-receptive afferent fibers in vagal or dorsal root neurons [147]. Proof of microbiota affecting emotional behavior was shown in various studies on mice. Mice that had a subdiaphragmatic vagotomy showed no changes in emotional behavior, irrespective of an increase in neurotransmitters produced by gut microbiota [148,149].

Studies conducted on mice have shown a drastic increase in the development of enteric neurons two to three weeks after treatment with a serotonin 5-HT4 agonist [150]. The authors have also shown that neurons of GF mice, unable to synthesize serotonin, were less developed. A separate study [151] has shown that neuronal dysfunctions in GF mice could be reversed by recolonization with gut microbiota. Several studies confirmed this and presented clear evidence that the synthesis of 5-HT is regulated by gut microbiota [88,146,150]. Reigstad et al. [152] have shown that sodium acetate (10–50 mM) significantly increased *TPH1* mRNA expression in a human-derived EC cell model (BON cells). Butyrate (0.5 and 1.0 mM) increased *TPH1* mRNA expression to similar levels (more than 3-fold). However, treatment with 2.0 mM butyrate did not significantly alter TPH1 expression. Higher levels of butyrate (8.0 and 16.0 mM) suppressed TPH1 expression 13.5- and 15.7-fold, respectively, which was below the expression levels recorded for untreated EC cells [152]. These findings clearly indicated that EC cells are stimulated by gut microbiota, specifically SCFAs, to produce 5-HT. Regulation of TPH1 expression is viewed as a rate-limiting step in the biosynthesis of DA, NAd and adrenaline [153]. Changes in 5-HT levels are also associated with IBD. In patients with ulcerative colitis (UC) and Crohn's disease (CD), a drastic increase in serotonin-immunoreactive cells was recorded in the colon [154]. In patients diagnosed with CD, colonic PYY, pancreatic polypeptide (PP), and oxyntomodulin-producing endocrine cells were much lower [154]. Patients with IBD have high levels of plasma chromogranin-A (CgA) [155], whereas patients with UC have high levels of fecal CgA [156]. CgA and its derived peptides, e.g., vasostatin (VS), catestatin (CST) and chromofungin (CHR), assist in the regulation of antimicrobial activity, suggesting that changes in CgA levels in EECs may lead to alterations in intestinal microbial composition and diversity [157]. Significant changes have also been recorded in other circulating EEC secretory products, such as PYY, CCK, GLP-1, 5-HT, somatostatin, gastrin, and motilin in individuals with IBD [158]. One of the consequences of chronic colitis is EC hyperplasia. In studies conducted on mice with colitis, an increase in 5-HT production was observed [159,160]. With an increase in 5-HT levels, expression of the serotonin type 7 (5-HT7) receptor on DCs is activated and a pro-inflammatory immune response is triggered [161]. Inhibition of the 5-HT7 receptor reduced intestinal inflammation [162]. Activation of 5-HT4 receptors plays a major role in maturation of the adult nervous system in that it regulates the formation of neurons and also protect the cells [88,150].

In germ-free mice, low levels of tyrosine led to reduced 5-HT levels [88]. A lack in communication between gut microbiota and the ENS is directly linked to dysbiosis and gastrointestinal disorders, as shown by several studies [88,163–165]. In a healthy gut, *Clostridium perfringens* modulates 5-HT synthesis by using TPH of the host [146].

5-HT also affects the host's immune system and behavior of glial cells in the ENS and CNS [166,167], whilst activation of the 5-HT4 receptor in the ENS displays neuro-

generative and neuroprotective properties [168]. Gut microbiota convert tryptophan to indole-3-acetic by using tryptophan monooxygenase (EC 1.13.12.3) and indole-3-acetamide hydrolase (EC 3.5.1.4), then decarboxylate indole-3-acetic to 3-methyl indole. Indole-containing compounds activate the aryl hydrocarbon receptor (AHR) on mucosal and immune cells [169,170]. The conversion of tryptophan to indole by *Lactobacillus reuteri, Lactobacillus johnsonii,* and *Lactobacillus murinus* helps in the differentiation of T cells [171] and prevents colitis [172]. Indole-3-aldehyde produced by *Lactobacillus* spp. induces AHR, which, in turn, induces IL-22 required for the secretion of antimicrobial peptides [169]. Since inflammation plays an important role in the development of Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, and Alzheimer's disease [173], production of indole by gut microbiota need to be investigated in more depth.

Another surprising finding is that 5-HT promotes the colonization of *Turicibacter sanguinis* in the human gut, a bacterium that expresses a neurotransmitter sodium symporter-related protein structurally similar to the mammalian serotonin reuptake transporter (SERT) [174]. A recent report [172] suggests that some neurotransmitters may serve as growth substrates for intestinal bacteria. High levels of 5-HT may decrease gut wall permeability, whilst low levels of 5-HT decrease the expression of occludin and weaken the gut wall, leading to increased permeability and the development of a leaky gut [143]. The latter was reported in patients diagnosed with irritable bowel syndrome (IBS) [143]. Excess 5-HT in the circulatory system is transported across the cell membrane by SERT and intracellularly inactivated by MAO [145]. Most of the currently available antidepressants prevent the synaptic re-uptake of biogenic amines [175]. On the other hand, patients with major depressive disorder (MDD) often develop resistance to antidepressants. This led to studies that investigated the relationship between gut microbiota and depression [176].

5.7. Histamine

Histamine facilitates homeostatic functions, endorses wakefulness, and controls feeding and motivational behavior [177]. Histamine produced by gut microbiota activates histamine receptors [178]. Bacteria that produce histamine include *Lactobacillus* spp., *Lactococcus lactis*, *Oenococcus oeni*, *Pediococcus parvulus*, *Streptococcus thermophilus*, *Morganella morganii*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Citrobacter freundii* and *Hafnia alvei* [3]. Cadaverine, putrescine and agmatine also activate histamine receptors [179]. Further research is required to identify neuron-signaling microbial metabolites. It is also important to determine if these metabolites are produced at high enough concentrations, and in active form, to communicate with receptors on neurons. An area that has up to now been ignored is the role viruses and bacteriophages play in neurotransmission. Bacteriophages can alter the levels of tryptamine and tyramine in the GIT [180], but we do not know if these changes have an impact on neuronal activity.

6. Role of Short Chain Fatty Acids in Neurotransmission

SCFAs, such as butyrate, acetate, lactate and propionate, are largely produced in the colon by *Bifodobacterium, Lactobacillus*, *Lachnospiraceae*, *Blautia*, *Coprococcus*, *Roseburia* and *Faecalibacterium* and provide energy to epithelial cells [181]. These SCFAs adhere to free fatty acid receptors (FFARs), e.g., GPR43(FFAR2) and GPR41 (FFAR3) located on the surface of IECs [182]. Receptors FFAR2 and FFAR3 are also expressed in the ENS, portal nerve and sensory ganglia [88]. GPR 41 in the ENS transfers signals induced by SCFAs directly to the CNS [183]. GPR43, expressed in white adipose tissue, communicates with SCFAs to stimulate energy expenditure in skeletal muscles and the liver [184]. SCFAs stimulate antimicrobial peptides through the cathelicidin LL-37 pathway and prevent Shigella infection [185]. Individuals with IBD have low levels of fecal SCFAs, accompanied by a decrease in Firmicutes and Bacteroidetes [186,187].

The crossing of IECs is facilitated by specialized monocarboxylate transporters [188,189]. Some SCFAs, however, diffuse across IEC membranes and enter the circulatory system un-ionized [190]. Butyrate protects intestinal barrier function by up-regulating the tight

junction protein claudin-1 [191] and is used by colonocytes as their main energy source [192]. Butyrate also induces apoptosis of colon cancer cells [192] and plays an essential role in the consumption of oxygen in epithelial cells. A balanced state of oxygen prevents dysbiosis [193]. Butyrate suppresses inflammatory responses by downregulating histone deacetylase EC 3.5.1.98 (also referred to as lysine deacetylase) inhibitors (HDACi) [194,195]. An increase in de-acetylated histones (due to the inhibition of HDACi), together with a decline in gene transcriptions, leads to autophagic cell death, the activation of extrinsic and/or intrinsic apoptotic pathways, an increase in the production of reactive oxygen species (ROS), and a decrease in the expression of pattern recognition receptors, kinases, transcription regulators, cytokines, chemokines, and growth factors [196,197]. Molecules released from dying cells, interpreted as damage-associated molecular patterns (DAMPs) and pathogens recorded as pathogen-associated molecular patterns (PAMPs), are recognized by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and form specific protein–protein interactions. These interactions, also prevalent in lymphocytes, macrophages and dendritic cells, play a key role in the regulation of cytokines, chemokines and the expression of genes coding for the production of antimicrobial compounds, collectively referred to as the innate immune response [183,198,199]. Downregulation of NLRs, thus, prevents the formation of multi-protein inflammasomes, the signaling of caspase-independent nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK, EC 2.7.11.24). These cascades of events counteract autoimmune and inflammatory disorders and as recently shown, repress the growth of cancerous cells [200].

Changes in butyrate levels may be due to an imbalance in butyrate-producing bacteria, or increased binding of butyrate to FFARs located on EECs [9,201]. The activation of G-protein-coupled receptors (GPCRs) by butyrate may cause several neurodegenerative disorders [9] and stimulation of regulatory T cells by butyrate induces the production of inflammatory cytokines [202]. Higher cytokine levels control the proliferation of *Proteobacteria* in the GIT [203]. Low levels of butyrate inhibit GPCRs and interrupt immune or hormonal responses [204]. Butyrate modifies the integrity of the BBB, which affects the CNS and maturation of microglia [204]. In germ-free (GF) mice, defective microglia could be stimulated by incorporating additional butyrate, propionate and acetate to the feed [205]. Acetate crosses the BBB and accumulates in the hypothalamus [124]. This activates the hypothalamic–pituitary–adrenal (HPA) axis, which sends signals to EECs [9].

Inhibition of HDACi in the frontal cortex and hippocampus of mice, brought about with the administration of sodium butyrate, alleviated depressive behavior [206], dementia and brain trauma [201]. High levels of HDAC have been reported in patients suffering from neurological disorders such as depression, Parkinson's disease, and schizophrenia [201]. Elevated levels of ACHs, however, increase the expression of *bdnf*, encoding brain-derived neurotrophic factor (BDNF) in the frontal cortex and hippocampus and stimulate brain development [207,208]. Low levels of BDNF are associated with depression and anxiety [144,209,210]. Neurological disorders may, thus, be prevented by keeping SCFAs and HDAC at optimal levels. One way of achieving this is to maintain a well-balanced gut microbiome. SCFAs, tryptophan precursors, and metabolites interact with receptors located on the gut wall, muscle layers surrounding the gut, liver, pancreas, adipose tissue and immune cells [58].

Subsequent studies have shown that SCFAs can modulate genes encoding the cAMP response element-binding (CREB) protein that regulates the synthesis of catecholamine neurotransmitters, such as DA [211,212]. SCFAs activated the expression of tyrosine hydroxylase, the first reaction in the production of DA, and decreased the expression of dopamine- β-hydroxylase (DBH; EC 1.14.17.1), and thus the conversion of DA to NE [213,214]. These studies clearly indicated that SCFA-producing gut microbiota play a crucial role in brain processes. Further evidence for this was found in a more recent study [4] on the role that SCFAs play in alleviating stress-induced symptoms. The hippocampus and striatum are especially susceptible to DA, which triggers a "neurotransmitter reward" response, and thus a change in mood. A diet that increases the production of

SCFAs may alleviate immune and metabolic dysfunctions, as shown in studies conducted on schizophrenic patients [215]. Despite the many positive attributes DA may have, the rate at which it is synthesized, including its conversion to NE, needs to be tightly regulated. A deficiency in DBH (thus elevated DA levels) may have a serious negative affect on the autonomic nervous system (ANS) that controls the regulation of blood pressure and body temperature [216]. Early symptoms of DBH deficiency include vomiting, dehydration, decreased blood pressure (hypotension), difficulty maintaining body temperature, low blood sugar (hypoglycemia) and extreme fatigue during exercise. In males, DBH deficiency (thus increased dopamine levels) may result in retrograde ejaculation, i.e., discharge of semen into the bladder [216].

Acetate reduces appetite by stimulating the secretion of ghrelin [124]. Consistent with this, propionate feeding induces fos (fos proto-oncogene, AP-1 transcription factor subunit) expression in the dorsal vagal complex of the brainstem, the hypothalamus, and the spinal cord [190], raising the question as to whether SCFA-induced stimulation of peripheral sensory neuronal activity could mediate the effects of SCFAs on host feeding behavior. SCFAs regulate several other physiological functions in the body, e.g., the maturation and functioning of microglia in the CNS [203], the transmission of signals generated by serotonin, GABA and DA signals to neurons [217,218] and the secretion of anions in the colon [192,219,220]. The latter is due to the stimulation of nicotinic Ach receptors in the colon that leads to an increase in Ach production and the stimulation of goblet cells to secrete more mucus [221].

In immune cells, SCFAs regulate the differentiation of T-cells [222,223]. In enteroendocrine cells, SCFAs stimulate the release of gut hormones [224]. Bile salts, combined with SCFAs, play an integral part in enterohepatic circulation, but are equally important in the regulation of neuronal pathways and signaling to the CNS [225]. Further research in this area may shed more light on the effects diets have on mood changes.

It was reported that individuals with Parkinson's disease (PD) had increased cells numbers of *Enterobacteria*, but fewer *Prevotella* spp. [226,227]. Other PD patients had fewer butyrate-producing species, such as *Blautia*, *Coprococcus*, and *Roseburia*, but higher numbers of potentially harmful proinflammatory Proteobacteria, especially *Ralstonia* [228]. These changes in composition of gut microbiota coincided with lower levels of SCFAs [227].

From all these studies, it is clear that SCFAs, produced by microorganisms, play a key role in microbiota–gut–brain axis communication, protection of the intestinal barrier and inflammatory responses. Levels of SCFAs, however, need to be carefully controlled, as several disadvantages have been reported. Acetate, for instance, promotes the production of intestinal IgA [229], stimulates the secretion of cytokine IL-6 and increases neutrophil recruitment [184]. Propionate administered to patients with obesity enhanced gut hormone secretion, while reducing adiposity and overall weight gain [230]. The mechanisms involved in the regulation of SCFA production and its impact on feeding behavior remain unclear. The exact mechanisms whereby SCFAs regulate appetite remains unclear. FFAR2 and FFAR3 are expressed in the ENS, portal nerve and sensory ganglia [192,231]. This suggests that SCFAs regulate the nervous system, a hypothesis supported by the induction of fos in the dorsal vagal complex of the brainstem, the hypothalamus, and spinal cord [192]. The expression of fos is induced by serum, growth factors, tumor promoters and cytokines. SCFAs are fundamental molecules involved in regulating energy homeostasis, and SCFARs are expressed by a wide variety of non-neuronal cell subtypes as well. In immune cells, for example, SCFAs can regulate T regulatory cell differentiation [182,222] and microglial maturation [223], whereas in EECs, SCFAs can stimulate the release of gut hormones [224].

Expression of *Tph1* is induced by SCFAs [142,145]. Alleviation of anxiety and depression is, thus, associated with an increase in SCFA levels [143]. The opposite is also true. Individuals suffering from anxiety and depression have low SCFA but may also have high blood pressure—all of which may lead to cardiovascular diseases, strokes, obesity and diabetes mellitus [143]. Studies conducted on rats have shown that hypertension could be prevented by restoring acetate levels in the cecum [88,232]. These studies have shown a

strong interaction between gut microbiota and the ENS, which may provide answers to the link between microbial dysbiosis and gastrointestinal disorders.

SCFAs produced by gut microbiota are transported via blood vessels to the brain and modulate functions of neurons, microglia and astrocytes, and affect the BBB [233]. Histone acetylation is regulated by SCFAs such as acetate and butyrate. It is, thus, important to control the production of SCFAs. A separate study has shown that acetate, produced in the colon by gut microbiota, crosses the BBB and concentrates in the hypothalamus, from where it stimulates GABA production in the brain [126]. Further research is required to understand how bacteria regulate neurotransmitters and lead to a sudden increase in pathogens.

7. Conclusions

Gut bacteria are not only sensitive to physiological variations in the GIT, but also to the signals received from the CNS via the VN and ENS. Minor activation of the VN results in drastic changes in the production of neurotransmitters, which affects digestion, intestinal permeability, gastric motility, and immune regulation. Signals received from the CNS and ENS change the microbial composition in the GIT and may benefit the survival and proliferation of certain species. In return, gut bacteria stimulate EECs to produce hormones such as 5-HT, CCK and PYY that communicate with the CNS via neural afferent fibers. Neurotransmitters such as Glu, GABA, DA, NE, 5-HT and histamine synthesized by gut bacteria communicate with the CNS, autonomic sympathetic and parasympathetic nervous systems and the HPA to control the release of growth-stimulating hormones. Intermediate compounds such as SCFAs, tryptophan and secondary bile acids also communicate with the CNS. Signals generated by the hypothalamus reach the pituitary and adrenal glands and communicate with EECs via the HPA. The intricate control of entero-endocrine signaling and immune responses keeps the gut microbiome in a balanced state. In future, stimulation of the VN by probiotic bacteria may be used in the treatment of neurological disorders, such as depression and anxiety, IBD and IBS.

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Abbreviations

CNS, central nervous system; SCFAs, short-chain fatty acids; Glu, glutamate; GABA, γ-aminobutyric acid; DA, dopamine; NE, norepinephrine; NAd, noradrenaline; 5-HT, serotonin; VN, vagus nerve; GIT, gastro-intestinal tract; ENS, enteric nervous system; HPA, hypothalamic pituitary adrenal axis; FFARs, free fatty acid receptors; GBA, gut–brain axis; Ach, acetylcholine; Epi, epinephrine; Oxt, oxytocin; ADH, antidiuretic hormone; ECCs, enterochromaffin cells; EECs, enteroendocrine cells; UC, ulcerative colitis; IBDs, inflammable bowel diseases; BBB, blood–brain barrier; HMP, Human Microbiome Project; MetaHIT, METAgenomics of the Human Intestinal Tract; NTS, nucleus tractus solitarii; LC, locus coeruleus; ENCCs, enteric neural crest cells; GDNF, glial cell line-derived nerve growth factor; NTN, neurturin; ART, artemin; PSP, persephin; TLRs Toll-like receptors; GFR, growth factor receptor; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide; CCK, cholecystokinin; GLP, glucagon-like peptide; GPBAR, G protein-coupled bile acid receptor; FXRs, farnesoid X receptors; AgRP, agouti-related protein; ACTH, adrenocorticotropin (corticotropin); GHSRs, ghrelin receptors; α-MSH, melanocyte-stimulation hormone; ECs, enterochromaffin cells; ClpB, caseinolytic protease B; DCs, dendritic cells; ITF, Intestinal trefoil factor; RELM-β, resistin-like molecule-β; Th, T-helper; Tuft, taste chemosensory epithelial; GF, germ-free; NSCs, neural stem cells; PLC-γ, phospholipase C gamma; RAS/MAPK, ras/mitogen activated protein kinase; LPS, lipopolysaccharide; Glu, glutamate; VACCs, voltage-activated calcium channels; CaMK, calcium calmodulin-

dependent kinase; ERK, extracellular signal-regulated kinase; CREB protein, cyclic AMP response element binding protein; VGLUT, vesicular glutamate; GAD, glutamic acid decarboxylase; SPF, specific pathogen-free; GABARs, γ-aminobutyric acid receptors; GABA-T, α-oxoglutarate transaminase; MAO, monoamine oxidase; NMDAR, N-methyl-D-aspartate receptor; L-DOPA, L-Dihydroxyphenylalanine; TPH, tryptophan hydroxylase; CD, Crohn's disease; PP, pancreatic polypeptide; CgA, chromogranin-A; VS, vasostatin; CST, catestatin; CHR, chromofungin; AHR, aryl hydrocarbon receptor; SERT, serotonin reuptake transporter; IBS, irritable bowel syndrome; MDD, major depressive disorder; HDACi, histone deacetylase (lysine deacetylase) inhibitors; ROS, reactive oxygen species; DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; NOD, nucleotide-binding oligomerization domain; NLRs, NOD-like receptors; NF-κB, nuclear factor kappa B; GPCRs, G-protein-coupled receptors; HPA, hypothalamic-pituitary-adrenal; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding; ANS, autonomic nervous system.

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Chapter 9

General Discussion, Conclusions, and the Future

So, what next?

General Discussion, Conclusions, and the Future

The human gut microbiome consisting of close to 4 trillion microorganisms co-evolved with humans over millions of years and supersedes the number of cells in our bodies 10-fold. With this in consideration, the human genome sequence published in 2003 is but a small drop in the ocean. A core group of autochthonous intestinal bacteria, ranging between 400 and 500 species, is always present. Most of these bacteria (90%) are represented by Firmicutes, Proteobacteria, Actinobacteria, *Fusobacteria, Cyanobacteria*, *Verrucomicrobia* and *Euryarchaeota*. Firmicutes are by far in the majority and is dominated by *Lactobacillus* spp. and Bacteroidetes. It is thus safe to conclude that most changes in the core bacteria gene pool will involve lactic acid bacteria (LAB). Based on results from a study on *Escherichia coli*, the mutation rate of intestinal bacteria is approximately 7×10^{-7} per base. It thus seems as if population changes are more driven by adaptations. The same species isolated from different individuals showed at least one variation in every hundred base pairs. This suggests that species that cannot adapt or compete are replaced by novices that are better equipped to regulate their own gene expressions or alter their genetic composition. This explains why the bacterial community in the GIT of individuals differ and are represented by strains with unique phenotypic and genotypic characteristics.

Although the GIT is a hostile environment, mucus, antimicrobial peptides (AMPs) and immunoglobulin A (IgA) produced by intestinal epithelial cells (IECs) provide some protection to commensal gut microbiota. The core group of autochthonous gut microbiota are generally less sensitive to AMPs compared to pathogenic bacteria. Pattern recognition receptors (PRRs) such as such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) recognise microbiota through microbe-associated molecular patterns (MAMP). AMPs such as defensins and cathelicidins, regenerating (Reg)-family proteins and ribonucleases, together with IgA, act as the first line of defence against bacteria, viruses, yeast, fungi, and cancer cells. Intestinal mucus layers are protected from microbial cells by lectins. Additional protection against microbial biofilm formation is provided by secretory immunoglobulin A (SIgA) produced by plasma cells in mucus layers. Dendritic cells (DCs) that penetrates the gut wall is in constant communication with the immune system.

Activation of TLRs induce strong immunity- and inflammation effects but is also involved in the secretion of hormones such as glucagon-like peptide-1 (GLP-1), 5-hydroxytryptamine (5-HT or serotonin) and peptide tyrosine tyrosine (PYY). Gut microbiota regulates the release of 5-HT, GLP-1, PYY, glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CCK), ghrelin, leptin, pancreatic polypeptide (PP), oxyntomodulin and neurotensin. Serotonin act as neurotransmitter, but also regulates diverse functions such as platelet aggregation, bone development, immune responses, cardiac functions, promote homeostasis and control enteric motor and secretory reflexes. Apart from serotonin, neurotransmitters such as glutamine (Glu), gamma-amino butyric acid (GABA), dopamine (DA), norepinephrine and histamine are synthesized by gut bacteria. These molecules communicate with the central nervous system (CNS), autonomic sympathetic and parasympathetic nervous systems, but also the hypothalamic-pituitary-adrenal axis (HPA) to control the release of growth-stimulating hormones. Intermediate compounds such as short-chain fatty acids (SCFAs), tryptophan and secondary bile acids also communicate with the CNS. Signals generated by the hypothalamus reach the pituitary and adrenal glands and communicate with entero-epithelial cells (EECs) via the HPA. The intricate control of entero-endocrine signaling and immune responses keeps the gut microbiome in a balanced state. The role of gut bacteria in the synthesis and degradation of neurotransmitters is reviewed by Dicks (2022). The modulation, development, and renewal of enteric nervous system (ENS) neurons are controlled by gut microbiota, especially those with the ability to produce and metabolize hormones. SCFAs produced by gut bacteria adhere to free fatty acid receptors (FFARs) on the surface of intestinal epithelial cells (IECs) and interact with neurons or enter the circulatory system. Gut bacteria are not only sensitive to physiological variations in the GIT, but also to the signals received from the CNS via the Vagus nerve (VN) and ENS. Minor activation of the VN results in drastic changes in the production of neurotransmitters, which affects digestion, intestinal permeability, gastric motility, and immune regulation. Fibers of the VN are not in direct contact with the gut wall or intestinal microbiota. Instead, signals reach the gut microbiota via 100 to 500 million neurons from the ENS in the submucosa and myenteric plexus of the gut wall.

Gut microbiota has an immense impact on the gut-brain axis (GBA) and overall mental health (reviewed by Dicks *et al*., 2021). Chemicals secreted by these bacteria, such as GABA, in addition to other metabolites, play an important role in anti-inflammatory responses and help alleviate psychiatric symptoms stemming from inflammation. Treatment of schizophrenic and bipolar patients with probiotics alleviated symptoms associated with IBD, autistic children

benefitted from probiotic treatment and OCD-like behavior could be controlled. The effect IBD has on depression, stress and anxiety requires in-depth studies. Our understanding of exactly how gut microorganisms control cognitive behavior, mood, and neuropsychiatric disorders remains limited. The deciphering of this complex, everchanging network between cells and neurons requires in-depth research by scientists from diverse disciplines. Although preclinical and clinical investigations have shown that treatment with probiotics may improve mood, extensive and carefully controlled clinical trials need to be performed to evaluate the effectiveness in treating mental disorders. Biomarkers need to be developed to identify differences in the gut microbiome of individuals suffering from psychological disorders. Interactions between drugs used in treatment and gut microbiota need to be studied in greater depth. Studies should include multi-omics of gut and oral microbiota to have a better understanding of the mutual interplay between phyla. Central to the control is quorum sensing, in the GIT defined as communication amongst gut microbiota and between microbial cells and gut epithelial cells.

QS molecules vary in type, structure, and specificity (reviewed by Dicks, 2022). Inter- and intra-species signalling systems have been well studied, but far less is known about interkingdom QS, especially between bacteria and intestinal epithelial cells (IECs) (Dicks, 2022). The auto-inducer 3 (AI-3)/epinephrine (Epi)/norepinephrine (NE) signalling system described for pathogenic *Escherichia coli, Salmonella typhimurium* and *Ctirobacter rodentium* that communicate with host cells (Moreira *et al*., 2010; Moreira and Sperandio, 2016) provides valuable information on the expression of virulence genes. *Escherichia coli, Salmonella, Citrobacter* and other pathogens such as *Pseudomonas aeruginosa, Enterobacter*, *Cronobacter*, *Klebsiella*, and *Shigella* that do not always have receptors to recognize signals produced by bacteria in the same niche, and are thus unable to communicate on an interspecies level. To circumvent the problem, they hey have developed “solo” LuxR-type receptors (e.g. SdiA, a LuxR homolog, and QscR) with a broader range of responsiveness (Styles and Blackwell, 2018; Sabag-Daigle and Ahmer, 2012). It is noteworthy that pathogens are exceptionally skilled at deploying “solo” signaling receptors. For some pathogens, the importance of communication is so high on their list that they have developed multiple QS signals. An example is *Pseudomonas aeruginosa* using the *las*, *rhl* and *pqs* systems simultaneously (although the latter two are induced by *las*) (Rutherford and Bassler, 2012). This is done to ensure control over the synthesis and secretion of virulence factors, bioluminescence, biofilm formation, ion uptake, antibiotic resistance, motility and enzyme

production. Another example is enterohemorrhagic *E. coli* (EHEC) that deploys at least five QS ignaling systems, i.e. AI-2, SdiA, AI-3/Epi/NE, indole and extracellular death factor (EDF) (Zohar and Kolodkin-Gal, 2015). The result of interspecies communication in the GIT is perfectly illustrated by the SdiAEC/AHL QS complex formed as result of communication between *E. coli* and *P. aeruginosa* (Hughes *et al*., 2010). The newly formed SdiAEC/AHL increases transcription of genes in the gad operon (*gadW*, *gadE*, *yhiD* and *hdeA*) of *E. coli*, rendering the species resistant to acidic conditions (Price *et al*., 2004). Whether other gut microbiota, and LAB, have developed similar strategies is uncertain. Although LAB are adapted to survive acidic conditions, their growth is repressed by stomach and bile acids. The study we have conducted on the probiotic strain *Lactobacillus plantarum* 423 may provide some answers. When exposed to pH 2.5 for an hour, the cells over-expressed 12 of the 97 proteins identified, as revealed using gel-free nanoLC−MS/MS proteomics analyses (Heunis *et al*., 2014). The acid-stressed cells produced several stress response proteins, metabolised a variety of carbohydrates (which implies that catabolite repression did not occur or was down regulated), redirected the metabolism of pyruvate, increased the production of lysine and ammonia, and developed a significant oxidative stress response. The accumulation of basic compounds also seemed to play an integral role in the response to acid stress. A marked decrease in proteins involved in cell wall and phospholipid biosynthesis, transcription, translation, and cell division were observed. The most abundant protein detected, JDM1_2142, remained uncharacterized. Although not proven, protein JDM1_2142 may be a proton pump, perhaps similar to F1F0-ATPase proton pumps described for other acid-resistant Gram-positive bacteria (Cotter and Hill, 2003; De Angelis and Gobbetti, 2004). It is tempting to think that, in the stomach, protein JDM1_2142 may be involved in some form of communication with epithelial cells. This is mere speculation, as various other acid-tolerating mechanisms have been identified, e.g. activation of glutamate decarboxylases and arginine deiminases, repair of damaged DNA and proteins, and changing of cell envelope structures (reviewed by Heunis *et al*., 2016).

The effect of QS molecules on the CNS is ill-researched. Several QSPs can diffuse through the intestinal mucosa and enter the circulatory system, from where they may penetrate the blood–brain barrier (BBB) (Wynendaele *et al*., 2015). Based on these findings, QSPs may play a key role in communication between the gut microbiome and the brain. If this is the case, QSPs may affect neurodevelopment and initiate neurodegenerative diseases. Janssens *et al*. (2018) screened 85 quorum sensing peptides on six different neuronal cell lines and found 22

peptides with a possible effect on the GBA. Of these, four peptides induced neurite outgrowth, two peptides inhibited nerve growth factor (NGF)-induced neurite outgrowth and eight peptides induced neurite outgrowth in human SH-SY5Y neuroblastoma cells. Two peptides killed SH-SY5Y cells and six peptides induced either IL-6 expression or nitric oxide (NO) production. Several reports have been published on the role that cell wall components such as lipopolysaccharides, polysaccharides and peptidoglycans play in neuron activation and the GBA (reviewed by Dicks, 2022). Cell wall components also induce the release of neuropeptides, ATP and cytokines. Short-chain fatty acids, tryptophan, trace amines and exotoxins also have neuromodulator properties. Serotonin and histamine excite mast cells in the proximity of nerve endings. Neuronal conditions such as Alzheimer's disease (AD), autism spectrum disorder (ASD), multiple sclerosis (MS), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are associated with dysfunctional microglia. Fecal transplants from humans with attention deficit hyperactivity disorder (ADHD), AD and PD to mice activated microglia in the brain and aggravated cognitive and physical impairments. These findings along with more evidence of a clear link between microbial dysbiosis and neurodevelopmental, neurodegenerative and psychiatric disorders such as ASD, schizophrenia, AD, major depressive disorder (MDD) and PD prompted researchers to have a closer look at the GBA. For more information on gut bacteria and neurotransmitters, the reader is referred to a recent review by Dicks (2022). The role that gut bacteria play in neuropsychiatric disorders has recently been reviewed by Dicks *et al*. (2021).

Exotoxins produced by *Staphylococcus aureus* increase intracellular calcium levels, leading to the activation of sensory neurons. This is especially true for phenolsoluble modulins (psms) attached to formyl peptide receptor-like proteins (FPRs). The expression of FPRs in sensory and dorsal root ganglia of the colon has been linked with QS-dependent pathways involved in the GBA (Hockley *et al*., 2018; Uhlig *et al*., 2020). The pore-forming toxin alpha-hemolysin (Hla) produced by *S. aureus* excites neurons by increasing the transfer of calcium (Chiu et al., 2013). The importance of exotoxins in GBA communication is unknown.

Little is known about QS molecules used by lactic acid bacteria (LAB) and other *Firmicutes*. It may be that LAB communicate using small linear or cyclized oligopeptides (QS peptides, QSPs) of 5 to 17 amino acids long, as reported for other Gram-positive bacteria (Coquant *et al*., 2021). The competence sporulation factor (CSF), a pentapeptide produced by *Bacillus* spp. (Fujiya *et al*., 2007) is the best studied. The peptide binds to the cation transporter OCTN2

and activates heat shock protein 27 (HSP-27), which increases the strength of intestinal barriers (Han *et al*., 1994). The PapRIV heptapeptide (SDLPFEH), produced by *Bacillus subtilis,* (Janssens et al., 2021; Yang et al., 2007) forms after cleaving of the inactive 48-amino-acid pre-peptide by NprB protease (Bouillaut et al., 2008; Pomerantsev et al., 2009). This is similar to the proteolytic cleaving of bacteriocin pre-peptides to form biologically active (mature) peptides. Are bacteriocins produced in the GIT and, if so, do they have a QS function and do they cross the gut-blood barrier (GBB)?

Several studies have shown that bacteriocins are produced in the GIT and protect the host against infection (Bron *et al*., 2004; Corr *et al*., 2007; Van Zyl, 2018). It is likely that bacteriocins interact with epithelial cells in the GIT and cross the GBB. Support for this comes from studies conducted by Spadoni *et al*. (2015). The authors have shown that molecules of 4 kDa can cross the gut-vascular barrier (GVB). Since most bacteriocins are smaller than 7 kDa, they may cross the GVB. We have shown that bacteriocins can indeed transverse epithelial (Caco-2) and endothelial (HUVECs) monolayers (Dreyer, 2018). Nisin A (3.35 kDa), plantaricin 423 (3.93 kDa), and bacST4SA (4.29 kDa), labeled with NHSfluorescein, crossed intestinal epithelial cells (IECs) and endothelial cells (ECs) without changing the integrity of the monolayers or being cytotoxic (Dreyer *et al*., 2019). Although the exact mechanism for crossing IECs and ECs was not examined, this study (albeit *in vitro*) provides evidence that bacteriocins can cross the GBB. Crossing of these peptides without eliciting a cytotoxic reaction suggests that they were transported paracellular. However, migration of cationic peptides via transcytosis (i.e., transcellular) cannot be ruled out and it may be the mechanism used by larger bacteriocins to cross the GBB. Botulinum and cholera toxins are transported by transcytosis. Once in the blood stream, bacteriocins may cross the blood-brain barrier (BBB). These peptides, being hydrophobic and positively charged, may adhere to lipophilic surfaces and negatively charged molecules. Bacteriocins may also bind to blood cells and plasma proteins, as indicated by Van Heel *et al*. (2011) and Dreyer *et al*., 2019). Since bacteriocins are membrane active cationic peptides, they may affect mammalian cell membranes. Minimal cytotoxic effects of bacteriocins against human cell lines have been reported (Sand *et al*., 2010; Begde *et al*., 2011; Kindrachuk *et al*., 2013; Dreyer *et al*., 2019). Given the concentration of bacteriocin required to induce significant cytotoxicity, these levels would most likely not be present if they do cross the GBB. This, however, does not discount the possibility of bacteriocins accumulating in organs such as the liver and causing membrane damage. If gut

wall permeability is severely changed, microbiota from the lumen may enter the blood stream and cause bacteremia. The fate of bacteriocins in the GIT is reviewed by Dicks *et al*. (2018).

The question whether bacteriocins survives the circulatory system has been dealt with in several studies. Bacteriocins injected intravenously did, to some extent, suppress intraperitoneal/subcutaneous infections (Goldstein *et al.*, 1998; Castiglione *et al*., 2007; Jabés *et al*., 2011). Peptidoglycan and bacterial flaggelins are recognised by Toll-like receptors (Asong *et al*., 2009; Gewirtz, 2006; Ren *et al*., 2006). A particular TLR may recognize more than one type of molecule, as in the case of TLR-2 recognizing different glycolipids and lipoproteins (Yan *et al*., 2007). Extracellular components produced by probiotic bacteria are recognised by the C-type lectin receptor (CLR) (Konstantinov *et al*., 2008). CLRs on the surface of immune cells, such as dendritic cells (DCs) and macrophages, recognize carbohydrate patterns and thus also glycoproteins (Benz and Schmidt, 2002). Glycolipids produced by lactobacilli are recognized by intestinal receptors (Iwamori *et al*., 2009). It is thus possible that bacteriocins will elicit an immune response. Further research on the fate of bacteriocins in the circulatory system and the responses they generate is important, as most intestinal bacteria have the ability to produce bacteriocins, as pointed out in a genome mining study conducted by Drissi *et al*. (2015). It is generally believed that the peptides will be degraded by proteolytic enzymes or destroyed by the immune system. It is almost certain that bacteriocins will be destroyed by macrophages should they cross the gut wall. Studies done on neurotherapeutic peptides and their crossing of the brain-blood barrier, along with other studies on small peptides intravenously injected, may provide some answers on the survival of bacteriocins in the circulatory system. Whether bacteriocins have the ability to cross the BBB remains uncertain. The heptapeptide PapRIV do cross the BBB, albeit in a one directional way (Janssens *et al*., 2021). It is possible that bacteriocins may react with enteric nerves. This has been shown for peptide hormones produced by enteroendocrine cells (EECs). Enteric nerves communicate with the central nervous system (CNS) via the vagus nerve (VN). It is also possible that bacteriocins may activate microglia and play a role in gut-brain interactions, similar to what has been described for PapRIV (Yang *et al*., 2007). Autoinducer peptides (AIPs) produced by *Clostridium acetobutylicum* and *Streptococcus pneumonia* cross the BBB (Wynendaele *et al*., 2015). To the best of our knowledge, we do not have evidence of bacteriocins crossing the BBB.

Several recent reports have been published on bacteriocins, non-ribosomal peptides, polyketides, toxins, antibiotics, phenylpropanoids, phenylflavonoids, purine nucleosides, short chain fatty acids (SCFAs) and enzymes with anticancer properties (reviewed by Dicks and Vermeulen, 2022). Most of these molecules target cancer cells in a selective manner, either directly or indirectly through specific pathways. The first report of a bacteriocin displaying anticancer properties was published in the late 1970s, but this was a crude extract (Cornut *et al*., 2008). Since then, several reports of bacteriocins with anticancer properties (also from our own group, unpublished), have been reported (Cotter *et al*., 2013). To the best of our knowledge only three bacteriocins have been patented for their anticancer properties, i.e., plantaricin A produced by *L. plantarum* C11, microcin E492 produced by *Klebsiella pneumoniae* and Pep27anal2, a 27-amino acid peptide produced by *Streptococcus pneumoniae* (reviewed by Dicks and Vermeulen, 2022). Anticancer properties have also been reported for bovicin HC5 produced by *Streptococcus bovus* HC5, colicins A, E1, E3, and U isolated from *E. coli*, pyocins from *P. aeruginosa*, nisin from *Lactococus lactis*, and pediocins from *Pediococcus* (Dicks and Vermeulen, 2022). Despite these reports, the interaction of bacteriocins with cancer cells is ill researched. Kaur and Kaur (2015) hypothesized that cancer cells increase the expression of negatively charged cell-surface molecules when exposed to bacteriocins and, by doing so, promote cytotoxicity and apoptotic cell death. Lessons can be learned from Azurin, an anticancer copper-containing bacteriocin of 14 kDa produced by *P. aeruginosa*. Azurin enters human cancer cells through receptor-mediated endocytosis or, as reported with studies on breast cancer cells lines MCF-7, ZR-75-1 and T47D, via caveolin-mediated pathways (reviewed by Dicks and Vermeulen, 2022). Once inside a cancer cell, azurin attaches to the DNA-binding domain (DBD) of the tumor-suppressor protein p53 and increases the intracellular level of the protein by inhibiting the binding of the E3 ubiquitin ligase COP1 to p53, leading to repression of cell growth and apoptosis. Plantaricin A (2.98 kDa), a peptide pheromone produced by *L. plantarum* C11, binds to negatively charged phospholipids and glycosylated proteins in cell membranes of cancerous and normal cells, leading to the destabilization of cytoplasmic membranes. The peptide also induces apoptosis and necrosis in Jurkat cells, accompanied by an increase in caspase 3 levels. Microcin E492 (7.8 kDa) causes cell shrinkage, DNA fragmentation, release of phosphatidylserine and calcium ions, and apoptosis of cancerous cells such as HeLa, Jurkat. RJ2.25 and colorectal carcinoma cells. Reports of nisin showing potential in the treatment of colorectal cancers and skin cancer, and pediocin PA-1, produced by *Pediococcus acidilactici* K2a2-3 cytotoxic towards human colon adenocarcinoma (HT29) and HeLa cells, provides hope that bacteriocins

may play a key role in prevention of, at least some, cancers. These findings need to be confirmed with *in vivo* studies. If successful, bacteriocin-producing strains with anticarcinogenic properties may be included in probiotic supplements. We are, however, still a long way from understanding the efficacy of bacteriocins in anticancer therapy, and whether these peptides should be used alone or in combination with chemotherapeutic agents. Progress in metagenomics, proteomics, heterologous gene expressions and nanotechnology, combined with the use of artificial intelligence software such as AlphaFold 2 (https://alphafold.ebi.ac.uk/) and Chemistry42 (https://arxiv.org/abs/2101.09050, accessed on 10 May 2022), may lead to the discovery and design of novel anticancer molecules.

Short chain fatty acids (SCFAs) are important neuro-immuno-endocrine regulators and controls peripheral activities such as adipose tissue activation, mitochondrial functions in the liver, energy levels, appetite, and sleep (reviewed by Dicks, 2022). While acetate is readily absorbed by the circulatory tissue for peripheral distribution, propionate is metabolized by hepatocytes and butyrate acts as fuel for colonocytes. SCFAs act as important signaling molecules for enteroendocrine cells by i) inhibiting nuclear histone deacetylase (HD) and ii) stimulating G-protein coupled free fatty acid receptors 2 & 3 (FFAR2 and FFAR3). Secondary bile acids, such as lithocholate (LCA) and deoxycholate (DCA) are important signaling molecules that have profound roles in peripheral metabolism through their action on two bile acid receptors expressed on EE cells, the G- protein coupled receptor TGR5 and the nuclear farnesoid receptor FXR.

From all these studies, it is evident the intestinal barrier is controlled by fine-tuned communications between gut microbes and the host immune system. The complexity of those interactions raise the question about the level of our current understanding and eventually explains why it has been, up to now, difficult to develop specific therapeutic targets. High-throughput sequencing of bacterial genomes and a multiomics approach will provide valuable information regarding the interactions between gut microbiome and host cells and will eventually open up new avenues to treat infectious and chronic diseases. The human gut microbiome is considered the second genome, yet its full potential has not been realized. In the near future, we should be able to use gut microbiota and their metabolites as biomarkers for many diseases, including cancer. I am confident that we will be developing probiotics to treat dysbiosis and neuropsychiatric abnormalities. Perhaps, in the not so near future, we will be considering the gut microbiome as an integral part in the developing of

dedicated/precision/personalised medicine with the assistance of machine-learning algorithms. To do this, we need a clear understanding of the factors influencing the composition and balance of the gut microbiome. We also need to have a better understanding of how gut microbiota deploy QS, understand how to inhibit (quench) QS signals, determine the *in vivo* stability (and safety) of quorum quenching (QQ) molecules and understand how QQ therapy will affect beneficial gut microbiota. These will undoubtfully lead to research on how combinations of neurotransmitters regulate synaptic targets, the discovery of new interneuronal, intraneuronal and glial signals, and a better understanding of the role lipids, steroids, and peptides (including bacteriocins) have on receptors. Within the same research discipline, we need to search for new pharmacological targets in treatment of neuropsychiatric disorders by using genetic tools. Hopefully this will provide some answers to obesity and diabetes often associated with the taking of psychotropic medicines. By learning from the behaviour of gut microbiota under stressful situations, we may be able to develop new psychiatric drugs or novel probiotics.

Gut microbiota, including LAB, show potential in the treatment of neuropsychiatric disorders and improve cognitive functions. Most of these studies have been performed on animals, with only a few clinical trials on humans. The limited number of in-depth studies that have been performed on humans are non-invasive, mostly reporting changes in blood analyses and organ functions. Studies performed on peripheral blood mononuclear cells (PBMCs) isolated from human volunteers to study cytokine production is perhaps closer to an *in vivo* approach but remains an *in vitro* experiment. Liu *et al*. (2016) used this approach to study IL-10 production in culture supernatants and reported higher production of the anti-inflammatory SCFA butyrate. Although valuable data were collected, the study suffer from limitations such as direct application of supernatant to PBMCs without passing through the GIT or liver. Similar other approaches suffer from the same shortcomings, e.g., mucin-coated beads and gastro-intestinal models such as the SHIME system (Duysburgh *et al*., 2021). These are indeed shortcomings in probiotic research. It is extremely difficult to obtain ethical approval to use humans in *in vivo* studies. Even pharmaceutical companies involved in probiotic trials are restricted to only certain experiments. Post-mortem studies on organ donors may be the closest we will get to study the direct, or real-time, effect of molecular changes brought about by probiotic bacteria. More longitudinal studies are needed. We also need to determine if medications taken for psychiatric and neurological disorders alter the composition of the gut microbiome. We also need to determine whether intestinal microorganisms have the ability to

metabolise these drugs. Although genetically engineered strains may not be used in treatment, research in this area must be initiated.

Recent studies suggest that most of the changes detected in immune responses are due to stimulation from the human genome. However, at least 10% of altering immune responses are caused by the gut microbiome (Schirmer *et al*., 2016). Studies such as these are performed under different conditions (e.g., different diets) which makes it difficult to compare findings. Furthermore, the human gut microbiome demonstrates enormous plasticity and is highly dynamic. This poses a serious challenge to the taking of a representative sample. This is also highlighted in the paper published by Gilbert *et al*. (2018).

More research is required to fully understand microbiome stability (resistance to change) and resilience (return to the initial state following perturbation). Information gained from longitudinal studies by serial collection of DNA sequence data from the gut microbiome, complemented by metabolite and gene expression profiling, may provide a better understanding of these changes. Almeida *et al*. (2019) identified 1 952 uncultured bacterial species by reconstructing 92 143 metagenome-assembled genomes (MAGs) from 11 850 human gut microbiomes. This expand our current knowledge on species variations in the human gut microbiome by approximately 280%. The authors hypothesized that these uncultured (candidate) species encode hundreds of unknown biosynthetic gene clusters that not only explains the elusive nature of these strains, but also opens a totally new scientific approach towards the human gut microbiome. Almost half of the putative species could not be classified at genus level, which suggests that a substantial degree of bacterial diversity remains unknown. The goal is to generate high-quality reference genomes from pure cultures to MAGs to serve as blueprint for metagenomic analyses of human gut microbiota. This will help us understand the impact bacteria has on human health and disease.

Markowitza *et al*. (2022) studied close to 1 000 gut microbiome-associated variants (MAVs) of phenotypes reported in electronic health records from tens of thousands of individuals. The authors used an unbiased framework that integrated gene transcriptions, and microbiome and evolutionary approaches to determine a link between MAVs and neurological, metabolic, digestive, and circulatory diseases. They concluded that human genetic influences may offer opportunities for precision diagnostics of microbiome-associated diseases. The most consistent and recurring gene-microbe associations have been reported between lactose digestion (*LCT/MCM6* genomic region) and *Bifidobacterium* spp. (Qin *et al*., 2022; Kolde *et al*., 2018; Lopera-Maya *et al*., 2022), but variations in these genomic regions did not result in drastic changes of species within the genus (Schmidt *et al*., 2020).

Several *in silico* approaches such as computational or machine learning (ML) models have been successfully used to study glycemic responses to variations in diet and may in future be applied in predicting individual responses to drugs, immunotherapy agents and cancer. Most of the statistics used in these models have been sourced from individuals in first-world countries with a specific lifestyle and may not be applicable to populations from other parts of the world. Mechanistic models that rely upon a network of validated, causal interactions do not require fitting into training data sets and are more robust than statistical models when applied across diverse populations. However, these models rely on information available in existing knowledge bases. To be fully productive and applicable, larger datasets need to be created, e.g., on immune-commensal interactions. Currently genome-scale metabolic models provide the best option to develop mechanistic models for host-microbe systems. The reader is referred to the review by Gibbons *et al*. (2022) for further information.

Future research should also involve tissue culture experiments and real-time observations with bioluminescent and other metabolite-specific markers. Fluorescence microscopy is a powerful research tool to visualize the dynamics of tissues, cells, organelles, molecular assemblies, and single molecules inside living cells. In many of these studies spatial and temporal resolutions are difficult to optimize simultaneously, thus hampering the visualization of detailed structures such as dendritic cells deep inside the brain. Multiphoton microscopy, a laser scanning fluorescence microscopy technique that exploits multiphoton excitation processes, is already used to study *in vivo* brain imaging at sub-cellular spatial resolution. The technique differs from that of confocal laser scanning fluorescence microscopy. Localized excitation allows diffusion of the photobleached fluorescent molecules to regions that are not in focus and allows for long-term imaging. An example of expressing the enhanced yellow fluorescent protein (eYFP) in the cortex and hippocampus of a living mouse is illustrated by Ishii *et al.* (2022). Similar real-time observations showing the effect of microbial cells and their metabolites in other organs should be possible.

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