BIOACTIVE COMPOUNDS FROM MICROBES

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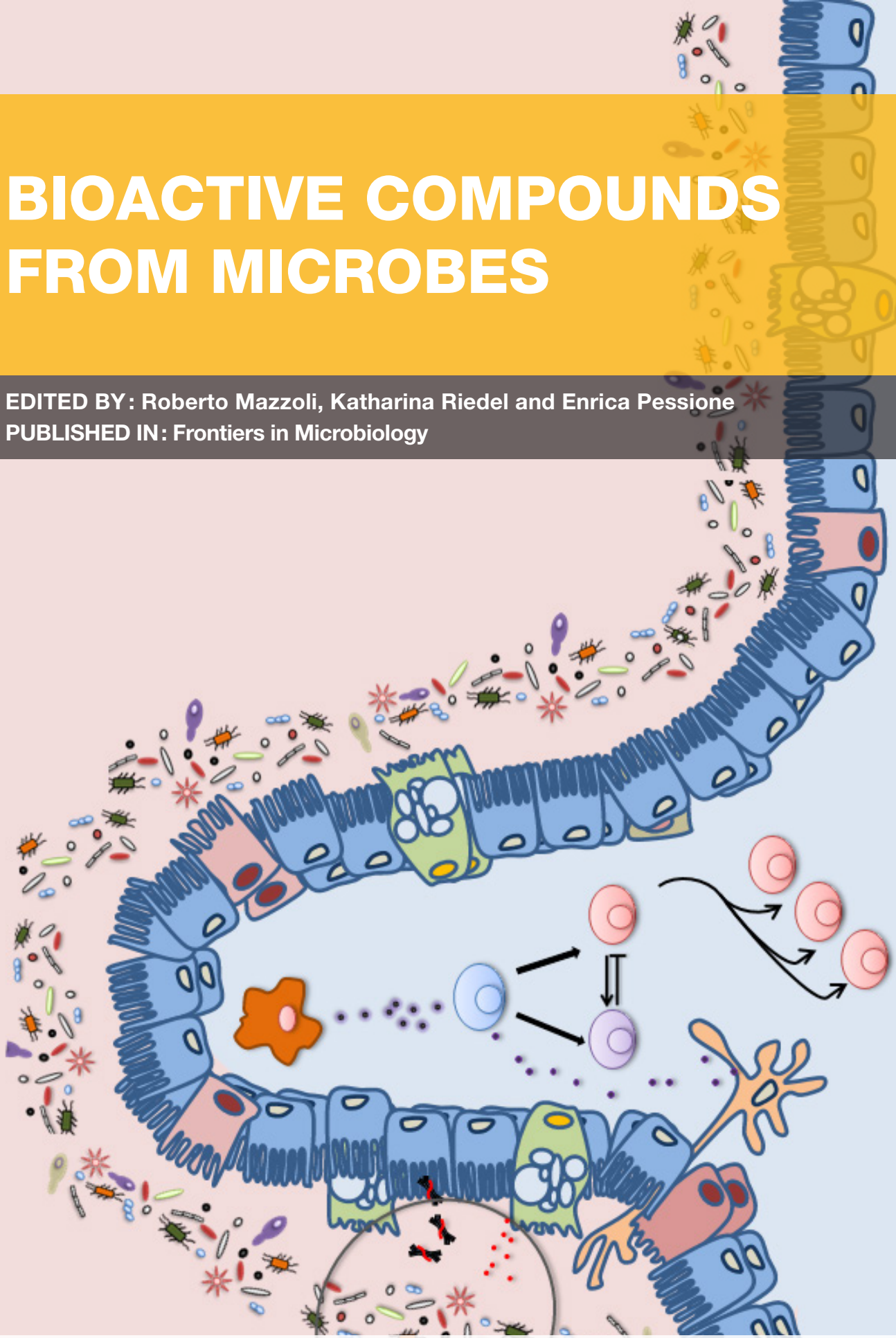

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BIOACTIVE COMPOUNDS FROM MICROBES

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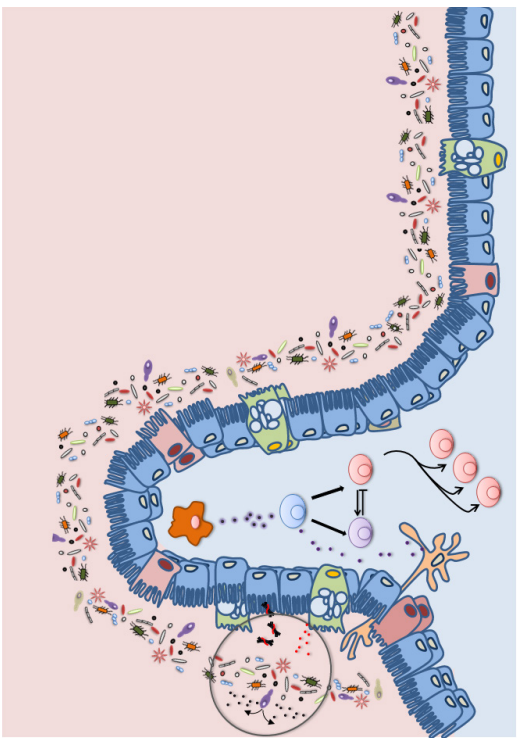

Schematic representation of the interactions established between the intestinal microbiota and the host immune system. General overview of the epithelium (enterocytes; M cells; Goblet cells; antigen presentation cells; dendritic cells and macrophages) in contact with multiple species of microorganisms that constitute the intestinal microbiota; defensins, bacteriocins, and secreted IgA also play an important role in controlling the levels of the different populations of microorganisms.

Image modified from: Hevia A, Delgado S, Sánchez B and Margolles A (2015) Molecular Players Involved in the Interaction Between Beneficial Bacteria and the Immune System. Front. Microbiol. 6:1285. doi: 10.3389/fmicb.2015.01285

Microorganisms have had a long and surprising history. They were "invisible" until invention of microscope in the 17th century. Until that date, although they were extensively (but inconsciously) employed in food preservation, beer and wine fermentation, cheese, vinegar, yogurt and bread making, as well as being the causative agents of infectious diseases, they were considered as "not-existing". The work of Pasteur in the middle of the 19th century revealed several biological activities performed by microorganisms including fermentations and pathogenicity. Due to the urgent issue to treat infectious diseases (the main cause of death at those times) the "positive potential" of the microbial world has been neglected for about one century. Once the fight against the "evil" strains was fulfilled also thanks to the antibiotics, industry began to appreciate bacteria's beneficial characteristics and exploit selected strains as starters for both food fermentations and aroma, enzyme and texturing agent production. However, it was only at the end of the 20th century that the probiotic potential of some bacteria such as lactic acid bacteria and bifidobacteria was fully recognized. Very recently, apart from the probiotic activity of *in toto* bacteria, attention has begun to be directed to the chemical mediators of the probiotic effect. Thanks also to the improvement of techniques such as transcriptomics, proteomics and metabolomics, several bioactive compounds are continuously being discovered. Bioactive molecules produced by bacteria, yeasts and virus-infected cells proved to be important for improving or impairing human health. The most important result of last years' research concerns the discovery that a very complex network of signals allows communication between organisms (from intra-species interactions to inter-kingdom signaling). Based on these findings a completely new approach has arisen: the system biology standpoind. Actually, the different organisms colonizing a certain environmental niche are not merely interacting with each other as individuals but should be considered as a whole complex ecosystem continuously exchanging information at the molecular level. In this context, this topic issue explores both antagonistic compounds (i.e. antibiotics) and "multiple function" cooperative molecules improving the physiological status of both stimulators and targets of this network. From the applicative viewpoint, these molecules could be hopefully exploited to develop new pharmaceuticals and/or nutraceuticals for improving human health.

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Chapter 4: Antibiotic compounds

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EDITORIAL
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Editorial: Bioactive Compounds from Microbes

Roberto Mazzoli[1], *Katharina Riedel*[2] *and Enrica Pessione*[1]*

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Keywords: gut-brain axis, antitumor activity, viral immune-escape, Bacteriocins, human-microbes cross talk

Editorial on the Research Topic

Bioactive Compounds from Microbes

Microorganisms are ubiquitous and essentially interact with all the other organisms present in the biosphere, sometimes creating a network of signals that constitutes the basis for life on the Earth. Clarifying the nature of these molecular signals, their targets and the pathways underlying their production, constitutes the essential pre-requisite for deciphering inter-kingdom communications, adaptive responses and systems biology (Nicholson et al., 2012; O'Mahony et al., 2015; Cani and Knauf, 2016). The insights acquired in the last two decades about human microbiota, and its fundamental role in maintaining a healthy physiological status, have opened the way to understanding the complex reciprocal talk between bacteria and humans (Hughes and Sperandio, 2008; Lyte and Freestone, 2010; Mayer, 2011; Cryan and Dinan, 2012; Mazzoli, 2014; Halang et al., 2015; Kelly et al., 2015). In parallel with these aspects, new bioactive molecules from the microbial world that interact with different cellular models continue to be discovered.

The times and the technological advances start now to be suitable for intercellular/interorganism communication to be studied at the molecular level. The aim of the present topic issue is to try to describing the mediator molecules of a network of signals which is still largely underexplored and underexploited.

As an example, some soil bacteria (such as *Serratia* spp.) can have antagonistic actions toward worms and the molecule involved, *zeamine*, is effective against yeasts and other biological systems, as well. *Prodigiosin*, the well-known pigment produced by the marine bacterium *Vibrio ruber*, has a broad antimicrobial spectrum and induces autolytic activity in the target cells (i.e., *Bacillus subtilis*). *Lantibiotics* are class I bacteriocins produced by Gram-positive bacteria that can be bioengineered to both enhance their effectiveness against a larger number of bacterial strains and to improve their stability during the gastric transit that is by rendering them protease-resistant. These overall data open new possibilities for antibiotic therapy in a period in which the phenomenon of antibiotic resistance is considered as a major threat to public health (World Health Organization, 2014) since it is widespread in pathogenic, commensal, and food bacteria (Laxminarayan et al., 2013). Furthermore, the appearance of multiresistant bacterial strains (the so-called superbugs), often causing death, clearly constitutes a serious problem to be solved. Exploiting the microbial world and its huge potential in finding new antimicrobial drugs is an urgent concern and some chapters of this topic issue deal with these aspects.

Other interesting molecules are produced by cytomegalovirus-infected cells: these compounds of viral origin (essentially *proteins*) can promote virus dissemination, persistence, and pathogenesis by counteracting host innate and adaptive immune responses. Conversely, some beneficial microbes, like Lactic Acid Bacteria (LAB) and Bifidobacteria can modulate the immune system controlling inflammation by means of proteins and non-proteinaceous compounds (Pessione, 2012).

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Several microbiota-derived compounds can contribute to control host physiological and pathological states: Metabolomic profiling of gut bacteria can allow to decipher several molecules (among which *short chain fatty acids*, for instance those produced by bacteria belonging to the clostridial cluster IV and XIV, vitamins and aromatic compounds) controlling cholesterol synthesis, obesity, cardiovascular diseases, metabolic syndrome. Curiously, some bioactive *peptides* or amino acids can be delivered by food in which LAB are present as fermentation starters. These bacteria can decrypt peptides from food proteins, which have anti-oxidant or metal chelating function, immunomodulatory properties as well as peptides controlling hyperglycemia, hypercholesterolemia, hypertension, cell cycle, apoptosis, and even having a refolding action on damaged proteins. An interesting tannin-degrading activity of bacteria (e.g., *Streptococcus* spp. and *Fusobacterium* spp.) can generate *gallic acid and pyrogallol*, both having an anti-carcinogenic role. Finally, microbial-derived *amines* can modulate a series of patho/physiological functions such as allergy, smooth muscle relaxation, anxiety, appetite, depression (Pessione et al., 2005). LAB are good producers of gamma-amino butyric acid (GABA) (Laroute et al., 2016) that contributes to gut-to-brain signaling through different pathways involving enteric neurons, entero-endocrine cells and immune cells. Yeasts, mainly *Saccharomyces cerevisiae*, can convert tryptophan into melatonin and serotonin that are informational molecules related to circadian rhythm but also promising agents for the prevention of neurodegenerative diseases.

This intense network of molecules, allowing communication among bacteria, viruses, and eukaryotic cells has evolved to guarantee optimal life in different ecological niches to each component of the ecosystem and is based upon effector-receptor model. In-depth knowledge of all these biochemical signals, as well as the underlying finalism are still far to be fully elucidated. Nevertheless, some mechanistic aspects highlighted in the present topic issue can open new perspectives in medicine but can also shed light on evolution strategies.

AUTHOR CONTRIBUTIONS

EP wrote the manuscript which was then reviewed by RM and KR.

REFERENCES

Cani, P. D., and Knauf, C. (2016). How gut microbes talk to organs: the role of endocrine and nervous routes. *Mol. Metab.* 5, 743–752. doi: 10.1016/j.molmet.2016.05.011

Cryan, J. F., and Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* 13, 701–712. doi: 10.1038/nrn3346

Halang, P., Toulouse, C., Geißel, B., Michel, B., Flauger, B., Müller, M., et al. (2015). Response of *Vibrio cholerae* to the catecholamine hormones Epinephrine and Norepinephrine. *J. Bacteriol.* 197, 3769–3778. doi: 10.1128/JB.00345-15

Hughes, D. T., and Sperandio, V. (2008). Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* 6, 111–120. doi: 10.1128/JB.00345-15

Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G., and Hyland, N. P. (2015). Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front. Cell. Neurosci.* 9:392. doi: 10.3389/fncel.2015.00392

Laroute, V., Yasaro, C., Narin, W., Mazzoli, R., Pessione, E., Cocaign-Bousquet, M., et al. (2016). GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate. *Front. Microbiol.* 7:1050. doi: 10.3389/fmicb.2016.01050

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., et al. (2013). Antibiotic resistance-the need for global solutions. *Lancet Infect. Dis.* 13, 1057–1098. doi: 10.1016/S1473-3099(13)70318-9

Lyte, M., and Freestone, P. P. E. (2010). *Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health*. New York, NY: Springer.

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut-brain communication. *Nat. Rev. Neurosci.* 12, 453–466. doi: 10.1038/nrn3071

Mazzoli, R. (2014). "Neuro-active compounds produced by probiotics. Towards a microbiota-(gut-) brain axis control?" in *Interactive Probiotics,* ed E. Pessione (Boca Raton, FL: CRC Press; Taylor & Francis Group), 148–176.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., and Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav. *Brain Res.* 277, 32–48. doi: 10.1016/j.bbr.2014.07.027

Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front. Cell. Infect. Microbiol.* 2:86. doi: 10.3389/fcimb.2012.00086

Pessione, E., Mazzoli, R., Giuffrida, M. G., Lamberti, C., Garcia-Moruno, E., Barello, C., et al. (2005). A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics* 5, 687–698. doi: 10.1002/pmic.200401116

World Health Organization (2014). *Antimicrobial Resistance: 2014 Global Report on Surveillance.* Geneva: World Health Organization.

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REVIEW
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Bioactive Molecules Released in Food by Lactic Acid Bacteria: Encrypted Peptides and Biogenic Amines

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Lactic acid bacteria (LAB) can produce a huge amount of bioactive compounds. Since their elective habitat is food, especially dairy but also vegetal food, it is frequent to find bioactive molecules in fermented products. Sometimes these compounds can have adverse effects on human health such as biogenic amines (tyramine and histamine), causing allergies, hypertensive crises, and headache. However, some LAB products also display benefits for the consumers. In the present review article, the main nitrogen compounds produced by LAB are considered. Besides biogenic amines derived from the amino acids tyrosine, histidine, phenylalanine, lysine, ornithine, and glutamate by decarboxylation, interesting peptides can be decrypted by the proteolytic activity of LAB. LAB proteolytic system is very efficient in releasing encrypted molecules from several proteins present in different food matrices. Alpha and beta-caseins, albumin and globulin from milk and dairy products, rubisco from spinach, beta-conglycinin from soy and gluten from cereals constitute a good source of important bioactive compounds. These encrypted peptides are able to control nutrition (mineral absorption and oxidative stress protection), metabolism (blood glucose and cholesterol lowering) cardiovascular function (antithrombotic and hypotensive action), infection (microbial inhibition and immunomodulation) and gut-brain axis (opioids and anti-opioids controlling mood and food intake). Very recent results underline the role of food-encrypted peptides in protein folding (chaperone-like molecules) as well as in cell cycle and apoptosis control, suggesting new and positive aspects of fermented food, still unexplored. In this context, the detailed (transcriptomic, proteomic, and metabolomic) characterization of LAB of food interest (as starters, biocontrol agents, nutraceuticals, and probiotics) can supply a solid evidence-based science to support beneficial effects and it is a promising approach as well to obtain functional food. The detailed knowledge of the modulation of human physiology, exploiting the health-promoting properties of fermented food, is an open field of investigation that will constitute the next challenge.

Keywords: opioid, antioxidant, chaperon-like, cell-cycle control, antimicrobial peptides, beta-phenylethylamine, tyramine

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INTRODUCTION

Human health is the result of a correct physiological status often resulting from the reciprocal interaction of gene-derived signals (genetics) and environment–generated information (epigenetics). Recognizing gene signals is relatively easy whereas environmental stimuli are often multiple, complex, and reciprocally interacting. Temperature, pH, redox balance, sleep, diet, drugs, and psychological status can deeply affect gene expression, metabolic pathways, and homeostasis (Carey, 2012). However, human genes and environment are not the only players: microorganisms can take part to the complex molecular cross-talk existing between external world and "self."

Firstly, endogenous symbiont microorganisms, the so-called microbiota, can modulate gene expression, induce preferential food intake (Aydin, 2007), influence pH, redox balance and the ratio between pro-inflammatory and anti-inflammatory cytokines (Belkaid and Hand, 2014). Briefly, control brain, metabolism, immune system, and several homeostatic routes.

Secondarily, food-derived bacteria and yeasts can exogenously affect nutritional parameters, metabolism, oxidative status, immunity, blood pressure, appetite, behavior, also controlling the endogenous microbiota (Arora et al., 2013) by altering the ratio among saccharolytic and proteolytic species, by modulating symbionts gene expression and several other functions (O'Flaerty, 2014). Hence, all fermented food, containing living organisms, can contribute to modulation of the host physiological balance and it constitutes an opportunity to enrich the diet with new bioactive molecules finally resulting in phenotypic effects (appetite, cholesterol and blood pressure lowering, improvement of mood, antioxidant, and immune defenses) on humans (Pessione, 2010). Actually, all types of modulations occur *via* a complex network of signals, among which proteinaceous compounds play a crucial role.

Microorganisms are able to synthesize a large number of metabolites with assessed beneficial or detrimental properties for human health. Among these, nitrogen-bearing molecules such as amino acids, amino acid derivatives and oligopeptides have received great attention since they can affect human physiology in multiple ways.

As an example, amino acid derivatives such as selenocysteines and selenomethionines, have recently been reported to be biosynthesized in both Lactobacilli (Lamberti et al., 2011) and yeasts (Porto et al., 2015). Although selenoaminoacids are not true "bioactive compounds," directly stimulating receptors on human cells, they can trigger effects deeply affecting human health. The bioactive role of seleno-fixing microorganisms lies in the fact that diet-derived inorganic selenium is toxic (selenate and selenite) or poorly active (elemental selenium) whereas fixed selenium forms (selenomethionines and selenocysteines) are the only bioavailable for humans. On the other hand, only bacteria and yeasts can produce seleno-amino acids from inorganic selenium. Once properly inserted into selenoproteins (i.e., glutathione peroxidase), they can counteract oxidative stress. Besides this well-known antioxidant function, there are data indicating that selenoproteins can modulate immune system (Huang et al., 2012) and activating anabolic circuits such as thyroid hormone biosynthesis (Mullur et al., 2014). Furthermore, epidemiological studies show an inverse correlation between selenium level in blood and cancer mortality, and laboratory experiments have shown a selenium protective effect against cancer initiation and development (Gromadzińska et al., 2008). In *Lactobacillus reuteri* exoproteome studies have demonstrated that two secreted proteins (GAPDH and Phosphoketolase) contain selenocysteines opening the way to employ this strain to supply organic bioavailable forms of selenium (Galano et al., 2013; Mangiapane et al., 2014a,b).

Among amino acid derivatives found in food, biogenic amines are worth of a special mention. Such compounds, although sometimes naturally present (especially in vegetal food) are often the result of the bacterial decarboxylative activity on free amino acids in food. Biogenic amines can be present in non-fermented food, like fish, due to spoilage bacteria that during protein putrefaction release free amino acids undergoing decarboxylation. *E. coli* can produce cadaverine from lysine and putrescine from ornithine (Applebaum et al., 1975). Proteus can produce putrescine from ornithine as a communication signal (Visick and Fuqua, 2005). However, also not-spoiled food, such as fermented food, can present the risk of biogenic amine accumulation. Although starters, exogenously added to perform controlled fermentations, are accurately typed to avoid any risk, autochthonous or contaminant lactic acid bacteria (LAB) can contribute to amine release. LAB are strong amine producers since they use this metabolic pathway (at the place of respiration) to both create a proton gradient and hence energy (for exhaustive review, see Pessione et al., 2010) and to alkalinize the environment, very acidic since their main fermentation products are acids (lactic acid for homofermenter LAB and lactic + formic + acetic acid in heterofermenters).

Many experimental evidences demonstrate that some LAB strains also produce anti-hypertensive, anti-thrombotic, cholesterol-lowering, metal-chelating, antimicrobial, anti-oxidant, immune-modulating, chaperone-like and opioid/opioid antagonist peptides from food proteins (Pessione, 2012), and they can modulate the concentration of opioid and cannabinoid receptors in the gut epithelium (Hayes et al., 2007b). The next sections will focus on some of the referred compounds and will illustrate the main effects exerted on human health.

ENCRYPTED BIOACTIVE PEPTIDES

Several bioactive peptides, lacking activity when protein-encrypted but acquiring their biological effects when proteolytically released, have been shown to have health-promoting properties as anti-microbials, hypocholesterolemics, opioid and opioid antagonists, angiotensin-converting enzyme inhibitors, anti-thrombotics, immunomodulators, cytomodulators, and anti-oxidants (Hayes et al., 2007b).

The human pool of digestive proteases and peptidases can liberate food-encrypted bioactive peptides that can be absorbed by the gut and then reach peripheral organs. However, the enzymatic activity of LAB largely contribute to their release, either into the food matrix (starter or autochthonous LAB) or in

the gut (endogenous microbiota or probiotics). LAB are ancient organisms adapted to an anoxic environment that never evolved the capability to biosynthesize heme and hence to have functional cytochromes, catalases, and peroxidases. They are very sensitive to oxygen and devote most of their genes to oxygen stress counteracting. Due to the limited length of the overall genome, the biosynthetic abilities of LAB are very limited especially in amino acid synthesis (Pessione, 2012). Therefore, LAB evolved a complex and sophisticated proteolytic system allowing them to get amino acids from the proteins present in the external environment. A schematic representation of this system, which includes proteases, peptidases and membrane transporters, is referred in **Figure 1**.

LAB Proteolytic System

Extracellular protein hydrolysis into various long oligopeptides is initiated by a cell-envelope proteinase (CEP). Oligopeptides generated by this first cleavage are subsequently taken up by cells *via* specific transport systems and they undergo further degradation into shorter peptides (bioactive or possible precursors of bioactive compounds) and amino acids in the cytoplasm (Savijoki et al., 2006). In LAB the oligopeptide transporter system (Opp) is the main transporter, belonging to a superfamily of highly conserved ATP-binding cassette transporters (Doeven et al., 2005). The Opp system of *L. lactis* transports peptides up to at least 18 residues (Savijoki et al., 2006). It should be underlined that most bioactive peptides are released into the external environment only when cells undergo autolysis, because only occasionally the longer peptides (originated by the first hydrolytic step) possess biological activity (Meisel and Bockelmann, 1999). Nevertheless, some authors reported that CEP from *Lactobacillus delbrueckii* subsp. *lactis* are suitable to liberate both metal-chelating and anti-hypertensive peptides directly from caseins and without necessity of prior cell autolysis. Maximum CEP activity was a little bit decreased by the addition of NaCl (1%) and glycerol (5%; Hébert et al., 2008). However, proteolytic enzymes released by LAB proved to be very different in the different LAB species and strains, giving rise to a different pool of bioactive peptides (Hébert et al., 1999).

Food Matrices Containing Encrypted Peptides

Lactic acid bacteria proteolytic system is suitable to produce bioactive peptides from several food proteins, especially caseins that constitute the main nitrogen substrate present in their habitat (milk and milk derivatives; Clare and Swaisgood, 2000). Casein consists of four main proteins (whose ratio is about 38:11:38:13): alpha s1 casein, alpha s2 casein, beta-casein and k-casein, differing in amino acid sequence, hydropathicity index, glycosylation and phosphorylation degree (Pessione, 2012). Usually, CEPs have a strong preference for hydrophobic caseins and *Lactococcus* CEPs have been classified into several types and subtypes depending on their substrate specificity for αS1-, β-, and κ-caseins (Kunji et al., 1996). Casein hydrolysis can give rise mainly to opioid/anti-opioid peptides, antithrombotic and antihypertensive, immunomodulatory, mineral-binding and antimicrobial peptides (Thakur et al., 2012) easily detectable by means of chromatographic techniques after microbial food digestion (Saraswat et al., 2012).

Hydrolytic cleavage of milk whey proteins (alpha-lacto-albumine, beta-lacto-globulin, lactoferrin, and immunoglobulins) can also generate bioactive molecules, such as hypocholesterolemic peptides, which can decrease absorption and enhance fecal extrusion of cholesterol. Actually, milk whey proteins display a greater cholesterol-lowering activity than casein, in particular beta-lactoglobulin (Nagaoka et al., 2001). Beta-lactoglobulin-released peptides also exhibit antioxidant (Hernández-Ledesma et al., 2005) and immune-modulating (Prioult et al., 2004) activity.

Bio-active molecules, especially ACE-inhibiting peptides, can also be found encrypted in bovine meat proteins, such as hemoglobin (i.e., hemorphins) and serum albumin (i.e., serorphin), collagen, elastine, fibrinogen (Minkiewicz et al., 2011; Lafarga et al., 2015).

However, also food of vegetal origin can be a source of bioactive peptides. In soy β-conglycinin β-subunit (Kaneko et al., 2010) and in the large subunit of spinach rubisco, both antihypertensive (Yang Y. et al., 2003) and opioid peptides (i.e., soymorphins-5,-6, and -7, and rubiscolin-5 and -6, respectively), displaying anxiolytic effects, food intake controlling action and enhancement of memory, have been described (Yang et al., 2001; Yang S. et al., 2003; Hirata et al., 2007).

A special mention needs gluten: opioid peptides (i.e., gluten exorphins A4, A5, B4, B5, and C), have been detected and characterized in wheat gluten since 1992 (Fukudome and Yoshikawa, 1992). Some of these peptides have analgesic action on the CNS (Takahashi et al., 2000) but some can also act on opioid receptors located outside the blood–brain barrier, inducing prolactin secretion (Fanciulli et al., 2005). Both antihypertensive (Rizzello et al., 2008) and antioxidant (Coda et al., 2012) peptides are the result of LAB activity on wheat and other cereals. Generally, the high proline content of gluten alpha gliadins prevent hydrolysis by enzymes of the gastrointestinal tract, whereas LAB possess proline-specific peptidase systems. Alternatively, peptide degradation is achieved by the combined action of peptidase from different LAB strains simultaneously present in fermented food (Gerez et al., 2008).

To date, LAB strains able to release bioactive peptides from food proteins, with particular reference to milk caseins, include *L. helveticus* CP790, *L. rhamnosus* GG, *L. bulgaricus* SS1, and *L. lactis* subsp. *cremoris* FT4 (Gobbetti et al., 2002). The hydrolytic ability is related to both the protein substrate, i.e., its amino acid sequence and the proteolytic enzyme panel of each microbial strain (Griffiths and Tellez, 2013). It is possible to enhance (100-fold) the proteolytic potential of LAB by growing them in milk (Wakai and Yamamoto, 2012). Actually it has been demonstrated that peptide-rich media like MRS supplying a ready-to-eat nitrogen source are unfavorable to the induction of most proteolytic enzymes for instance those encoded by Pep N and PepX (Smeianov et al., 2007). On the other hand, CEP are able to hydrolyze caseins only after growth on skim milk but not after MRS pre-culture (Jensen et al., 2009). Generally, lactobacilli genome encodes a larger number of proteases, peptidases, amino

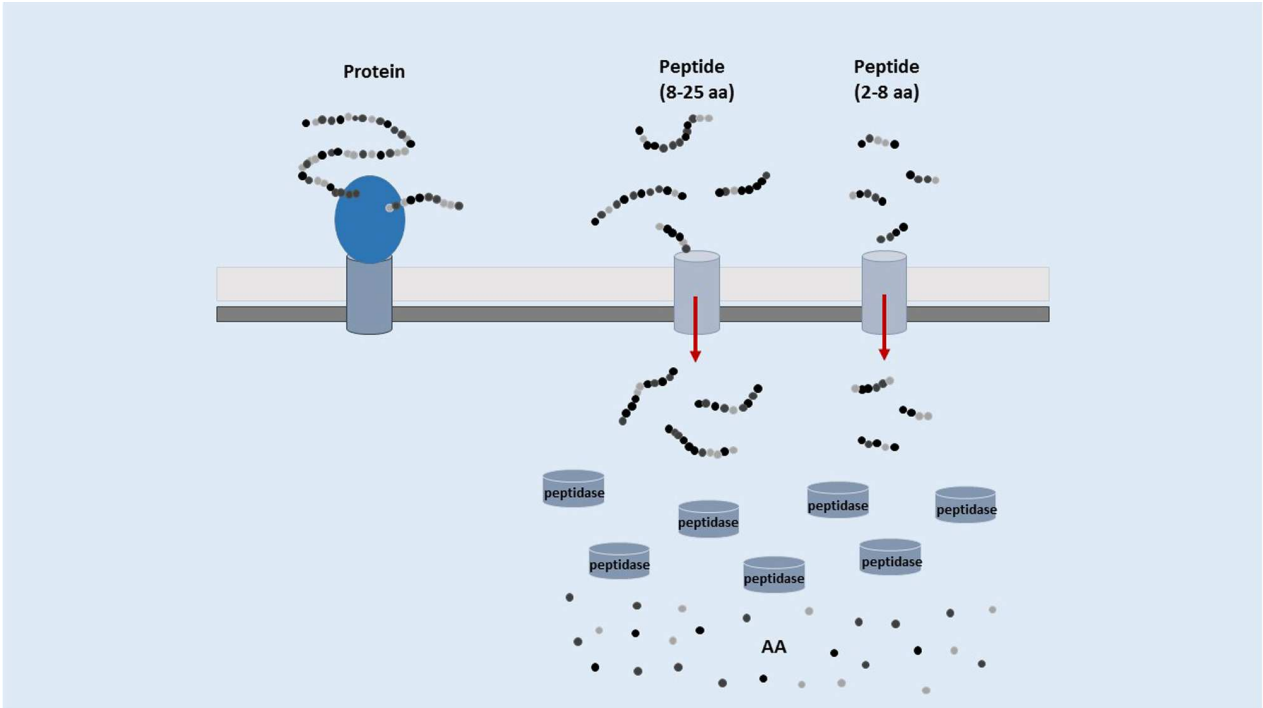

FIGURE 1 | Schematic representation of the proteolytic system of LAB.

acid permeases, and Opp transport systems than lactococci (Klaenhammer et al., 2005). However, more studies on a larger number of species and strains are necessary to establish the true potential of each strain.

Finally, it is worth considering that most information on LAB proteolytic system has been referred to food matrix isolated microflora and to its action on bovine milk casein (Korhonen and Pihlanto, 2003). However, endogenous or probiotic or food – derived LAB can also produce bioactive peptides from the different protein pool present in the host gut, opening interesting perspectives for improving human health (Sharon et al., 2014). In the following paragraphs, the best known bioactive molecules generated from LAB proteases and peptidases are described.

Antimicrobial Peptides

Lactic acid bacteria proteolytic activity on caseins gives rise to small peptides displaying antimicrobial (both bactericidal and bacteriostatic) activity. Alpha s1 casein and K-casein hydrolysis gives rise to isracidin and k-casecidin, respectively (McCann et al., 2006). Both peptides display inhibitory action on *S. aureus* growth (Meisel and Bockelmann, 1999; Atanasova et al., 2014). K-casein can also originate kappacin, a long bioactive peptide active against *Streptococcus mutans*. This peptide, although deriving from K-casein, is phosphorylated but not glycosylated, and displays the ability to prevent bacterial adhesion to gingival mucosa and to bind enterotoxins (Malkoski et al., 2001). The alpha-s-2 casein fragment 165-203, called casocidin-I can inhibit both *S. carnosus* and *E. coli* (Zucht et al., 1995). *Lactobacillus helveticus* can hydrolyze beta casein, by means of a PR4 proteinase, decrypting an antimicrobial peptide active toward several strains of Gram-positive bacteria (including *S. aureus* and *Listeria innocua*) but also against Gram-negative pathogens such as *E. coli, Salmonella, Yersinia,* (Minervini et al., 2003). Homology studies revealed that this peptide has a similar length to isracidin, but a stronger hydrophobic nature (lacking positive charges) allowing a better activity on Gram-negative bacteria. Furthermore, it bears some proline residues near the C-terminal, which can prolong its half-life rendering this molecule more resistant to the peptidolytic action (Gobbetti et al., 2004). The critical question concerning this peptide is that it has been decrypted from human milk but not from other caseins present in food (cow, buffalo, goat, ship).

Different peptides produced from caseins by different *Lactobacillus* species (*L. acidophilus, L. helveticus, L. plantarum, and L. rhamnosus*) and by *Lactococcus lactis* are active against Gram-negative rods such as *Enterobacter sakazakii* (Hayes et al., 2006). An interesting antimicrobial peptide, named Lactoferricin B, having broad spectrum of action including Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi, has been described as the result of the hydrolytic action on bovine lactoferrin (Dionysius and Milne, 1997; Kitts and Weiler, 2003). This cationic peptide alters membrane permeability with consequent dissipation of the proton gradient and has therefore bactericidal action (FitzGerald and Murray, 2006). It was cloned and expressed in *E. coli*, showing activity against *Klebsiella pneumoniae, Streptococcus Mutans*, and *S. aureus* (Luo et al., 2007). Lactoferricin B is especially active toward the enterohemorrhagic strain of *E. coli* 0157h:7 (Muro-Urista

et al., 2011) and proved to be active also against wine-spoilage LAB such as *Oenococcus oeni, Pediococcus damnosus,* and *Lactobacillus brevis* (Enrique et al., 2009). It is difficult to establish the real LAB potential in hydrolyzing lactoferrin. Some experiments were performed by using both microbial-derived enzymes and fermentation with proteolytic starter cultures (Sharma et al., 2011), however, the true potential of each strain has to be experimentally proved since a great variability was observed among strains (Korhonen, 2009). More recently, the proteolytic activity of different LAB strains on goat milk beta casein and beta-lactoglobulin were tested. *Lactococcus lactis l598, Lactobacillus lactis 1043, Streptococcus thermophilus t3D1, Dt1*and *Lb. delbrueckii* subsp. *bulgaricus b38, b122* and *b24* revealed a significant potential in producing antimicrobial peptides inhibiting *S. aureus, L. monocytogenes, Listeria innocua, Enterobacter aerogenes,* and *Salmonella enteritidis* (Atanasova et al., 2014).

Concerning the mechanism of action of such antibacterial compounds, several models have been proposed. Being amphipathic molecules, bearing a hydrophobic moiety and a strongly positively charged domain, this peptides can firstly bind to teichoic acids in Gram-positive-, or to LPS in Gram-negative bacteria (Vorland, 1999) and then interact with the negatively charged bacterial membranes. Most of them act as proton gradient perturbing molecules, (causing membrane depolarization like polymyxin B and colistin; Lohner and Blondelle, 2005), however, some authors suggest that also removal/chelating of membrane-bound iron can affect bacterial viability (Sharma et al., 2011). For what concern lactoferrin-derived peptides, however, it has long been established (Bellamy et al., 1992) that the domains of lactoferrin involved in the antibacterial activity are different from those involved in iron binding. Complex mechanism, including inhibition of the synthesis of macromolecules (Ulvatne et al., 2004) as well as synergic action with host innate immunity compounds were also reported (Brogden, 2005).

These antimicrobial compounds are appreciated in the food industry (especially dairy industry) as natural preservatives counteracting undesired contamination. This allows to reduce the amount of sugar and salt with excellent benefits for diabetics, obese, and hypertensive subjects. Furthermore, their stability in blood and serum render them promising infection control agents. Some of them are also good candidates to be tested for their anti-viral potential. Bojsen et al. (2007) reported the antiviral effect of bovine whey proteins, whereas Civra et al. (2015) very recently identified from donkey milk lactadherin a peptide displaying anti-Rotavirus activity. The actual role of LAB proteolysis in the decrypting of the antiviral potential is far to be fully elucidated. An overview of antibacterial peptides is proposed in **Table 1**.

Metal-Binding Peptides

Some LAB strains digest casein, releasing casein phosphopeptides (CPP) phosphorylated on Ser residues. CPP have a strong anionic character and hence are very resistant to further proteolytic degradation (Sharma et al., 2011). They have been identified during cheese ripening due to microbial protease activity (Singh et al., 1997). The typical cluster is Ser(P)-Ser(P)Ser(P)-Glu-Glu, but they may contain also phosphorylated cysteines. Even if this motif is crucial for metal-binding, various phosphopeptide fractions revealed significant differences in this capability, which may be due to variant amino acid composition around the phosphorylated region. Hence, other factors can affect CPP-metal interaction, namely the total number of amino acids and the total negative charge (Cross et al., 2005).

It has long been established (Gagnaire et al., 1996) that beta and k-caseins (being poor in hydroxylated amino acids and therefore less suitable for phosphorylation) generate peptides less active in metal binding, whereas the Ser/Thr rich alpha s1 and alpha s2 caseins are the most suitable for chelating cations by means of CPP. However, alpha s1 casein-derived peptide (59–79), although bearing five phosphate groups, is less active in allowing mineral uptake by the human tumor cells HT29 than beta casein-derived peptides (1–25). Actually, besides the typical cluster sequence, specific secondary structure motifs in the bioactive peptide (such as beta-turn and loop), as well as the degree of aggregation of CPP in presence of divalent cations, are required for a correct mineral absorption (Ferraretto et al., 2003).

Casein phosphopeptides can chelate up to 250 mgCa/g with a dissociation constant in the mM range, with an affinity even higher for trace elements like zinc, iron, and copper (FitzGerald, 1998). Their mechanism of action rely on the fact that they can form soluble complexes with calcium even at alkaline pH (Berrocal et al., 1989). This results in increased absorption of calcium in the intestine, useful in the treatment of osteoporosis. Re-calcification of dental enamel and prevention of dental caries were also reported (Reynolds et al., 2008). Enhanced gut bioavailability and potential higher absorption of enzyme co-factors such as iron, zinc, copper, selenium, magnesium, and manganese, due to CPP was also described (Meisel and Olieman, 1998). On the other hand, controversial clinical results concerning both calcium (Korhonen and Pihlanto, 2006) zinc (Miquel and Farré, 2007) and calcium and iron absorption obtained on patients receiving CPP supplementation and controls, underline the difficulty to standardize the different supplements (Teucher et al., 2006). Finally, calcium availability is strongly dependent on meal composition (i.e., phytates) and complex interactions between different foods ingested.

In spite of all these considerations, all described peptides may constitute interesting tools for microelements nutritional improvement. In our aging western society bone disease is the third cause of nursery care and osteoporosis is not fully controlled by a healthy lifestyle (active movement and diet; Wei et al., 2003). Therefore, calcium-enriching supplements such as CPP open new nutraceutical perspectives. However, the need to use purified and well-characterized CPP (for having a reproducible nutritional impact) highly affects the costs. Hence, the use of LAB as probiotics or food supplements to obtain functional food, especially milk based drinks and yogurts will be a promising strategy. *Lactobacillus helveticus* LA, is a LAB adapted to the cheese environment displaying good proteolytic activity. It can decrypt from alpha casein a peptide showing calcium-binding activity (Dimitrov, 2009). *Lactobacillus helveticus* LBK16H can decrypt the tripeptides Ile-Pro-Pro and

TABLE 1 | Main antibacterial peptides from LAB.

Bacteria	Peptide	Protein source	Target	Author
LAB	Isracidin	α s1-Casein	*S. aureus*	Meisel and Bockelmann, 1999
LAB	k-Casedicin	k-Casein	*S. aureus*	McCann et al., 2006
LAB	Kappacin	k-Casein	*S. mutans*	Malkoski et al., 2001
LAB	Casocidin I	α s2-Casein	*E. coli* *S. carnosus*	Zucht et al., 1995
L. helveticus	Isracidin-like	β-Casein	*S. aureus* *L. innocua* *Yersinia* *Salmonella*	Minervini et al., 2003
L acidophilus *L. helveticus* *L. plantarum* *L. rhamnosus*	ND	In toto casein	*Entobacteria sakazakii*	Hayes et al., 2006
ND	Lactoferricin	Cow lactoferrin	*K. pneumoniae* *S. aureus* *S. mutans* *E. coli ol57*	Luo et al., 2007; Muro-Urista et al., 2011
L lactis *L bulgaricus* *L delbrueckii* *S. thermophilus*	ND	Goat casein	*S. aureus* *L. monocytogenes* *L. innocua* *Salmonella* *Enterobacteria*	Atanasova et al., 2014
L lactis *L bulgaricus* *L delbrueckii* *S. thermophilus*	ND	β-lactoglobulin	*S. aureus* *L. monocytogenes* *L. innocua* *Salmonella* *Enterobacteria*	Atanasova et al., 2014

ND, not determined.

Val-Pro-Pro, both exerting anabolic effect on bones (Griffiths and Tellez, 2013).

Antioxidant Peptides

The existence of antioxidant peptides in vegetal food (especially cereals and legumes) is a well-established concept, recently reviewed by Malaguti et al. (2014). Marine food, such as algae (Sheih et al., 2009) and squid (Rajapakse et al., 2005b), as well as unusual proteins like royal jelly-derived (Guo et al., 2009), yak milk casein (Mao et al., 2011), goat milk casein (Li et al., 2013), donkey milk (Piovesana et al., 2015), but also egg white lysozyme (Rao et al., 2012), can be a source of antioxidant peptides. Bacteria have the capability to decrypt antioxidant peptides from several food proteins. Antioxidant activities were described in fermented mussel sauce (Rajapakse et al., 2005a), in LAB-fermented milk whey (Virtanen et al., 2007), in fermented shrimps (Faithong et al., 2010), during sourdough fermentation of wheat, rye, and kamut flours (Coda et al., 2012) and in peptides derived from bovine casein after 24 h hydrolysis with *Bifidobacterium longum* (Chang et al., 2013). Antioxidant efficacy was also found in cell-free extracts of *Lactobacillus plantarum* isolated from traditional Chinese food (tofu and kefir) but it is rather related to pseudo catalase enzymes, exopolysaccharides and lipoteichoic acids than to bioactive peptides (Li et al., 2012). Recently, mixed cultures of LAB and yeast proved to be able to produce antioxidant peptides from cow milk, however, the peptide sequences having activity were not described (Li et al., 2015).

Antioxidant peptides can control both enzymatic and non-enzymatic peroxidation of fatty acids thus preventing membrane lipid peroxidation (Hartmann and Meisel, 2007). They can act as direct radical scavengers, non-radical oxygen quenchers or metal chelators, but they also can eliminate radical precursors, hence controlling ROS damage to cells and tissues, resulting in a beneficial action on the whole organism. The redox balance between ROS and antioxidant endogenous defenses is highly impaired during chronic inflammation (Zhang et al., 2015) thus, antioxidants are essential in controlling age-related chronic degenerative diseases concerning both the cardiovascular and the central nervous system.

Natural antioxidant peptides such as GSH (Cys-Glu-Gly) or carnosine (beta-Ala-His) play important roles in radical scavenging (Power et al., 2013). Antioxidant properties have been demonstrated for Tyr, Trp, His, and Lys, in particular when Tyr and Trp are in the C-terminus and the hydrophobic residues Val and Leu at the N-terminus of a peptide. Trp and Tyr behave as antioxidant molecules since their indolic and phenolic groups can act as hydrogen donors (Hernández-Ledesma et al., 2008). Basic amino acids can chelate metallic ions and cysteine acts as a proton donor, thanks to its thiolic group but the highest antioxidant activity was demonstrated for the tripeptide Pro, His, His (Malaguti et al., 2014). Furthermore it was demonstrated that Leu-His-His and Pro-His-His can both act synergically with non-peptide antioxidants such as butylated hydroxyanisole (Kitts and Weiler, 2003).

Immunomodulatory Peptides

It is well established that some milk proteins (caseins, whey proteins, lactoferrin, lactoperoxidase) are able to control lymphocyte proliferation (Möller et al., 2008). Milk-derived peptides such as the C-terminus sequence of beta casein (193–209) can increase proliferation of rat lymphocytes (Meisel and Bockelmann, 1999), whereas alpha casein C-terminal hexapeptide (194–199) and beta casein fragments (63–68 and 91–93) induce macrophage maturation and phagocytosis enhancement *in vitro* (Fiat and Jollès, 1989). In agreement with these data, studies *in vivo* show that alpha casein-derived peptides can stimulate macrophages phagocytosis exerting a protective effect against *Klebsiella pneumoniae* infection in mice (Migliore-Samour et al., 1989). Short-chain peptides from milk whey proteins also stimulate the proliferation of murine spleen lymphocytes *in vitro* (Mercier et al., 2004). More detailed studies report that proliferation of human peripheral blood lymphocytes is induced by the k-casein and alpha lactalbumin fragment Tyr-Gly-Gly (Kayser and Meisel, 1996; Sharma et al., 2011). Natural killer (NK) cell activity and antibody synthesis can also be enhanced by food–derived bioactive peptides (Hartmann and Meisel, 2007). However, milk-derived peptides do not act only as simply stimulators of the immune system: immune-modulating activities including cytokines regulation and attenuation of allergic reactions has also been described (Korhonen and Pihlanto, 2003; FitzGerald and Murray, 2006).

Transferring clinical data to food is not so simple. Actually, in this scenario, LAB (especially probiotic LAB) play a very central role: beta-casein medium fermented with LAB gives rise to bioactive peptides acting on monocytes, macrophages and T helper cells, particularly with Th1-like cells (Laffineur et al., 1996). *Lactobacillus paracasei* hydrolyzes beta-lactoglobulin originating peptides stimulating Interleukin 10 (IL-10) production and downregulation of IL-4 and gamma interferon secretion (Prioult et al., 2004). Casein hydrolysated by *Lactobacillus rhamnosus GG* displays modulating effects (both stimulation and suppression) on lymphocyte proliferation (Möller et al., 2008). Furthermore, casein hydrolysates from *Lactobacillus rhamnosus GG* can induce enhancement of anti-inflammatory cytokines from Th1 lymphocytes and a parallel decrease of pro-inflammatory cytokines and immunoglobulins produced by Th2 lymphocytes, thus controlling allergic reactions (Delcenserie et al., 2008). *Lactobacillus helveticus* proteolysis products down-regulate cytokines production and stimulate macrophage phagocytosis (Matar et al., 2001) and also show protective effects against *E. coli O157* (LeBlanc et al., 2004) and *Salmonella typhimurium* (Tellez et al., 2011) infection. All the non-proteolytic strains of *Lactobacillus helveticus* failed to trigger immune system modulation. Three immune-active peptides derived both from beta casein and from alpha lactalbumin after milk fermentation with proteolytic strains of *Lactobacillus helveticus* were analyzed: they contain high percentage of proline histidine and lysine (Tellez et al., 2010). Establishing how, when and in what extent proteolysis occurs in food (and in the gut) is still an open question.

Cell Cycle and Apoptosis Modulating Peptides

Milk-derived peptides, sometimes resulting from microbial proteolytic activity (Roy et al., 1999), can regulate cell growth, differentiation, and apoptosis (Roy et al., 2002). De Simone et al. (2008) demonstrated that waste milk whey is able to induce apoptosis in CaCo2 cancer cells, but also interfere with the cell cycle (reducing the number of cells in S-phase and enhancing the number of cells in G1 phase), thus inhibiting proliferation. Cooked milk whey loose this property probably due to irreversible denaturation of proteases decrypting the peptides of interest. The endogenous microflora of milk (both starters and contaminating bacteria) seems to be the main responsible of the phenomenon observed, since fresh milk does not exhibit this behavior (De Simone et al., 2008). Although the involved peptides were not characterized, it is very well known that some sequences such as Arg-Gly-Asp-Asp-Asp-Asp-Asp-Asp-Asp-Asp-Asp can have anti-proliferative effects on cancer cells models: the triplet Arg-Gly-Asp can account for adhesion to the extracellular matrix whereas the eight Asp residues can act intracellularly by binding chromatin (Galvez and de Lumen, 1999). Meisel and FitzGerald (2003) also demonstrated that normal cells are less sensitive to the apoptotic induction than malignant cells. Tumor cells apoptosis was also detected in an *in vivo* model in which mice were treated with milk that underwent fermentation by a highly proteolytic strain of *Lactobacillus helveticus.* On the contrary, the use of low-proteolytic strains did not gave satisfactory results in terms of breast cancer control in the same mice (De LeBlanc et al., 2005). On the other hand, not all anticancer effects of milk are related to proteolysis. It has been demonstrated that alpha lactalbumin can form a complex with oleic acid in acid conditions. This modified protein can induce apoptosis in cancer cells *in vitro* (Svensson et al., 2000). Therefore, is it tempting to hypothesize that acidification caused by LAB metabolism can also contribute to these effects, and further experiments proving this will be crucial to confirm such hypothesis and opening new perspectives for cancer control. Taken together all these results suggest that it is possible to increase host defenses against tumor degeneration by using food fermented with LAB.

Opioid and Anti-opioid Peptides

These molecules constitute a promising frontier for treating both stress-related behaviors such as anxiety and depression and season-related mood disorders. They act on the appetite/satiety as well, as demonstrated by Pfluger et al. (2012) who named these molecules "nutropioids." In general, these peptides are able to control the gut–brain axis at several levels, including gut–brain communication, brain cognitive function, and behavior (Cryan and Dinan, 2012). Their mechanism of action includes stimulation of receptors (K, delta, and mu) present in both the central and the peripheral nervous system and subsequent inhibition on adenylate cyclase activity. Some of these compounds are summarized in **Table 2**.

The best studied myorelaxant peptides are beta-casomorphins (which act on the mu receptors) and alpha s1 casein-derived peptide (which acts on the delta receptor). Their primary

TABLE 2 | Overview of the opioid and anti-opioid peptides.

Peptide	Protein source	Effect	Receptor	Author
β-Casomorphin	β-Casein	Opioid	μ	Loukas et al., 1983
ND	α S1-Casein	Opioid	δ	Loukas et al., 1983
Casoxin	k-Casein	Anti-opioid	μk	Chiba and Yoshikawa, 1986
Exorphin B5l	Gluten	Opioid	Out of the BBB	Fukudome and Yoshikawa, 1992
Soymorphine	Soy	Opioid	μ	Kaneko et al., 2010
Rubiscolin	Rubisco	Opioid	δ	Yang et al., 2001

sequences consist of 4–10 amino acids whose N-terminal residue, (essential to trigger biological activity), is Arg in alpha s1 casein-derived peptide and Tyr in beta-casomorphins (Loukas et al., 1983). This difference, together with the presence of six proline residues in beta-casomorphins, can account for the higher resistance to enzymatic digestion of the latter, resulting in enhanced half-life and absorption in human digestive tract. Once in blood, beta-casomorphins can reach receptors in the brain and in the peripheral tissues thus exerting a relaxing action inducing calmness and sleeping (Chabance et al., 1998). Another interesting peptide derived from alpha s1casein is alpha-casozepine, displaying anxiolytic action but not directly interacting with the mu and delta receptors. It has been demonstrated that the biological effect is mediated by activation of serotonin and $GABA_A$ receptors, causing release of endogenous serotonin, dopamine, and GABA (Mizushige et al., 2013). Conversely, casoxins peptides (originated from k-casein hydrolysis) behave as opioid-antagonists over both the mu and k-type receptors. Due to this different physiological role, casoxins can be employed to counteract depression (Chiba and Yoshikawa, 1986).

Peptides produced with the contribution of LAB protease system on dairy proteins are named exorphins and casoxins, the former having opioid–like and the latter opioid-antagonist function (Chabance et al., 1998). However, other foods may contain opioid/anti-opioid peptides. It has long been established that wheat gluten is a good source of opioid peptides (Fukudome and Yoshikawa, 1992), and more recently, it has been shown that gluten exorphin B5 can enhance prolactine secretion by acting on receptors present outside the blood brain barrier. In this case, the biological effect is linked to a reduced dopaminergic tone (Fanciulli et al., 2005). Soymorphins are very interesting opioid peptides acting on mu-receptors present in the gut and connected with the serotonin, dopamine, and GABA receptors systems. It was shown that soy-derived opioid peptides act as anorexigenics and also reduce gastrointestinal motility (Kaneko et al., 2010). A delta-opioid peptide called rubiscolin was decrypted from the large subunit of spinach rubisco (Yang et al., 2001). The rubiscolin amino acid sequence is Tyr-Pro-Leu-Asp-Leu-Phe and it displays an analgesic effect besides being involved in memory consolidation (Yang S. et al., 2003). Hirata et al. (2007) also report anxiolytic action of this natural peptide by activation of the sigma1 and D1 dopamine receptors, when delivered orally (100 mg/Kg). The replacement of Leu^3 with Ile or Met enhances by a factor four the opioid activity, whereas the replacement of Phe with Val potentiates the opioid activity more than 10-fold. The overall activity of the sequence Tyr-Pro-Met-Asp-Leu-Val is 20 times higher than the natural rubisco derivative (Yang S. et al., 2003).

Antithrombotic Peptides

Blood coagulation occurs through a complex system of proteins undergoing a sequential proteolytic cascade. These proteins include pro-enzymes such as fibrinogen that is proteolytically activated to fibrin by means of thrombin. Bovine k-casein derived oligopeptides (fragment 106–116, 11 amino acids long) named casoplatelins (or thrombin inhibitory peptides), can inhibit the binding of human fibrinogen (gamma chain) on the platelet surface receptor, due to sequence homology between fibrinogen gamma chain and K-casein. Actually, the mechanism involved in milk clotting, defined by interaction of k-casein with chymosin bear a remarkable similarity to the process involved in blood clotting, defined by interaction of fibrinogen with thrombin (Qian et al., 1995). As a result, they prevent aggregation of ADP-activated platelets. Also the glycopeptide present on the N-terminus of k-casein, a smaller molecule called casopiastrin, displays inhibiting activity on fibrinogen binding on the platelet membrane (Ricci-Cabello et al., 2012). Although these molecules are mainly the result of human proteolysis on milk, LAB proteolytic activity on casein can contribute to their release both in food and *in vivo*. Casoplatelins are present in high amounts in the gut but, after absorption, significant concentrations are still bio-available in blood thus contributing to thrombosis control in humans.

Antihypertensive Peptides

Lactobacillus helveticus, *Lactobacillus delbrueckii* subsp. *Bulgaricus* SS1, *Lactobacillus delbrueckii* subsp. *lactis*, and *Lactococcus lactis* subsp. *cremoris* FT4 can modulate blood pressure by producing angiotensin 1-converting enzyme inhibitory peptides (ACE inhibitors) from milk proteins. ACE is a carboxypeptidase converting angiotensin I (a decapeptide) into angiotensin II (an octapeptide) having a strong vasoconstrictor action (Hartmann and Meisel, 2007). *Lactobacillus delbrueckii* subsp. *lactis* hydrolyzes both alpha s1 and beta casein (but not k-casein) by means of a cell envelope proteinase releasing antihypertensive peptides: the proteolytic activity was maximal during the logarithmic growth and addition of NaCl and glycerol prevents correct proteolysis (Hébert et al., 2008). Casein-derived anti-hypertensive peptides are 2–6 amino acids long oligopeptides. Generally, LAB peptidases, by shortening the poly/oligopeptide chain, contribute to enhance the anti ACE potential. Actually the 6 (alpha s1 casokinin-6 = Thre-Thre-Met-Pro-Leu-Trp) and the 5 (alpha s1 casokinin-5 = Phe-Phe-Val-Ala-Pro and betacasokinin = Lys-Val-Leu-Pro-Val) amino acids long oligopeptides are less active than the tripeptides made up of Val-Pro-Pro and Ile-Pro-Pro. A very small dipeptide Tyr-Pro, proved to be effective in blood pressure control as well

(Yamamoto et al., 1999). The final active short peptides are resistant to both pH variations and human digestive enzymes thus opening interesting applicative perspectives (Gobbetti et al., 2004).

Cholesterol-Lowering Peptides

Hypocholesterolemic peptides can be originated by beta-lactoglobulin, casein and soy proteins proteolysis (Hori et al., 2001). They can lower the total cholesterol of rats *in vivo*. Their mechanism of action probably consists in the reduction of the micellar cholesterol solubility or also to an enhanced ability to bind taurocholate (Nagaoka et al., 2002). They overall action prevents cholesterol absorption by CaCo2 cells *in vitro* and enhances fecal steroid excretion *in vivo*. The sequences displaying such activities are Ile-Ile-Ala-Glu-Lys, Ala-Leu-Pro-Met-His and Gly-Leu-Asp-Ile-Gln-Lys (Nagaoka et al., 2001).

Glucose-Uptake Stimulating Peptides

It has been demonstrated that dipeptides containing branched chain amino acids (Ile-Ile, Leu Leu, Ile-Leu, Leu-Ile, Ile-Val, Leu-Val, Val-Leu) can enhance glucose uptake in skeletal muscle cells, favoring glycogen synthesis and thus controlling hyperglycemia (Morifuji et al., 2010). Generally, glucose uptake by skeletal muscle cells is induced by exercize-training by means of increased expression or activity of key-signaling proteins (Goodyear and Kahn, 1998). Branched-chain amino acids are the main nitrogen source for skeletal muscles and milk whey proteins are particularly rich in branched chain amino acids (22.3%) as compared to caseins (20.3%), soy proteins (17.5%), or wheat gluten (14.1%; Morifuji et al., 2010). The dipeptide Ile-Leu is the most abundant after milk whey hydrolysis. Therefore, assessing the proteolytic activity of LAB toward milk whey proteins (that constitute natural nitrogen substrates for LAB), is a promising strategy to get nutritional supplements improving health. Proteomic approaches using LC-MS-MS can be a valuable help for identifying small nutritionally relevant peptides and a new discipline, called food peptidomics, is now expanding (Lahrichi et al., 2013).

Chaperone-Like Peptides

A very interesting class of protein-encrypted peptides are those displaying chaperone-mimetic action (Bumagina et al., 2009; Artemova et al., 2010). Synthetic peptides were constructed on the model of bovine hemorphin-6, wheat gluten exorphin C, and spinach rubiscolin-5 (all opioid peptides), and their ability in refolding model proteins was investigated. The target proteins to be refolded were heat-treated or DTT-aggregated carbonic anhydrase, ADH and bovine milk alpha lactoglobulin. The results clearly indicate that the peptides are able to refold damaged proteins thus opening the way to the interesting hypothesis that food proteins, besides their nutritional role, can act as systems involved in quality control of proteins especially during stress (Artemova et al., 2010).

Mixed Function Peptides

Some food encrypted peptides, in case decrypted by LAB proteolytic action, possess mixed function. In **Table 3** are reported the peptides displaying two or more biological activities.

As an example the tripeptides Val-Pro-Pro and Ile-Pro-Pro, released from beta and K-casein by *Lactobacillus helveticus* are immunomodulatory and hypotensive (Hartmann and Meisel, 2007). This effect can be explained by the fact that ACE inhibition favors bradykinin production, and bradykinin plays a crucial role in the inflammation process by stimulating macrophages and increasing lymphokines secretion by lymphocytes (Sharma et al., 2011).

Some immunostimulatory peptides also exhibit antimicrobial features: beta-lactoglobulin-derived peptides can improve phagocytosis but also stimulate the microbial autolytic system. This is not only restricted to bacteria but also to fungi and both naturally autolyzing and non-autolyzing strains are sensitive to this effect (Hernández-Ledesma et al., 2008).

It has long been established that opioid peptides such as casomorphin agonists also decrease cell proliferation by acting on somatostatin receptors (Hatzoglou et al., 1996a,b). Since opioid and somatostatin receptors are present on different cells including central nervous, endocrine and immune system (De Simone et al., 2008) the action of such peptides is more complex than expected.

Several peptides are opioid and hypotensive, especially those derived from bovine milk proteins (casomorphins, lactorphins) and beta-casomorphin 7 is opioid, hypotensive, and immunomodulatory: this last function is targeted on lymphocyte proliferation that is sometimes stimulated and sometimes inhibited depending on the peptide concentration (Meisel and Bockelmann, 1999).

Lactoferricin, the lactoferrin-derived antimicrobial peptide, besides being an antibacterial, antifungal and antiviral molecule, can also control carcinogenesis by means of its anti-inflammatory and immune-modulating properties. This effect seems to be due to a positively charged region of the peptide that is very rich in tryptophan and arginine residues (Sharma et al., 2011)

The reason of these mixed functions lies in the fact that some regions in the primary structure of caseins contain overlapping peptide sequences, exerting different physiological functions (Meisel and Bockelmann, 1999). These domains, highly hydrophobic and rich in proline residues, have been defined as "strategic zones" and are resistant to proteolytic attack (Fiat and Jollès, 1989).

In conclusion, of this paragraph it is important to underline that not all bioactive peptides released by the combined action of LAB proteolytic system and digestive proteases in food and in the gut can be available for humans. Actually, bioactive peptides may be degraded during digestion, may be poorly absorbed and hence reach the target tissues at a concentration lower than the one necessary to exert their biological function. As far as degradation is concerned, supplying bioactive peptides by means of GRAS bacteria (like LAB are) plus protein complements is simpler than encapsulate them and this constitutes therefore a promising strategy to transport the molecules to the intestine, preventing degradation

TABLE 3 | Overview of peptides with mixed functions.

Peptide	Protein source	Activity					Author
		Immuno-modulator	Cell cycle	Anti-ACE	Opioid	Anti-microbial	
β-Casomorphin-7 (YPFPGPI)	β-CN	+	+		+		Hatzoglou et al., 1996a,b
β-Casokinins-10 (YQQPVLGPVR)	β-CN	+		+			Hartmann and Meisel, 2007
α-Lactorphin (YGLF)	α-LA			+	+		Meisel and Bockelmann, 1999
β-Lactorphin (YLLF)	β-LG			+	+		Meisel and Bockelmann, 1999
Antimicrobial pept ide (AGTWY)	β-LG	+				+	Hernández-Ledesma et al., 2008
Immunopeptide (YGG)	α-LA	+	+			+	Sharma et al., 2011

in the upper digestive tract. Efforts have been made also to assess the real *in vivo* absorption of bioactive peptides: blood levels of antihypertensive peptides have been quantified (by LC ESI triple) after ingestion by human volunteers (van Platerink et al., 2006) but also *in vitro* studies measuring epithelial translocation across CaCo2 monolayers by means of ESI-LC-MS-MS can supply reasonable information about absorption. One challenging aspect of these approaches is the need of improved "omics" methods aimed to identify low-molecular weight bioactive peptides. MALDI (matrix-assisted laser desorption ionization) TOF-MS has proved to be inadequate because of matrix interference (Saavedra et al., 2013) and matrix-free methods such as NALDI (nanostructure-assisted-laser desorption ionization; Kütt et al., 2011) have been re-proposed.

BIOACTIVE AMINES

A number of microorganisms synthesize, as decarboxylation products of precursor amino acids, biogenic amines. In LAB, expression (by transcriptional induction) and/or activation (by catalytic modulation) of amino acid decarboxylation systems are non-essential adaptive responses to energy depletion but also strategies to counteract acid stress (Pessione, 2012).

However, not all biogenic amines are bioactive molecules since most of them only act as spoilage compounds (putrescine, cadaverine) sometimes enhancing the toxicity of true bioactive molecules (Medina et al., 2003). On the contrary, the decarboxylation of certain amino acids, as tyrosine and histidine, can give rise to bioactive molecules (tyramine and histamine) involved in several pathogenic syndromes, more extensively described in the following paragraphs. On the other hand, not all bioactive compounds negatively affect human health: as an example, beta-phenylethylamine derived from phenylalanine or tryptamine originated from tryptophan can exert beneficial actions (such as mood control and appetite/satiety balance regulation) when administered to humans (Shimazu and Miklya, 2004). Similarly, it has been reported that several LAB (*L. bulgaricus, L. acidophilus, L. casei, L. plantarum*) can synthesize melatonin, a bioactive molecule, deriving from tryptophan decarboxylation and serotonin metabolism, acting on sleep and reproductive behavior but also controlling immunity, inflammation and carcinogenesis (Tan et al., 2014). A particular case is the glutamate product gamma amino butyrate (GABA): this molecule can be naturally present in food but also artificially added to enhance the nutraceutical value of a certain food (GABA-tea, GABA-rice; Abe et al., 1995; Oh, 2003) due to its relaxing action on muscles and to its overall beneficial effects on the nervous system.

Bioamines in Food

Cheese is the food most frequently associated with a too high biogenic amine content. It is very rich in free amino acids due to the desired proteolytic action performed by both bacteria and fungi during "maturation," when colonized by decarboxylase-positive LAB (either autochthonous or contaminant bacteria) it can be a source of tyramine, histamine, putrescine, and beta-phenylethylamine (Pessione et al., 2009). Tyramine and beta phenylethylamine are particularly abundant in food from animal origin (including fermented sausages) since these matrices are very rich in the precursor amino acids, tyrosine, and phenylalanine, used by animals to synthesize catecholamines. Both tyramine and beta-phenylethylamine are bioactive compounds whose action will be better described in the following paragraphs.

As far as fermented meat products are concerned, tyramine, cadaverine, putrescine, and histamine can be found (Singh et al., 2012). Poor quality processing favoring contamination is the main cause of a too high bio-amine content of meat, however starter strains possessing the capability to form biogenic amines, like *Lactobacillus curvatus*, have been described as well (Singh et al., 2012). To prevent such a risk, selection of particular starter cultures possessing either amino oxidase activity (Alvarez and Moreno-Arribas, 2014) or bacteriocin synthesis capability (Somda et al., 2011) should be taken into concern to contain the undesired consequences of spontaneous fermentations.

Other fermented foods are alcoholic beverages such as beer, ciders, and wine, that being of vegetal origin, are generally poor of aromatic amino acid precursors (Lehtonen, 1996). At the end of alcoholic fermentation the only amino acid present in wine in significant amount is arginine (2,4 g/L): this can be converted by the arginine decarboxylase into agmatine and then putrescine, or rather be directed to the arginine deiminase pathway (ADI) generating ammonia, ornithine (generating putrescine) and carbamoyl phosphate (**Figure 2**) (Costantini et al., 2013). Both putrescine and ornithine are not true bioactive compounds even if they can

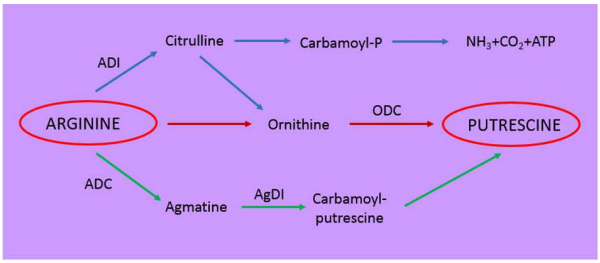

FIGURE 2 | Schematic representation of the metabolic pathways involved in putrescine formation. ADI, arginine deiminase; ADC, arginine decarboxylase; AgDI, agmatine deiminase; ODC, ornithine decarboxylase.

alter the organoleptic properties of wine and enhance the toxicity of other biogenic amines (Costantini et al., 2013). Carbamoyl phosphate can combine with ethanol in wine generating ethyl carbamate a carcinogenic molecule (Jiao et al., 2014).

However, if the oenological procedures are not correctly performed, other bioactive amines can be generated. Actually, at the end of the alcoholic fermentation when all the grape sugars are exhausted, yeast cells die and undergo autolysis, releasing their own proteins and proteases (from lysosomes). Hence, if dead yeasts cells are not immediately separated from wine, free amino acids (from yeast proteins) become available for autochthonous LAB able to perform malolactic fermentation (MLF; Coton et al., 1999). This secondary fermentation is widely appreciated for its improvement of wine flavor, and it is usually performed by the predominant species *Oenococcus oeni* or, more occasionally, by other LAB species belonging to the genera *Lactobacillus* and *Pediococcus,* which may develop during the winemaking process (Lonvaud-Funel, 1999). Unfortunately, some LAB, besides TPP-dependent-acid decarboxylases, also bear PLP-or pyruvoil-dependent amino acid decarboxylases that can generate undesired amines, depending on the precursor amino acid present at this fermentation stage (Recsei and Snell, 1985). Therefore, histamine, cadaverine, tyramine, and beta-phenylethylamine (whose action will be better described in the following paragraphs) can be produced.

The total content of biogenic amines in food varies from 1 to 50 mg/Kg (alcoholic and no-alcoholic beverages, soy products) to 20–1,500 mg/Kg [sausages, cheeses; EFSA Panel on Biological Hazards (BIOHAZ), 2011] At high concentrations, BA are risk factors for food intoxication, while moderate levels may lead to food intolerance (Ladero et al., 2010) It is very difficult to establish a uniform maximum limit for ingested BA since their toxic effect depends on the type of BA in question. The unique BA for which the European Food Safety Authority (EFSA) has legally set maximum limits is histamine and only related to fish and fish products (Linares et al., 2016). Furthermore, the toxic effects exerted by amines possessing biological activity in humans, strongly depend on individual sensitivity to them (Santos, 1996). As an example, tyramine tolerance is lower in patients treated with antidepressant mono-amino-oxidase inhibitory drugs (IMAO), which interact with amino-oxidase enzymatic systems (MAO, DAO), since these patients have an impaired ability to oxidize amines. For the same reason, the toxic effects of bioactive amines in wine consumers is enhanced by the presence of ethanol, which is one of the most effective inhibitors of amine oxidases (Sessa et al., 1984).

Factors Affecting Amines Production in LAB

When considering the risk of bioactive amine release into food, it is important to underline that maximum energy depletion and acidification occurs during the stationary growth phase. If (theoretically) LAB can be removed from food before resting, no amine accumulation takes place. This statement is supported by proteomic investigations that revealed that, in *L. hilgardii,* histamine production is induced only during stationary phase (Pessione et al., 2005). On the contrary, northern blotting experiments showed activation of the HDC (histidine decarboxylase) gene already during exponential growth phase in a different *L. hilgardii* strain (Landete et al., 2008). However, the same authors clearly demonstrated an inhibitory effect of both glucose and fructose (still high during exponential growth phase) over the genes coding for HDC in *L. hilgardii*, *O. oeni*, and *P. parvulus* (Landete et al., 2008). Furthermore, in both *L. hilgardii* and *L. brevis*, it has been demonstrated that tyramine accumulation is maximal in presence of either fructose or fructose plus L-malic acid (Moreno-Arribas et al., 2003) thus supporting the idea that it is not necessary to reach the stationary phase to trigger amine accumulation. Actually, also in a different bacterial model (*E. faecalis*) studies involving sub-proteomes profiling demonstrated a high tyramine content already in the exponential phase when sugars are not yet consumed (experiments performed in chemically defined medium having glucose as sugar carbon source; Pessione et al., 2009). Therefore, the evidences are still controversial and in part related to specific amino acid/amine reaction, in part linked to a strain difference.

A further strong evidence is the fact that amine production is related to the availability of the precursor amino acid. Supplements of tyrosine in the medium can increase about 10-fold tyramine accumulation and about twofold tyrosine decarboxylase (TDC) activity in *L. brevis* (Moreno-Arribas et al., 2000). Similarly, in *Enterococcus faecalis*, TDC was found to be more abundant in the membrane extracts when the strain was exposed to free tyrosine (Pessione et al., 2009). Histamine is biosynthesized only in presence of histidine: the cytosolic enzyme HDC was found more abundant in cells grown in histidine medium (stimulated condition) in *Lactobacillus hilgardii*, and *Lactobacillus 30a* (Pessione et al., 2005; Mazzoli et al., 2009) as compared to control conditions.

The only exception to this rule seems to be the GAD enzyme that can allow GABA accumulation even in the absence of glutamate. However, the chemically defined-medium medium (CDM) used for growing *L. lactis* contains glutamine, so it is

possible that part of it is converted to glutamate that can undergo decarboxylation to GABA. On the other hand, a transcriptional control exists since addition of glutamate can trigger a light enhance of GABA biosynthesis (Mazzoli et al., 2010).

A critical concern connected with the necessity of free amino acid availability is how bacteria can get them. As far as LAB are concerned, often in the same ecological niche where they live proteins are available (cheese) or microbial autolysis (wine) generates free proteins that can undergo proteolytic breakdown by LAB (Lamberti et al., 2011).

Interestingly, other factors can affect bioactive amine production and, among these, acids (that can undergo or not a similar decarboxylative process). It has been demonstrated that L-lactic acid does not influence both histamine accumulation and HDC biosynthesis (Landete et al., 2008). Similarly, malate has no effect on histamine accumulation (Mangani et al., 2005) or histidine consumption (Mazzoli et al., 2009). In agreement with these data, Moreno-Arribas et al. (2000) showed that tyramine production was not affected by either malate or citrate, whereas Lonvaud-Funel (2001) observed that both tyramine and histamine accumulation was lower during MLF and higher after it. The utilization of the amino acids tyrosine and histidine only after malate does not necessarily indicate malate inhibition, but simply that bacteria prefer utilize carbon sources before consuming nitrogen compounds.

Other factors affecting amine accumulation in food include temperature, spices and salt content, generally controlling microbial growth. The optimum growth for most amine-producing bacteria is 20–37°C. Below this temperature, the risk to find biogenic amines in food decreases (Karovičová and Kohajdová, 2005). The sausage diameter as well as the addition of spices can control a too high amine content in meat: the larger the size, the higher the risk of amines due to higher water activity and lower salt content. However, NaCl not only negatively affects bacterial growth, proteolytic activity and histidine decarboxylase activity but also stimulates both tyrosine and glutamate decarboxylase (GAD) synthesis acting as operon inducer (Singh et al., 2012).

Histamine

The ingestion of histamine-rich food may cause heterogeneous symptoms such as diarrhea, arrhythmia, headache, and allergic syndromes such as respiratory distress (asthma), rhino-conjunctivitis, urticaria, pruritus, and flushing (Manzotti et al., 2016). Histamine is a strong hypotensive compound also acting on many receptors present in the gastro intestinal tract (Santos, 1996). Hence, high content of histamine is responsible of the toxicity of spoiled or not properly stored fish. In black carp fillets, for example, the contents of BAs remained invariable during the early stages of storage and began to increase at the later stages of storage occurs during food storage. In particular, a significantly higher concentration of histamine (132.05 mg/kg on the third day) was detected in the black carp filets stored at 20°C, indicating that this temperature favors the formation of histamine (Hongbing et al., 2016). In wines, especially red wines undergoing spontaneous MLF by LAB, are frequently responsible of these syndromes. Amino acids generating bioactive amines (histidine, tryptophan, glutamate) can be released during the, post-alcoholic fermentation, by yeasts autolysis as referred above (Alexandre et al., 2001). The concentration of free amino acids in wine is also dependent on the proteolytic activity of the strain concerned with MLF (Alexandre et al., 2001).

Tyramine

Tyramine is a bioactive molecule acting both at vascular level (hypertension, vasal-constriction) and on the central nervous system (headache). Despite it is generally assumed that histamine is the most toxic BA, a recent work reveals that unexpectedly, tyramine is more cytotoxic than histamine on an *in vitro* model of the human intestinal epithelium. Furthermore, the concentrations found to be toxic are commonly reached in BA-rich foods (Linares et al., 2016). Severe syndromes have been described in patients lacking a correct amino-oxidase activity because in treatment with mono-amino-oxidase inhibitory drugs (MAOI). MAOI are used as antidepressant because they prevent the oxidative catabolism of brain-active amines, such as dopamine, serotonin, tryptamine, and β-phenylethylamine. Unfortunately, MAO-inhibitors cannot discriminate among the different MAOs, and their overall effect results in high concentrations of tyramine that can cause hypertensive crises, brain hemorrhage and sometimes death (Pessione et al., 2009). Since tyramine is the biogenic amine most frequently found in cheese (due to the referred high tyrosine content of dairy products) this syndrome is also called "cheese reaction." For the reasons explained above, MAOI development has led to compounds with improved tolerability profiles compared to the initial irreversible MAOIs, particularly in regard to tyramine-induced hypertension and dietary restrictions (Yanez et al., 2012). The huge number of non-controlled autochthonous bacteria involved in cheese manufacturing and the accidental contamination by unwanted strains during storage render cheese a risky food for tyramine ingestion, although starters are accurately typed. TDC activity has been described in several LAB strains of the genera *Lactobacillus, Carnobacterium, and Leuconostoc.* The enzyme from *Lactobacillus brevis* and *Enterococcus faecalis* has been fully characterized. Proteomic studies reveal the membrane location of such a protein in *Enterococcus faecalis* (Pessione et al., 2009), thus confirming the high hydrophobic nature of this enzyme (previously predicted on the basis of gene analyses suggesting large hydrophobic domains but also because of the harsh procedures needed to purify it). Interestingly, *Enterococcus faecalis* TDC proved to be able to produce not only tyramine from tyrosine but also beta-phenylethylamine from phenylalanine (Pessione et al., 2009).

Beta-Phenylethylamine

β-Phenylethylamine, the decarboxylation product of phenylalanine, behaves like an endogenous amphetamine. It can control appetite/satiety thus being useful to treat obesity and related syndromes as well as in weight-control diets. Like

amphetamine it can also positively affect mood. However, controversial effects including insomnia, anxiety, and a MAO-blocking activity that may extend the half-life of tyramine thus potentiating its toxicity (Millichap and Yee, 2003) have been described. Evidence of a specific phenylalanine decarboxylase enzyme in bacteria, has been reported only for *Bacillus cohnii*, a *species* taxonomically distant from LAB. Nevertheless, both Lactobacilli and Enterococci, frequently found in fermented food, display a clear β-phenylethylamine accumulation. Originally, some authors (Millichap and Yee, 2003) suggested that the β-phenylethylamine found in fermented food is the result of the activity of tyrosine decarboxylating bacteria. It is interesting to underline that in mammals and in several eukaryotic cells models a low-selective aromatic L-amino acid decarboxylase (EC 4.1.1.28) exists. In bacteria, enzymatic studies proved that TDC from *E. faecalis* is active also toward DOPA, but not toward other amino acids like phenylalanine while *Lactobacillus* bears a highly tyrosine selective TDC. More recently, the presence in *E. faecalis* of phenylalanine-decarboxylating TDC (EC 4.1.1.25) has been clearly demonstrated by a bi-phasic kinetic of tyramine and β-phenylethylamine production (Pessione et al., 2009). Tyrosine is consumed first and it is fully converted into tyramine (during logarithmic growth). Then phenylalanine is decarboxylated to β-phenylethylamine, although with lower conversion rate and yield. These results are consistent with a double catalytic activity of the same protein (TDC) having higher specificity for tyrosine and only low affinity for phenylalanine. On the other hand, Marcobal et al. (2006) observed β-phenylethylamine accumulation in a recombinant *E. coli* expressing the TDC gene of *E. faecium*.

GABA

γ-Aminobutyric acid is one of the bioactive amines having true positive effects on human health. It acts as a neurotransmitter in the central nervous system of most vertebrates and plays additional roles on the overall human physiology such as lowering the blood pressure in mild hypertensive patients and acting as smooth muscles relaxation system (Mazzoli et al., 2010). Bacteria can produce GABA by the decarboxylation of glutamate through GAD. Glutamate and GABA act as opposite molecules having excitatory and inhibitory roles, respectively (Hyland and Cryan, 2010). *Lactococcus lactis* NCDO 2118 can obtain GABA from glutamine opportunely converted into glutamate (Mazzoli et al., 2010). It has long been established that *L. lactis* possess only one gene encoding GAD and that this enzymes is highly specific for glutamate (apparent Km 0.51 mM). Its molecular weight is about 54 kD and it has maximal activity at pH 4.7, whereas no activity is present at neutral pH (Nomura et al., 1999). Differences have been reported between *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*: both possess gadCB genes encoding for GAD, however, one-base deletion of adenine and one-base insertion of thymine were detected in the coding region of the latter rendering it unable to synthesize a functional GAD enzyme (Nomura et al., 2000). Lactobacilli are the best GABA-producers, however, Lactococci, Streptococci, and Bifidobacteria can synthesize GABA as well (Lyte, 2011). Several strategies have been proposed to obtain high GABA amounts from cheap substrates through LAB sourdough fermentation (Coda et al., 2012), batch fermentation using immobilized (Huang et al., 2007) or entrapped (Choi et al., 2006) *Lactobacillus brevis* cells for the construction of functional food. To achieve maximum GABA production, temperature and pH are crucial (Dhakal et al., 2012). Nowadays, several nutraceutical preparations, such as GABA tea (Abe et al., 1995), GABA rice (Oh, 2003), GABA soymilk (Tsai et al., 2006), and GABA chocolate (Nakamura et al., 2009) are available, especially in Asia. GABA natural content of cacao is about 0.009%, however, this value is lower when considering the market-available chocolate also containing milk, sugar, and other ingredients. Functionalized chocolate, containing 0.28% GABA, proved to be able to lower the level of salivary chromogranin A and cortisol, thus reducing psychological stress (Nakamura et al., 2009).

CONCLUSION

The knowledge on how, when and where bioactive amines are produced by LAB present in fermented food will be helpful by one side to prevent food borne diseases but on the other side also to use food as a nutraceutical delivery system to control mood, appetite and smooth muscle relaxation.

The knowledge about food-encrypted peptides and their potential can offer, in the coming decades, new tools for valuable therapies for the treatment of infectious diseases as well as metabolic, nutritional and psychiatric disorders, cardiovascular diseases, allergies and cancer, as suggested by Lien and Lowman (2003). The increasing availability of mass spectrometry facilities together with homology-based identification of potential bioactive peptide domains on protein sequences allows *in silico* prediction of encrypted peptides and will in the future be essential for targeting the right proteins. Several data banks are available for the bio-informatics analysis of potential bioactive peptides (Iwaniak et al., 2005; Lahrichi et al., 2013), especially antibacterial peptides, due to the urgent issue to face antibiotic resistance (Khaldi, 2012; Mooney et al., 2013). APD2 and CAMP are databases set up to classify not only antibacterial but also antifungal and antiviral peptides (Wang et al., 2009; Thomas et al., 2010). An even larger target is supplied by BIOPEP, Pep Bank, and Peptide DB, also classifying peptide hormones, growth factors and cytokines (Shtatland et al., 2007).

A new term, "nutritional peptidomics" has been proposed by Panchaud et al. (2012) to underline the importance of characterizing (mainly by mass spectrometry) food-encrypted peptides displaying health effects. These approaches allow to set up procedures to liberate the hidden bioactive molecules by means of different proteolytic enzymes (pepsin, trypsin, chymotrypsin) but also to synthesize artificial peptides displaying bioactivity (Zambrowicz et al., 2013). Microbial fermentation is one of the most promising strategies to generate bioactive peptides, hence genomic and proteomic characterization of new strains to predict

their proteolytic profile is a challenging approach in view of obtaining functional food. As far as LAB are concerned, combining the substrate specificity and the cleavage pattern of LAB proteases can highlight the strain potential to release health-promoting molecules (or possible dangerous compounds) in food (Hayes et al., 2007a,b). This is a valuable strategy for selecting the right starters and to enhance the number of LAB strains to be used as food additives or probiotics. Moreover, the use of industrial by-products, such as milk whey, as growth substrates can lower the cost of functional food production and furtherly improve nutraceutical food availability.

AUTHOR CONTRIBUTIONS

EP: Overall organization of the paper and content. SC: Figure and table management and critical reading.

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REFERENCES

Abe, Y., Umemura, S., Sugimoto, K., Hirawa, N., Kato, Y., Yokoyama, N., et al. (1995). Effect of green tea rich in γ-aminobutyric acid on blood pressure of Dahl salt-sensitive rats. *Am. J. Hypertens.* 8, 74–79. doi: 10.1016/0895-7061(94)00141-W

Alexandre, H., Heintz, D., Chassagne, D., Guilloux-Benatier, M., Charpentier, C., and Feuillat, M. (2001). Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. *J. Ind. Microbiol. Biotechnol.* 26, 235–240. doi: 10.1038/sj.jim.7000119

Alvarez, M. A., and Moreno-Arribas, M. V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends Food Sci. Technol.* 39, 146–155. doi: 10.1016/j.tifs.2014.07.007

Applebaum, D., Sabo, D. L., Fischer, E. H., and Morris, D. R. (1975). Biodegradative ornithine decarboxylase of *Escherichia coli*. Purification, properties, and pyridoxal 5'-phosphate binding site. *Biochem* 14, 3675–3681. doi: 10.1021/bi00687a025

Arora, T., Singh, S., and Sharma, R. K. (2013). Probiotics: interaction with gut microbiome and antiobesity potential. *Nutrition* 29, 591–596. doi: 10.1016/j.nut.2012.07.017

Artemova, N. V., Bumagina, Z. M., Kasakov, A. S., Shubin, V. V., and Gurvits, B. Y. (2010). Opioid peptides derived from food proteins suppress aggregation and promote reactivation of partly unfolded stressed proteins. *Peptides* 31, 332–338. doi: 10.1016/j.peptides.2009.11.025

Bumagina, Z. M., Kasakov, A. S., Shubin, V. V., and Gurvits, B. Y. (2009). Opioid peptides derived from food proteins suppress aggregation and promote reactivation of partly unfolded stressed proteins. *Peptides* 31, 332–338. doi: 10.1016/j.peptides.2009.11.025

Atanasova, J., Moncheva, P., and Ivanova, I. (2014). Proteolytic and antimicrobial activity of lactic acid bacteria grown in goat milk. *Biotechnol. Biotechnol. Equip.* 28, 1073–1078. doi: 10.1080/13102818.2014.971487

Aydin, S. (2007). Ghrelin may be a universal peptide in all living organisms. *Turk. J. Med. Sci.* 37, 123–124.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* 1121, 130–136. doi: 10.1016/0167-4838(92)90346-F

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–139. doi: 10.1016/j.cell.2014.03.011

Berrocal, R., Chanton, S., Juillerat, M. A., Favillare, B., Scherz, J. C., and Jost, R. (1989). Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium. *J. Dairy Res.* 56, 335–341. doi: 10.1017/S0022029900028776

Bojsen, A., Buesa, J., Montava, R., Kvistgaard, A. S., Kongsbak, M. B., Petersen, T. E., et al. (2007). Inhibitory activities of bovine macromolecular whey proteins on rotavirus infections in vitro and in vivo. *J. Dairy Sci.* 90, 66–74. doi: 10.3168/jds.S0022-0302(07)72609-7

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3, 238–250. doi: 10.1038/nrmicro1098

Carey, N. (2012). *The Epigenetics Revolution: How Modern Biology is rewriting Our Understanding of Genetics, Disease, and Inheritance*. New York City, NY: Columbia University Press.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., et al. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* 80, 155–165. doi: 10.1016/S0300-9084(98)80022-9

Chang, O. K., Seol, K.-H., Jeong, S.-G., Oh, M.-H., Park, B.-Y., Perrin, C., et al. (2013). Casein hydrolysis by *Bifidobacterium longum* KACC91563 and antioxidant activities of peptides derived therefrom. *J. Dairy Sci.* 96, 5544–5555. doi: 10.3168/jds.2013-6687

Chiba H., and Yoshikawa M. (1986). "Biologically functional peptides from food proteins: new opioid peptides from milk proteins," in *Protein Tailoring for Food and Medical Uses*, eds R. E. Feeney and J. R. Whitaker (New York: Marcel Dekker Inc.), 123–153.

Choi, S. I., Lee, J. W., Park, S. M., Lee, M. Y., Ge, J. Y., Park, M. S., et al. (2006). Improvement of gamma-aminobutyric acid (GABA) production using cell entrapment of *Lactobacillus brevis* GABA 057. *J. Microbiol. Biotechnol.* 16, 562–568.

Civra, A., Giuffrida, M. G., Donalisio, M., Napolitano, L., Takada, Y., Coulson, B. S., et al. (2015). Identification of Equine Lactadherin-derived peptides that inhibit rotavirus infection via integrin receptor competition. *J. Biol. Chem.* 290, 12403–12414. doi: 10.1074/jbc.M114.620500

Clare, D. A., and Swaisgood, H. E. (2000). Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 83, 1187–1195. doi: 10.3168/jds.S0022-0302(00)74983-6

Coda, R., Rizzello, C. G., Pinto, D., and Gobbetti, M. (2012). Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. *Appl. Environ. Microbiol.* 78, 1087–1096. doi: 10.1128/AEM.06837-11

Costantini, A., Pietroniro, R., Doria, F., Pessione, E., and Garcia-Moruno, E. (2013). Putrescine production from different amino acid precursors by lactic acid bacteria from wine and cider. *Int. J. Food Microbiol.* 165, 11–17. doi: 10.1016/j.ijfoodmicro.2013.04.011

Coton, E., Torlois, S., Bertrand, A., and Lonvaud-Funel, A. (1999). Biogenic amines and wine lactic acid bacteria. *Bullettin* 816, 22–34.

Cross, K. J., Huq, N. L., Palamara, J. E., Perich, J. W., and Reynolds, E. C. (2005). Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *J. Biol. Chem.* 280, 15362–15369. doi: 10.1074/jbc.M413504200

Cryan, J. F., and Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior. *Nat. Rev. Neurosci.* 13, 701–712. doi: 10.1038/nrn3346

De LeBlanc, A. M., Matar, C., Thériault, C., and Perdigón, G. (2005). Effects of milk fermented by *Lactobacillus helveticus* R389 on immune cells associated to mammary glands in normal and a breast cancer model. *Immunobiology* 210, 349–358. doi: 10.1016/j.imbio.2005.05.024

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., Vanacore, D., et al. (2008). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *J. Pept. Sci.* 15, 251–258. doi: 10.1002/psc.1093

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., and Roy, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. *Curr. Issues Mol. Biol.* 10, 37–54.

Dhakal, R., Bajpai, V. K., and Baek, K.-H. (2012). Production of gaba (γ - aminobutyric acid) by microorganisms: a review. *Braz. J. Microbiol.* 43, 1230–1241. doi: 10.1590/S1517-83822012000400001

Dimitrov, Z. (2009). Characterization of bioactive peptides with calcium-binding activity released by specially designed cheese starter. *Biotechnol. Biotechnol. Equip.* 23, 927–930. doi: 10.1080/13102818.2009.10818574

Dionysius, D. A., and Milne, J. M. (1997). Antibacterial peptides of bovine lactoferrin: purification and characterization. *J. Dairy Sci.* 80, 667–674. doi: 10.3168/jds.S0022-0302(97)75985-X

Doeven, M. K., Kok, J., and Poolman, B. (2005). Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms. *Mol. Microbiol.* 57, 640–649. doi: 10.1111/j.1365-2958.2005.04698.x

EFSA Panel on Biological Hazards (BIOHAZ) (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA J.* 9:2393. doi: 10.2903/j.efsa.2011.2393

Enrique, M., Manzanares, P., Yuste, M., Martínez, M., Vallés, S., and Marcos, J. F. (2009). Selectivity and antimicrobial action of bovine lactoferrin derived peptides against wine lactic acid bacteria. *Food Microbiol.* 26, 340–346. doi: 10.1016/j.fm.2008.11.003

Faithong, N., Benjakul, S., Phatcharat, S., and Binsan, W. (2010). Chemical composition and antioxidative activity of Thai traditional fermented shrimp and krill products. *Food Chem.* 119, 133–140. doi: 10.1016/j.foodchem.2009.06.056

Fanciulli, G., Dettori, A., Demontis, M. P., Tomasi, P. A., Anania, V., and Delitala, G. (2005). Gluten exorphin B5 stimulates prolactin secretion through opioid receptors located outside the blood-brain barrier. *Life Sci.* 76, 1713–1719. doi: 10.1016/j.lfs.2004.09.023

Ferraretto, A., Gravaghi, C., Fiorilli, A., and Tettamanti, G. (2003). Casein-derived bioactive phosphopeptides: role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells. *FEBS Lett.* 551, 92–98. doi: 10.1016/S0014-5793(03)00741-5

Fiat, A.-M., and Jollès, P. (1989). Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. *Mol. Cell. Biochem.* 87, 5–30. doi: 10.1007/BF00421079

FitzGerald, R. J. (1998). Potential uses of caseinophosphopeptides. *Int. Dairy J.* 8, 451–457. doi: 10.1016/s0958-6946(98)00068-5

FitzGerald, R. J., and Murray, B. A. (2006). Bioactive peptides and lactic fermentations. *Int. J. Dairy Technol* 59, 118–125. doi: 10.1111/j.1471-0307.2006.00250.x

Fukudome, S., and Yoshikawa, M. (1992). Opioid peptides derived from wheat gluten: their isolation and characterization. *FEBS Lett.* 296, 107–111. doi: 10.1016/0014-5793(92)80414-C

Gagnaire, V., Pierre, A., Molle, D., and Leonil, J. (1996). Phosphopeptides interacting with colloidal calcium phosphate isolated by tryptic hydrolysis of bovine casein micelles. *J. Dairy Res.* 63, 405–422. doi: 10.1017/S0022029900031927

Galano, E., Mangiapane, E., Bianga, J., Palmese, A., Pessione, E., Szpunar, J., et al. (2013). Privileged incorporation of selenium as selenocysteine in *Lactobacillus reuteri* proteins demonstrated by selenium-specific imaging and proteomics. *Mol. Cell. Proteomics* 12, 2196–2204. doi: 10.1074/mcp.M113.027607

Galvez, A. F., and de Lumen, B. O. (1999). A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. *Nat. Biotechnol.* 17, 495–500. doi: 10.1038/8676

Gerez, C. L., Cuezzo, S., Rollán, G., and Font de Valdez, G. (2008). Lactobacillus reuteri CRL 1100 as starter culture for wheat dough fermentation. *Food Microbiol.* 25, 253–259. doi: 10.1016/j.fm.2007.10.011

Gobbetti, M., Minervini, F., and Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *Int. J. Dairy Technol.* 69, 5297–5305. doi: 10.1111/j.1471-0307.2004.00139.x

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., and Di Cagno, R. (2002). Latent Bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Food Sci. Nutr.* 42, 223–239. doi: 10.1080/10408690290825538

Goodyear, L. J., and Kahn, B. B. (1998). Exercise, glucose transport, and insulin sensitivity. *Annu. Rev. Med.* 49, 235–261. doi: 10.1146/annurev.med.49.1.235

Griffiths, M. W., and Tellez, A. M. (2013). *Lactobacillus helvictus*: the proteolytic system. *Front. Microbiol.* 4:30. doi: 10.3389/fmicb.2013.00030

Gromadzińska, J., Reszka, E., Bruzelius, K., Wasowicz, W., and Akesson, B. (2008). Selenium and cancer: biomarkers of selenium status and molecular action of selenium supplements. *Eur. J. Nutr.* 47, 29–50. doi: 10.1007/s00394-008-2005-z

Guo, H., Kouzuma, Y., and Yonekura, M. (2009). Structures and properties of antioxidative peptides derived from royal jelly protein. *Food Chem.* 113, 238–245. doi: 10.1016/j.foodchem.2008.06.081

Hartmann, R., and Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Curr. Opin. Biotechnol.* 18, 163–169. doi: 10.1016/j.copbio.2007.01.013

Hatzoglou, A., Bakogeorgou, E., and Castanas, E. (1996a). The antiproliferative effect of opioid receptor agonists on the T47D human breast cancer cell line, is partially mediated through opioid receptors. *Eur. J. Pharmacol.* 296, 199–207. doi: 10.1016/0014-2999(95)00703-2

Hatzoglou, A., Bakogeorgou, E., Hatzoglou, C., Martin, P. M., and Castanas, E. (1996b). Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line. *Eur. J. Pharmacol.* 310, 217–223. doi: 10.1016/0014-2999(96)00339-1

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., and Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. *Appl. Environ. Microbiol.* 72, 2260–2264. doi: 10.1128/AEM.72.3.2260-2264.2006

Hayes, M., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2007a). Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview. *Biotechnol. J.* 2, 426–434.

Hayes, M., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2007b). Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions. *Biotechnol. J.* 2, 435–449. doi: 10.1002/biot.200600246

Hébert, E. M., Mamone, G., Picariello, G., Raya, R. R., Savoy, G., Ferranti, P., et al. (2008). Characterization of the pattern of αs1- and β-casein breakdown and release of a bioactive peptide by a cell envelope proteinase from *Lactobacillus delbrueckii* subsp. lactis CRL 581. *Appl. Environ. Microbiol.* 74, 3682–3689. doi: 10.1128/AEM.00247-08

Hébert, E. M., Raya, R. R., and de Giori, G. S. (1999). Characterisation of a cell-envelope proteinase from *Lactobacillus helveticus*. *Biotechnol. Lett.* 21, 831–834.

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., and Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-Lactalbumin and β-Lactoglobulin. Identification of Active Peptides by HPLC-MS/MS. *J. Agric. Food Chem.* 53, 588–593. doi: 10.1021/jf048626m

Hernández-Ledesma, B., Recio, I., and Amigo, L. (2008). β-Lactoglobulin as source of bioactive peptides. *Amino Acids* 35, 257–265. doi: 10.1007/s00726-007-0585-1

Hirata, H., Sonoda, S., Agui, S., Yoshida, M., Ohinata, K., and Yoshikawa, M. (2007). Rubiscolin-6, a δ opioid peptide derived from spinach Rubisco, has anxiolytic effect via activating σ1 and dopamine D1 receptors. *Peptides* 28, 1998–2003. doi: 10.1016/j.peptides.2007.07.024

Hongbing, F., Xiaochang, L., Hui, H., Song, S., Qian, X., Ligeng, F., et al. (2016). Quality changes and biogenic amines accumulation of black carp (*Mylopharyngodon piceus*) fillets stored at different temperatures. *J. Food Prot.* 79, 524–586. doi: 10.4315/0362-028X.JFP-15-373

Hori, G., Wang, M.-F., Chan, Y.-C., Komtatsu, T., Wong, Y., Chenc, T.-H., et al. (2001). Soy Protein hydrolyzate with bound phospholipids reduces serum cholesterol levels in hypercholesterolemic adult male volunteers. *Biosci. Biotechnol. Biochem.* 65, 72–78. doi: 10.1271/bbb.65.72

Huang, J., Mei, L., Wu, H., and Lin, D. (2007). Biosynthesis of γ-aminobutyric acid (GABA) using immobilized whole cells of Lactobacillus brevis. *J. Microbiol. Biotechnol.* 23, 865–871. doi: 10.1007/s11274-006-9311-5

Huang, Z., Rose, A. H., and Hoffmann, P. R. (2012). The role of selenium in inflammation and immunity: from molecular mechanisms to therapeutic opportunities. *Antioxid. Redox. Signal.* 1 16, 705–743. doi: 10.1089/ars.2011.4145

Hyland, N. P., and Cryan, J. F. (2010). A gut feeling about GABA: focus on GABAB receptors. *Front. Pharmacol.* 1:124. doi: 10.3389/fphar.2010.00124

Iwaniak, A., Dziuba, J., and Niklewicz, M. (2005). The BIOPEP database - a tool for the in silico method of classification of food proteins as the source

of peptides with antihypertensive activity. *Acta Aliment.* 34, 417–425. doi: 10.1556/AAlim.34.2005.4.9

Jensen, M. P., Vogensen, F. K., and Ardö, Y. (2009). Variation in caseinolytic properties of six cheese related *Lactobacillus helveticus* strains. *Int. Dairy J.* 19, 661–668. doi: 10.1016/j.idairyj.2009.04.001

Jiao, Z., Dong, Y., and Chen, Q. (2014). Ethyl carbamate in fermented beverages: presence, analytical chemistry, formation mechanism, and mitigation proposals. *Compr. Rev. Food Sci. Food Saf.* 13, 611–626. doi: 10.1111/1541-4337.12084

Kaneko, K., Iwasaki, M., Yoshikawa, M., and Ohinata, K. (2010). Orally administered soymorphins, soy-derived opioid peptides, suppress feeding and intestinal transit via gut μ1-receptor coupled to 5-HT1A, D2, and GABAB systems. *Am. J. Physiol.* 299, G799–G805. doi: 10.1152/ajpgi.00081.2010

Karovičová, J., and Kohajdová, Z. (2005). Biogenic amines in food. *Chem. Pap.* 59, 70–79.

Kayser, H., and Meisel, H. (1996). Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Lett.* 383, 18–20. doi: 10.1016/0014-5793(96)00207-4

Khaldi, N. (2012). Bioinformatics approaches for identifying new therapeutic bioactive peptides in food. *Funct. Foods Health Dis.* 2:10.

Kitts, D. D., and Weiler, K. (2003). Bioactive proteins and peptides from food sources. applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* 9, 1309–1323. doi: 10.2174/1381612033454883

Klaenhammer, T. R., Barrangou, R., Logan Buck, B., Azcarate-Peril, M. A., and Altermann, E. (2005). Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol. Rev.* 29, 393–409. doi: 10.1016/j.fmrre.2005.04.007

Korhonen, H. (2009). Milk-derived bioactive peptides: from science to applications. *J. Funct. Foods* 1, 177–187. doi: 10.1016/j.jff.2009.01.007

Korhonen, H., and Pihlanto, A. (2003). A. food-derived bioactive peptides - opportunities for designing future foods. *Curr. Pharm. Des.* 9, 1297–1308. doi: 10.2174/1381612033454892

Korhonen, H., and Pihlanto, A. (2006). A. Bioactive peptides: production and functionality. *Int. Dairy J.* 16, 945–960. doi: 10.1016/j.idairyj.2005.10.012

Kunji, E. R. S., Mierau, I., Hagting, A., Poolman, B., and Konings, W. N. (1996). The proteotytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek* 70, 187–221. doi: 10.1007/BF00395933

Kütt, M.-L., Malbe, M., and Stagsted, J. (2011). Nanostructure-assisted laser desorption/ionization (NALDI) for analysis of peptides in milk and colostrum. *Agron. Res.* 9, 415–420.

Ladero, V., Calles-Enriquez, M., Fernandez, M., and Alvarez, M. A. (2010). Toxicological effects of dietary biogenic amines. *Curr. Nutr. Food Sci.* 6, 145–156. doi: 10.2174/157340110791233256

Lafarga, T., Rai, D. K., O'Connor, P., and Hayes, M. (2015). A bovine fibrinogen-enriched fraction as a source of peptides with in vitro renin and angiotensin-i-converting enzyme inhibitory activities. *J. Agric. Food Chem.* 63, 8676–8684. doi: 10.1021/acs.jafc.5b03167

Laffineur, E., Genetet, N., and Leonil, J. (1996). Immunomodulatory activity of β-Casein permeate medium fermented by lactic acid bacteria. *J. Dairy Sci.* 79, 2112–2120. doi: 10.3168/jds.S0022-0302(96)76585-2

Lahrichi, S. L., Affolter, M., Zolezzi, I. S., and Panchaud, A. (2013). Food peptidomics: large scale analysis of small bioactive peptides — A pilot study. *J. Proteomics* 88, 83–91. doi: 10.1016/j.jprot.2013.02.018

Lamberti, C., Purrotti, M., Mazzoli, R., Fattori, P., Barello, C., Coïsson, J. D., et al. (2011). ADI pathway and histidine decarboxylation are reciprocally regulated in *Lactobacillus hilgardii* ISE 5211: proteomic evidence. *Amino Acids* 41, 517–527. doi: 10.1007/s00726-010-0781-2

Landete, J. M., Pardo, I., and Ferrer, S. (2008). Regulation of hdc expression and HDC activity by enological factors in lactic acid bacteria. *J. Appl. Microbial.* 105, 1544–1551. doi: 10.1111/j.1365-2672.2008.03865.x

LeBlanc, J., Fliss, I., and Matar, C. (2004). Induction of a humoral immune response following an *Escherichia coli* O157:H7 Infection with an immunomodulatory peptidic fraction derived from lactobacillus helveticus-fermented milk. *Clin. Vaccine Immunol.* 11, 1171–1181. doi: 10.1128/CDLI.11.6.1171-1181.2004

Lehtonen, P. (1996). Determination of amines and amino acids in wine - A review. *Am. J. Enol. Vitic.* 47, 127–133.

Li, S., Zhao, Y., Zhang, L., Zhang, X., Huang, L., Li, D., et al. (2012). Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. *Food Chem.* 135, 1914–1919. doi: 10.1016/j.foodchem.2012.06.048

Li, Y., Liu, T.-J., and He, G.-Q. (2015). Antioxidant activity of peptides from fermented milk with mix culture of lactic acid bacteria and yeast. *Adv. J. Food Sci. Technol.* 7, 422–427.

Li, Z., Jiang, A., Yue, T., Wang, J., Wang, Y., and Su, J. (2013). Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. *J. Dairy Sci.* 96, 4242–4251. doi: 10.3168/jds.2012-6511

Lien, S., and Lowman, H. B. (2003). Therapeutic peptides. *Trends Biotechnol.* 21, 556–562. doi: 10.1016/j.tibtech.2003.10.005

Linares, D. M., del Rio, B., Redruello, B., Ladero, V., Martin, M. C., Fernandez, M., et al. (2016). Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine. *Food Chem.* 197, 658–663. doi: 10.1016/j.foodchem.2015.11.013

Lohner, K., and Blondelle, S. E. (2005). Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics. *Comb. Chem. High Throughput Screen.* 8, 241–256. doi: 10.2174/1386207053764576

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie Van Leeuwenhoek* 76, 317–331.

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiol. Lett.* 199, 9–13. doi: 10.1111/j.1574-6968.2001.tb10643.x

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., and Klee, W. A. (1983). Opioid activities and structures of alpha-casein-derived exorphins. *Biochemistry* 22, 4567–4573. doi: 10.1021/bi00288a034

Luo, H., Chen, S., Ren, F., Guo, H., Lin, S., and Xu, W. (2007). In vitro reconstitution of antimicrobial pathogen activity by expressed recombinant bovine lactoferrin N-terminal peptide in *Escherichia coli*. *J. Dairy Res.* 74, 233–238. doi: 10.1017/S0022029907002531

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design, and use of probiotics. *Bioessays* 33, 574–581. doi: 10.1002/bies.201100024

Malaguti, M., Dinelli, G., Leoncini, E., Bregola, V., Bosi, S., Cicero, A. F. G., et al. (2014). Bioactive peptides in cereals and legumes: agronomical, biochemical and clinical aspects. *Int. J. Mol. Sci.* 15, 21120–21135. doi: 10.3390/ijms151121120

Malkoski, M., Dashper, S. G., O'Brien-Simpson, N. M., Talbo, G. H., Macris, M., Cross, K. J., et al. (2001). Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrob. Agents Chemother.* 45, 2309–2315. doi: 10.1128/AAC.45.8.2309-2315.2001

Mangani, S., Guerrini, S., Granchi, L., and Vincenzini, M. (2005). Putrescine accumulation in wine: role of *Oenococcus oeni*. *Curr. Microbiol.* 51, 6–10. doi: 10.1007/s00284-004-4425-1

Mangiapane, E., Lamberti, C., Pessione, A., Galano, E., Amoresano, A., and Pessione, E. (2014a). Selenium effects on the metabolism of a Se-metabolizing *Lactobacillus reuteri*: analysis of envelope-enriched and extracellular proteomes. *Mol. Biosyst.* 10, 1272–1280. doi: 10.1039/c3mb70557a

Mangiapane, E., Pessione, A., and Pessione, E. (2014b). Selenium and selenoproteins: an overview on different biological systems. *Curr. Protein Pept. Sci.* 15, 598–607. doi: 10.2174/1389203715666140608151134

Manzotti, G., Breda, D., Di Gioacchino, M., and Burastero, S. E. (2016). Serum diamine oxidase activity in patients with histamine intolerance. *Int. J. Immunopathol. Pharmacol.* 29, 105–111. doi: 10.1177/0394632015617170

Mao, X.-Y., Cheng, X., Wang, X., and Wu, S.-J. (2011). Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chem.* 126, 484–490. doi: 10.1016/j.foodchem.2010.11.025

Marcobal, A., De las Rivas, B., and Muñoz, R. (2006). First genetic characterization of a bacterial β-phenylethylamine biosynthetic enzyme in *Enterococcus faecium* RM58. *FEMS Microbiol. Lett.* 258, 144–149. doi: 10.1111/j.1574-6968.2006.00206.x

Matar, C., Carlos Valdez, J., Medina, M., Rachid, M., and Perdigon, G. (2001). Immunomodulating effects of milks fermented by *Lactobacillus*

helveticus and its non-proteolytic variant. *J. Dairy Res.* 68, 601–609. doi: 10.1017/S0022029901005143

Mazzoli, R., Lamberti, C., Coisson, J. D., Purrotti, M., Arlorio, M., Giuffrida, M. G., et al. (2009). Influence of ethanol, malate and arginine on histamine production of *Lactobacillus hilgardii* isolated from an Italian red wine. *Amino Acids* 36, 81–89. doi: 10.1007/s00726-008-0035-8

Mazzoli, R., Pessione, E., Dufour, M., Laroute, V., Giuffrida, M. G., Giunta, C., et al. (2010). Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. *Amino Acids* 39, 727–737. doi: 10.1007/s00726-010-0507-5

McCann, K. B., Shiellb, B. J., Michalskib, W. P., Leec, A., Wanc, J., Roginskia, H., et al. (2006). Isolation and characterization of a novel antibacterial peptide from bovine αS1-casein. *Int. Dairy J.* 16, 316–323. doi: 10.1016/j.idairyj.2005.05.005

Medina, M. A., Urdiales, J. L., Rodríguez-Caso, C., Ramírez, F. J., and Sánchez-Jiménez, F. (2003). Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications. *Crit. Rev. Biochem. Mol. Biol.* 38, 23–59. doi: 10.1080/713609209

Meisel, H., and Bockelmann, W. (1999). Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* 76, 207–215. doi: 10.1023/A:1002063805780

Meisel, H., and FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* 9, 1289–1295. doi: 10.2174/1381612033454847

Meisel, H., and Olieman, C. (1998). Estimation of calcium-binding constants of casein phosphopeptides by capillary zone electrophoresis. *Anal. Chim. Acta* 372, 291–297. doi: 10.1016/S0003-2670(98)00335-3

Mercier, A., Gauthier, S. F., and Fliss, I. (2004). Immunomodulating effects of whey proteins and their enzymatic digests. *Int. Dairy J.* 14, 175–183. doi: 10.1016/j.idairyj.2003.08.003

Migliore-Samour, D., Floc'ha, F., and Jollès, P. (1989). Biologically active casein peptides implicated in immunomodulation. *J. Dairy Res.* 56, 357–362. doi: 10.1017/S0022029900028806

Millichap, J. G., and Yee, M. M. (2003). The diet factor in pediatric and adolescent migraine. *Pediatr. Neurol.* 28, 9–15. doi: 10.1016/S0887-8994(02)00466-6

Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., and Gobbetti, M. (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. *Appl. Environ. Microbiol.* 69, 5297–5305. doi: 10.1128/AEM.69.9.5297-5305.2003

Minkiewicz, P., Dziuba, J., and Michalska, J. (2011). Bovine meat proteins as potential precursors of biologically active peptides - a computational study based on the BIOPEP database. *Food Sci. Technol. Res.* 1, 39–45. doi: 10.1177/1082013210368461

Miquel, E., and Farré, R. (2007). Effects and future trends of casein phosphopeptides on zinc bioavailability. *Trends Food Sci. Technol.* 18, 139–143. doi: 10.1016/j.tifs.2006.11.004

Mizushige, T., Sawashi, Y., Yamada, A., Kanamoto, R., and Ohinata, K. (2013). Characterization of Tyr-Leu-Gly, a novel anxiolytic-like peptide released from bovine αS-casein. *FASEB J.* 27, 2911–2917. doi: 10.1096/fj.12-225474

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., and Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *Eur. J. Nutr.* 47, 171–182. doi: 10.1007/s00394-008-0710-2

Mooney, C., Haslam, N. J., Holton, T. A., Pollastri, G., and Shields, D. C. (2013). Peptide Locator: prediction of bioactive peptides in protein sequences. *Bioinformatics* 29, 1120–1126. doi: 10.1093/bioinformatics/btt103

Moreno-Arribas, M. V., Polo, M. C., Jourganes, F., and Munoz, R. (2003). Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* 84, 117–123. doi: 10.1016/S0168-1605(02)00391-4

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., and Lonvaud Funel, A. (2000). Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. *J. Appl. Microbiol.* 88, 584–593. doi: 10.1046/j.1365-2672.2000.00997.x

Morifuji, M., Kanda, A., Koga, J., Kawanaka, K., and Higuchi, M. (2010). Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats. *Amino Acids* 38, 1109–1115. doi: 10.1007/s00726-009-0321-0

Mullur, R., Liu, Y.-Y., and Brent, G. A. (2014). Thyroid hormone regulation of metabolism. *Physiol. Rev.* 94, 355–382. doi: 10.1152/physrev.00030.2013

Muro-Urista, C., Alvarez-Fernandez, R., Riera-Rodriguez, F., Arana Cuenca, A., and Tellez Jurado, A. (2011). Production and functionality of active peptides from milk. *Food Sci. Technol. Int.* 17, 293–317. doi: 10.1177/1082013211398801

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., et al. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochem. Biophys. Res. Commun.* 281, 11–17. doi: 10.1006/bbrc.2001.4298

Nagaoka, S., Masaoka, M., Zhang, Q., Hasegawa, M., and Watanabe, K. (2002). Egg ovomucin attenuates hypercholesterolemia in rats and inhibits cholesterol absorption in Caco-2 cells. *Lipids* 37, 267–272. doi: 10.1007/s11745-002-0890-6

Nakamura, H., Takishima, T., Kometani, T., and Yokogoshi, H. (2009). Psychological stress-reducing effect of chocolate enriched with γ-aminobutyric acid (GABA) in humans: assessment of stress using heart rate variability and salivary chromogranin A. *Int. J. Food Sci. Nutr.* 60, 106–113. doi: 10.1080/09637480802558508

Nomura, M., Kobayashi, M., and Ohmomo, T. (2000). Inactivation of the glutamate decarboxylase gene in *Lactococcus lactis* subsp. Cremoris. *Appl. Environ. Microbiol.* 66, 2235–2237. doi: 10.1128/AEM.66.5.2235-2237.2000

Nomura, M., Nakajima, I., Fujita, Y., Kobayashi, M., Kimoto, H., Suzuki, I., et al. (1999). *Lactococcus lactis* contains only one glutamate decarboxylase gene. *Microbiology* 145, 1375–1380. doi: 10.1099/13500872-145-6-1375

O'Flaerty, S. (2014). "Probiotic-mediated modulation of host gene expression," in *Interactive Probiotics,* ed. E. Pessione (Boca Raton, FL: CRC Press).

Oh, S. H. (2003). Stimulation of γ-aminobutyric acid synthesis activity in brown rice by a chitosan/glutamic acid germination solution and calcium/calmodulin. *BMB Rep.* 36, 319–325. doi: 10.5483/BMBRep.2003.36.3.319

Panchaud, A., Affolter, M., and Kussman, M. (2012). Mass spectrometry for nutritional peptidomics: how to analyze food bioactives and their health effects. *J. Proteomics* 75, 3546–3559. doi: 10.1016/j.jprot.2011.12.022

Pessione, A., Lamberti, C., and Pessione, E. (2010). Proteomics as a tool for studying energy metabolism in lactic acid bacteria. *Mol. Biosyst.* 8, 1419–1430. doi: 10.1039/C001948H

Pessione, E. (2010). "Biochemical aspects of nutrition: probiotics," in *Biochemical Aspects in Human Nutrition*, eds L. Avigliano and L. Rossi (Kerala: Transworld research network).

Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front. Cell Infect. Microbiol.* 2:86. doi: 10.3389/fcimb.2012.00086

Pessione, E., Mazzoli, R., Giuffrida, M. G., Lamberti, C., Garcia-Moruno, E., Barello, C., et al. (2005). A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics* 5, 687–689. doi: 10.1002/pmic.200401116

Pessione, E., Pessione, A., Lamberti, C., Coïsson, J. D., Riedel, K., Mazzoli, R., et al. (2009). First evidence of a membrane-bound, tyramine and beta-phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a two-dimensional electrophoresis proteomic study. *Proteomics* 9, 2695–2710. doi: 10.1002/pmic.200800780

Pfluger, P. T., Schriever, S. C., and Tschöp, M. H. (2012). Nutropioids, hedonism in the gut? *Cell Metab.* 16, 137–139. doi: 10.1016/j.cmet.2012.07.011

Piovesana, S., Capriotti, A. L., Cavaliere, C., La Barbera, G., Samperi, R., Zenezini Chiozzi, R., et al. (2015). Peptidome characterization and bioactivity analysis of donkey milk. *J. Proteomics* 119, 21–29. doi: 10.1016/j.jprot.2015.01.020

Porto, B., Mangiapane, E., Pessione, A., Neves, M. J., and Pessione, E. (2015). Martins FS The effects of sodium selenite on the probiotic *Saccharomyces cerevisiae* UFMG A-905: a physiological and proteomic analysis. *J. Funct. Food* 17, 828–836. doi: 10.1016/j.jff.2015.06.048

Power, O., Jakeman, P., and FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids* 44, 797–820. doi: 10.1007/s00726-012-1393-9

Prioult, G., Pecquet, S., and Fliss, I. (2004). Allergenicity of acidic peptides from bovine β-lactoglobulin is reduced by hydrolysis with *Bifidobacterium lactis* NCC362 enzymes. *Int. Dairy J.* 15, 439–448. doi: 10.1016/j.idairyj.2004.09.001

Qian, Z.-Y., Jollès, P., Migliore-Samour, D., Schoentgen, F., and Fiat, A.-M. (1995). Sheep κ-casein peptides inhibit platelet aggregation. *Biochim. Biophys. Acta* 1244, 411–417. doi: 10.1016/0304-4165(95)00047-F

Rajapakse, N., Mendis, E., Jung, W.-K., Je, J.-Y., and Kim, S.-K. (2005a). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.* 38, 175–182. doi: 10.1016/j.foodres.2004.10.002

Rajapakse, N., Mendisa, E., Byunb, H.-G., and Kima, S.-K. (2005b). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutri. Biochem.* 16, 562–569. doi: 10.1016/j.jnutbio.2005.02.005

Rao, S., Sun, J., Liu, Y., Zeng, H., Su, Y., and Yang, Y. (2012). ACE inhibitory peptides and antioxidant peptides derived from in vitro digestion hydrolysate of hen egg white lysozyme. *Food Chem.* 135, 1245–1252. doi: 10.1016/j.foodchem.2012.05.059

Recsei, P. A., and Snell, E. E. (1985). Pyruvoyl-dependent histidine decarboxylases. Mechanism of cleavage of the proenzyme from *Lactobacillus buchneri*. *J. Biol. Chem.* 260, 2804–2806.

Reynolds, E. C., Cai, N. J. F., Cochrane, P., Shen, G. D., Walker, M. V., and Morgan, C. (2008). Fluoride and casein phosphopeptide-amorphous calcium phosphate. *J. Dent. Res.* 87, 344–348. doi: 10.1177/154405910808700420

Ricci-Cabello, I., Herrera, M. O., and Artacho, R. (2012). Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. *Nutr. Rev.* 70, 241–255. doi: 10.1111/j.1753-4887.2011.00448.x

Rizzello, C. G., Cassone, A., Di Cagno, R., and Gobbetti, M. (2008). Synthesis of angiotensin i-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. *J. Agric. Food Chem.* 56, 6936–6943. doi: 10.1021/jf800512u

Roy, M. K., Kuwabara, Y., Hara, K., Watanabe, Y., and Tamai, Y. (2002). Peptides From the N-terminal End of Bovine Lactoferrin Induce Apoptosis in Human Leukemic (HL-60) Cells. *J. Dairy Sci.* 85, 852065–852074. doi: 10.3168/jds.S0022-0302(02)74284-7

Roy, M. K., Watanabe, Y., and Tamai, Y. (1999). Induction of apoptosis in HL-60 cells by skimmed milk digested with a proteolytic enzyme from the yeast *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 88, 426–432. doi: 10.1016/S1389-1723(99)80221-7

Saavedra, L., Hébert, E. M., Minahk, C., and Ferranti, P. (2013). An overview of "omic" analytical methods applied in bioactive peptide studies. *Food Res. Int.* 54, 925–934. doi: 10.1016/j.foodres.2013.02.034

Santos, S. M. H. (1996). Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* 29, 213–231. doi: 10.1016/0168-1605(95)00032-1

Saraswat, S., Snyder, B., and Isailovic, D. (2012). Quantification of HPLC-separated peptides and proteins by spectrofluorimetric detection of native fluorescence and mass spectrometry. *J. Chromatogr. B* 902, 70–77. doi: 10.1016/j.jchromb.2012.06.018

Savijoki, K., Ingmer, H., and Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 71, 394–406. doi: 10.1007/s00253-006-0427-1

Sessa, A., Desiderio, M. A., and Perin, A. (1984). Effect of acute ethanol administration on diamine oxidase activity on the upper gastrointestinal tract of rat. *Alcohol. Clin. Exp. Res.* 8, 185–190. doi: 10.1111/j.1530-0277.1984.tb05835.x

Sharma, S., Singh, R., and Rana, S. (2011). Bioactive peptides: a review. *Intc J. BIOautomation* 15, 223–250.

Sharon, G., Garg, N., Debelius, J., Knight, R., Dorrestein, P. C., and Mazmanian, S. K. (2014). Specialized metabolites from the microbiome in health and disease. *Cell Metab.* 20, 719–730. doi: 10.1016/j.cmet.2014.10.016

Sheih, I.-C., Wub, T.-K., and Fang, T. J. (2009). Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. *Bioresour. Technol.* 100, 3419–3425. doi: 10.1016/j.biortech.2009.02.014

Shimazu, S., and Miklya, I. (2004). Pharmacological studies with endogenous enhancer substances: beta phenylethylamine, tryptamine and their synthetic derivatives. *Prog. Neuropsychopharmacol. Biol. Psichiatry* 28, 421–427. doi: 10.1016/j.pnpbp.2003.11.016

Shtatland, T., Guettler, D., Kossodo, M., Pivovarov, M., and Weissleder, R. (2007). PepBank - a database of peptides based on sequence text mining and public peptide data sources. *BMC Bioinf.* 8:280. doi: 10.1186/1471-2105-8-280

Singh, T. K., Fox, P. F., and Healy, A. (1997). Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *J. Dairy Res.* 64, 433–443. doi: 10.1017/S0022029997002227

Singh, V. P., Pathak, V., and Verma, A. K. (2012). Fermented meat products: organoleptic qualities and biogenic amines–a review. *Am. J. Food Technol.* 7, 278–288. doi: 10.3923/ajft.2012.278.288

Smeianov, V. V., Wechter, P., Broadbent, J. R., Hughes, J. E., Rodríguez, B. T., Christensen, T. K., et al. (2007). Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Appl. Environ. Microbiol.* 73, 2661–2672. doi: 10.1128/AEM.00005-07

Somda, M. K., Savadogo, A., Barro, N., Thonart, P., and Traore, A. S. (2011). Effect of mineral salts in fermentation process using mango residues as carbon source for bioethanol production. *Asian J. Ind. Eng.* 3, 29–38. doi: 10.3923/ajie.2011.29.38

Svensson, M., Håkansson, A., Mossberg, A.-K., Linse, S., and Svanborg, C. (2000). Conversion of α-lactalbumin to a protein inducing apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4221–4226. doi: 10.1073/pnas.97.8.4221

Takahashi, M., Fukunaga, H., Kaneto, H., Fukudome, S., and Yoshikawa, M. (2000). Behavioral and pharmacological studies on gluten exorphin a5, a newly isolated bioactive food protein fragment, in mice. *Jpn. J. Pharmacol.* 84, 259–265. doi: 10.1254/jjp.84.259

Tan, D. X., Zheng, X., Kong, J., Manchester, L. C., Hardeland, R., Kim, S. J., et al. (2014). Fundamental issues related to the origin of melatonin and melatonin isomers during evolution: relation to their biological functions. *Int. J. Mol. Sci.* 15, 15858–15890. doi: 10.3390/ijms150915858

Tellez, A., Corrediga, M., Brovkoa, L. Y., and Griffith, M. W. (2010). Characterization of immune-active peptides obtained from milk fermented by *Lactobacillus helveticus*. *J. Dairy Res.* 77, 129–136. doi: 10.1017/S002202990999046X

Tellez, A., Corredig, M., Turner, P. V., Morales, R., and Griffiths, M. (2011). A peptidic fraction from milk fermented with *Lactobacillus helveticus* protects mice against *Salmonella* infection. *Int. Dairy J.* 21, 607–614. doi: 10.1016/j.idairyj.2011.03.011

Teucher, B., Majsak-Newman, G., Dainty, J. R., McDonagh, D., FitzGerald, R., and Fairweather-Tait, S. J. (2006). Calcium absorption is not increased by caseinophosphopeptides1,2,3. *Am. J. Clin. Nutr.* 84, 162–166.

Thakur, N., Qureshi, A., and Kumar, M. (2012). AVPpred: collection and prediction of highly effective antiviral peptides. *Nucleic Acids Res.* 40, W199–W204. doi: 10.1093/nar/gks450

Thomas, S., Karnik, S., Barai, R. S., Jayaraman, V. K., and Idicula-Thomas, S. (2010). CAMP: a useful resource for research on antimicrobial peptides. *Nucleic Acids Res.* 38, D774–D780. doi: 10.1093/nar/gkp1021

Tsai, J. S., Lin, Y. S., Pan, B. S., and Chen, T. J. (2006). Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochem.* 41, 1282–1288. doi: 10.1016/j.procbio.2005.12.026

Ulvatne, H., Samuelsen, Ø., Haukland, H. H., Krämer, M., and Vorland, L. H. (2004). Lactoferricin B inhibits bacterial macromolecular synthesis in *Escherichia coli* and *Bacillus subtilis*. *FEMS Microbiol.* 237, 377–384. doi: 10.1111/j.1574-6968.2004.tb09720.x

van Platerink, C. J., Janssen, H.-G. M., Horsten, R., and Haverkamp, J. (2006). Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography–mass spectrometry. *J. Chromatogr. B* 830, 151–157. doi: 10.1016/j.jchromb.2005.10.036

Virtanen, T., Pihlanto, A., Akkanen, S., and Korhonen, H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *J. Appl. Microbiol.* 102, 106–115. doi: 10.1111/j.1365-2672.2006.03072.x

Visick, K. L., and Fuqua, C. (2005). Decoding microbial chatter: cell-cell communication in bacteria. *J. Bacteriol.* 187, 5507–5519. doi: 10.1128/JB.187.16.5507-5519.2005

Vorland, L. H. (1999). Lactoferrin: a multifunctional glycoprotein. *APMIS* 107, 971–981. doi: 10.1111/j.1699-0463.1999.tb01499.x

Wakai, T., and Yamamoto, N. (2012). "Antihypertensive peptides specific to *Lactobacillus helveticus* fermented milk," in *Biotechnology – Molecular Studies and Novel Applications for Improved Quality of Human Life, Biochemistry, Genetics and Molecular Biology,* ed. R. H. Sammour (Rijeka: InTech), 159–172.

Wang, G., Li, X., and Wang, Z. (2009). APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic. Acids Res.* 37(Suppl. 1), D933–D937. doi: 10.1093/nar/gkn823

Wei, G. S., Jackson, J. L., Hatzigeorgiou, C., and Tofferi, J. K. (2003). Osteoporosis management in the new millennium. *Prim. Care* 30, 711–741. doi: 10.1016/S0095-4543(03)00090-3

Yamamoto, N., Maeno, M., and Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* 82, 1388–1393. doi: 10.3168/jds.S0022-0302(99)75364-6

Yanez, M., Padin, J. F., Arranz-Tagarro, J. A., Camina, M., and Laguna, R. (2012). History and therapeutic use of MAO-A inhibitors: a historical perspective of mao-a inhibitors as antidepressant drug. *Curr. Top. Med. Chem.* 12, 2275–2282. doi: 10.2174/156802612805220011

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., et al. (2001). Rubiscolin, a δ selective opioid peptide derived from plant Rubisco. *FEBS Lett.* 509, 213–217. doi: 10.1016/S0014-5793(01)03042-3

Yang, S., Kawamura, Y., and Yoshikawa, M. (2003). Effect of rubiscolin, a δ opioid peptide derived from Rubisco, on memory consolidation. *Peptides* 24, 325–328. doi: 10.1016/S0196-9781(03)00044-5

Yang, Y., Marczak, E. D., Yokoo, M., Usui, H., and Yoshikawa, M. (2003). Isolation and antihypertensive effect of angiotensin i-converting enzyme (ace) inhibitory peptides from spinach rubisco. *J. Agric. Food Chem.* 51, 4897–4902. doi: 10.1021/jf026186y

Zambrowicz, A., Timmer, M., Polanowski, A., Lubec, G., and Trziszka, T. (2013). Manufacturing of peptides exhibiting biological activity. *Amino Acids* 44, 315–320. doi: 10.1007/s00726-012-1379-7

Zhang, H., Hu, C. A., Kovacs-Nolan, J., and Mine, Y. (2015). Bioactive dietary peptides and amino acids in inflammatory bowel disease. *Amino Acids* 47, 2127–2141. doi: 10.1007/s00726-014-1886-9

Zucht, H.-D., Raida, M., Adermann, K., Mägert, H.-J., and Forssmann, W. G. (1995). Casocidin-I: a casein-αs2 derived peptide exhibits antibacterial activity. *FEBS Lett.* 372, 185–188. doi: 10.1016/0014-5793(95)00974-E

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Identification of novel esterase-active enzymes from hot environments by use of the host bacterium *Thermus thermophilus*

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Functional metagenomic screening strategies, which are independent of known sequence information, can lead to the identification of truly novel genes and enzymes. Since *E. coli* has been used exhaustively for this purpose as a host, it is important to establish alternative expression hosts and to use them for functional metagenomic screening for new enzymes. In this study we show that *Thermus thermophilus* HB27 is an excellent screening host and can be used as an alternative provider of truly novel biocatalysts. In a previous study we constructed mutant strain BL03 with multiple markerless deletions in genes for major extra- and intracellular lipolytic activities. This esterase-diminished strain was no longer able to grow on defined minimal medium supplemented with tributyrin as the sole carbon source and could be used as a host to screen for metagenomic DNA fragments that could complement growth on tributyrin. Several thousand single fosmid clones from thermophilic metagenomic libraries from heated compost and hot spring water samples were subjected to a comparative screening for esterase activity in both *T. thermophilus* strain BL03 and *E. coli* EPI300. We scored a greater number of active esterase clones in the thermophilic bacterium than in the mesophilic *E. coli*. From several thousand functionally screened clones only two thermostable α/β-fold hydrolase enzymes with high amino acid sequence similarity to already characterized enzymes were identifiable in *E. coli*. In contrast, five further fosmids were found that conferred lipolytic activities in *T. thermophilus* only. Four open reading frames (ORFs) were found which did not share significant similarity to known esterase enzymes but contained the conserved GXSXG motif regularly found in lipolytic enzymes. Two of the genes were expressed in both hosts and the novel thermophilic esterases, which based on their primary structures could not be assigned to known esterase or lipase families, were purified and preliminarily characterized. Our work underscores the benefit of using additional screening hosts other than *E. coli* for the identification of novel biocatalysts with industrial relevance.

Keywords: *Thermus thermophiles*, novel screening host, functional metagenomics, comparative screening, novel metagenomic esterases

Introduction

As only a small fraction of all microorganisms from environmental samples can be isolated and studied by classic microbiological methods, culture-independent strategies, such as metagenomics, have been employed to overcome these restrictions (Torsvik et al., 1990; Amann et al., 1995; Handelsman et al., 1998). The cloning and analysis of environmental DNA has led to the identification of various new genes for valuable enzymes (for a recent review see Leis et al., 2013). Sequence-based metagenomics strategies have been successful in many cases, but sequences without similarity to known genes cannot be identified using this approach (Steele et al., 2009; Liebl, 2011; Liebl et al., 2014). Function-based strategies on the other hand rely on the direct detection of functional gene products via diverse screening setups. Until today, *E. coli* is the most commonly used bacterial host in functional metagenomics. Since only a fraction of the genes from random (meta)genomic gene libraries are expressed (Gabor et al., 2004; Sorek et al., 2007), the identification of truly novel genes from metagenomes is hampered when a single host is used (Jenney and Adams, 2008). Other hosts than *E. coli* (e.g., diverse Proteobacteria, Actinobacteria, and eukaryotic fungi) have been shown to be good alternatives and new broad-range expression vectors have been developed for use in functional metagenomics (Courtois et al., 2003; Martinez et al., 2004; Aakvik et al., 2009; Craig et al., 2010; Damon et al., 2011; Kakirde et al., 2011; Kellner et al., 2011; Parachin and Gorwa-Grauslund, 2011; Liebl et al., 2014).

In previous work we have shown that *Thermus thermophilus* HB27 can be used for screening of genomic libraries using the shuttle fosmid pCT3FK. These studies resulted in the identification of several genes that would not have been found by using the mesophilic *E. coli* host alone (Angelov et al., 2009; Leis et al., 2013).

In this study we present the generation of large-insert metagenomic libraries and their subsequent screening for lipolytic activities in *E. coli* and *T. thermophilus* HB27. For the screenings in *T. thermophilus*, a multiple clean deletion mutant was used (termed BL03) which lacks several characterized extracellular and putative esterase encoding genes (Leis et al., 2014). The knockout strain had a substantially lower esterase activity than the wild type and the deletions abolished its ability to grow on SH minimal medium supplemented with tributyrin. Therefore, we decided to use this esterase-deficient strain in a high-throughput screening and selection setup where complementation of growth on tributyrin minimal medium by cloned heterologous metagenomic genes served to select for new environmental genes encoding tributyrin-cleaving esterases.

Materials and Methods

Bacterial Strains and Growth Conditions

Transformation and propagation of recombinant plasmids were performed using *Escherichia coli* XL1-Blue, XL10 GOLD® ultracompetent cells (Stratagene, La Jolla, USA) and DH10B (Invitrogen, Carlsbad, USA). *E. coli* EPI300-T1® (Epicentre, Madison, USA) was used for the generation and screening of metagenomic libraries cloned in pCT3FK large-insert fosmids (Angelov et al., 2009) based on pCC1FOS) upon addition of fosmid autoinduction solution (Epicentre). *E. coli* strain BL21(DE3) was used for the expression of target proteins in pET21a(+) (Novagen, Merck KGaA, Darmstadt, Germany). Bacterial cultures were grown in lysogeny broth (LB) or on LB agar plates supplemented with ampicillin (100 μg/ml), kanamycin (20 μg/ml) and chloramphenicol (12.5 μg/ml) as necessary. For *T. thermophilus* HB27 (DSMZ 7039) and *T. thermophilus* BL03 (genotype ΔTT_P0042, ΔTT_C0340-1, ΔTT_C0904, ΔTT_C1787), culturing and transformations were done as described in Angelov et al. (2009), using TB complex medium (8 g/l trypticase peptone, 4 g/l yeast extract, and 3 g/l NaCl) (Ramírez-Arcos et al., 1998) and SH minimal medium (Leis et al., 2014) with high-carbonate mineral water Aqua Purania (TSI, Zeven, Germany) at pH 7.5. Cells were cultured at 70°C or at 60°C when using kanamycin (final concentration of 20 μg/ml) for the propagation of transformants. For the growth selection and complementation assay, bacterial suspensions from overnight-grown liquid cultures were washed three times in 50 mM phosphate buffer (pH 7.5) and adjusted to equal optical densities before spotting 5 or 10 μl on complex and minimal medium. For screening and selection purposes on solid medium, tributyrin with a final concentration of 1.0% (v/v) was emulsified with an Ultra-Turrax homogenizer (IKA, Staufen, Germany) immediately before autoclaving of the medium.

Generation of a Large-Insert Fosmid Library and Functional Screenings

Water, sediment and biofilms were sampled from hot springs in the town of Furnas Azores, Portugal (37°46′21.78″N 25°18′14.673″W). Compost heap samples were collected from a composting plant (Bioenergiezentrum GmbH, Göttingen, Germany). After separation of microorganisms from the samples' matrices (filtration and scraping off cells from organic matter), the metagenomic DNA was prepared using the PowerSoil, PowerMax Soil and UltraClean Fecal DNA Isolation Kit (MO BIO, Carlsbad, USA). The generation of a large-insert DNA library was done using the pCC1FOS-derived pCT3FK *T. thermophilus*/*E. coli* shuttle fosmid (Angelov et al., 2009) according to the instructions of the pCC1FOS Fosmid Library Production Kit (Epicentre, Madison, USA). Arrayed clones from *E. coli* were cultured in 96-well microtiter plate format. To overcome labor-intensive transfer of single fosmid clones from *E. coli* to *T. thermophilus*, a high-throughput screening strategy was chosen based on growth complementation (Leis et al., 2014). Therefore, each pool of 96 single fosmids from *E. coli* was transferred in the screening strain *T. thermophilus* BL03 by a single transformation reaction. The transformants of each reaction were grown on TB plates containing kanamycin as antibiotic. After 2 days of incubation at 60°C, colonies were suspended from the plates and washed three times using 50 mM phosphate buffer (pH 7.5). The washed cell suspensions (each comprising a pool of 96 *T. thermophilus* transformants obtained by transformation of strain BL03 with pooled fosmids from 96 *E. coli* clones) were spotted onto SH minimal medium containing tributyrin (1% v/v) and kanamycin. Candidate clone pools were able to grow on tributyrin due to heterologous growth complementation after 2–3 days of incubation at 60°C. In order to determine single clone(s) whose fosmid-derived

inserts conferred tributyrase activity within each positive pool, *T. thermophilus* BL03 was transformed individually with each of the 96 *E. coli* fosmids in deep well plate format as described before (Angelov et al., 2009). Suspensions of the transformants were washed several times and spotted on minimal medium containing tributyrin. *T. thermophilus* clones with candidate fosmid inserts grew after several days of incubation at 60°C. The stability of each phenotype was assessed by repeated streaking and growth of the clones on SH with tributyrin.

Each candidate clone was analyzed for its ability to hydrolyze the substrate in comparison to the control containing only the empty fosmid DNA. The corresponding halo formation was calculated as the area of the clearing zone without the area of the corresponding colony size according to the following equation:

$$\text{Halo area formed} = [\text{diameter(halo)}^2 - \text{diameter(colony)}^2] \times 0.25 \times \pi$$

Sequencing and *in silico* Analysis of Sequence Data

Fosmids conferring significant halo formation were sequenced using Roche's GS-FLX series 454 pyrosequencer at the Göttingen Genomics laboratory (G_2L) and LGC Genomics (Berlin, Germany). For phylogenetic analysis, bacterial and archaeal 16S rDNA sequences were amplified by PCR using degenerate primer pairs 616Valt, 100K, AC165, and AC1601, respectively (Supplementary Table S1). Phylogenetic analysis was done using ARB (Ludwig et al., 2004) and the Ribosomal Database Project II (RDP-II) server (Cole et al., 2009). Assembled contig sequences were analyzed *in silico* using SEED (Overbeek et al., 2005). BlastP (Altschul et al., 1990), conserved domain database (CDD, Marchler-Bauer et al., 2011), and the Pfam database (Punta et al., 2012) were used for similarity searches and annotation. The presence of predicted signal peptides was analyzed with the SignalP server (Petersen et al., 2011). Regular expression algorithm was used to unravel potential esterase candidate genes by GXSXG conserved motif searches. For the generation of multiple sequence alignments and similarity tree reconstruction, representative protein sequences from classified esterases/lipases (Chow et al., 2012) were used. Tree calculations based on multiple sequence alignments were performed with the T-REX webserver (Boc et al., 2012) and the resulting Newick-formatted data was visualized with MEGA Version 5.2 (Tamura et al., 2011).

Cloning, Expression, and Purification of Metagenomic Esterases

Candidate genes were PCR amplified with Phusion DNA polymerase (ThermoFisher Scientific, Waltham, USA) using primers listed in Supplementary Table S1 (oligonucleotides EstA2_for and EstA2_rev for cloning EstA2, EstB1_for and EstB1_rev for EstB1, respectively). The PCR products were cloned using a CloneJET vector (Fermentas) backbone. Insertion of the polyhistidine (6xHis) sequence was performed by the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix, Santa Clara, USA) with 5′-phosphorylated oligonucleotides (EstA2_PHO_F and EstB1_PHO_F) according to the manufacturer's instructions. Metagenomic esterase-encoding genes (EstA2 and EstB1) were cloned in pET21a(+) (Novagen) using *Nde*I and *Nco*I, and the same strategy was used for cloning in the *T. thermophilus* vector pMK18 (de Grado et al., 1999) by isothermal DNA assembly using the Gibson Assembly Master Mix (New England Biolabs, Ipswich, USA) with vector primers pMK18_RBS_for and pMK18_RBS_rev. The insert was amplified with Est_rev and corresponding forward primers of the esterases. For expression, *E. coli* BL21(DE3) Star cultures (250 ml) were grown in Erlenmeyer flasks to mid-log phase (absorbance at 600 nm ranging from 0.6 to 0.7) and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1.0 mM. After 4 h at 37°C, the cultures were harvested by centrifugation and disrupted by sonication (Hielscher Ultrasonics GmbH, Teltow, Germany). After removal of the cell debris, the lysate was subjected to heat treatment (at least 60°C for 20 min). The supernatant containing the thermostable and soluble protein of interest was purified using Protino Ni-IDA 2000 protein purification system (Macherey-Nagel) according to the manufacturer's instructions. Protein separation and purity was determined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on Laemmli (1970).

Enzyme Assays

E. coli and *T. thermophilus* cells from 5 ml overnight grown cultures were harvested by centrifugation and washed twice in 50 mM Tris-HCl (pH 7.5). All steps were performed on ice. Cells were resuspended in half volume of Tris buffer and disrupted by sonication until the turbid suspensions became clear. Cell debris was removed by centrifugation (21,000 × g, 10 min at 4°C) and supernatants were used for protein quantification and measurement of intracellular lipolytic activities. The *para*-nitrophenyl (*p*NP) assays were performed according to Leis et al. (2014) by using 0.1 ml of crude lysates incubated in 50 mM Tris-HCl (pH 7.5) buffer with 1.25 mM *p*NP-caprylate, -laurate, and palmitate at 60°C. After stopping the reaction with 0.25 ml of 2 M sodium carbonate and incubation on ice for 15 min, the mixture was centrifuged at 21,000 × g for 5 min at 4°C. Esterase activity measurements for purified enzyme preparations were performed using 1.25 mM final concentrations of different *para*-nitrophenyl-substrates (*p*NP-propionate (C_3), -butyrate (C_4), -valerate (C_5), -caproate (C_6), -caprylate (C_8), caprate (C_{10}), -laurate (C_{12}), -myristate (C_{14}), and -palmitate (C_{16}), all obtained from Sigma-Aldrich). The influence of the additives NaCl, KCl, $CaCl_2$, $MgCl_2$ (salts), and inhibitor EDTA (chelating agent) and phenylmethylsulfonylfluorid (PMSF), an agent known to inhibit serine/cysteine proteases as well as acetylcholinesterases, was tested at final concentrations of 1 mM. Other *p*NP-substrates were derived by esterification with 2-methyldecanoate (Reetz et al., 1997; Franken et al., 2010; Wu et al., 2013), Ibuprofen, Naproxen (Reetz et al., 2010; Sandström et al., 2012), 6-methyl-2-(*ortho*-tolyl)hept-5-enoate (Gaich and Mulzer, 2009), and indancarboxylic acid (Pietruszka et al., 2009) and the activity assays were performed at 60, 70, and 80°C under the conditions reported by López et al. (2014) and Chow et al. (2012).

One unit of specific enzymatic activity was defined as 1.0 μmol of *para*-nitrophenol per minute and mg protein released from the

substrate. All measurements were performed at least in duplicates ($n = 2$).

Synthesis of Assay Substrates—Representative Procedure

Synthesis of 4-nitrophenyl 6-methyl-2-(*ortho*-tolyl)hept-5-enoate: 4-Nitrophenol (538 mg, 3.87 mmol), 4-dimethylaminopyridine (47.3 mg, 387 μmol), and dicyclohexylcarbodiimide (798 mg, 3.87 mmol) were added to a solution of 6-methyl-2-(*ortho*-tolyl)hept-5-enoic acid (900 mg, 3.87 mmol) in 25 mL of dichloromethane under nitrogen. The mixture was stirred at room temperature. After 24 h the precipitate was filtered off and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (petroleum ether/ethyl acetate, 99/1) to give the product as a yellow oil (1.07 g, 3.03 mmol, 78%). ^{1}H-NMR (600 MHz, $CDCl_3$,): δ_H [ppm] = 1.55 (s, 3 H, 1-H), 1.71 (s, 3 H, 2-H), 1.88 [dddd, $^2J_{(6a,6b)}$=13.7 Hz, $^3J_{(6a,5b)}$ =7.2 Hz, $^3J_{(6a,5a)}$ = 7.2 Hz, $^3J_{(6a,7)}$ = 6.9 Hz, 1 H, 6-H_a], 2.07 [dd,$^3J_{(5,6)}$ = 7.2 Hz, $^3J_{(5,4)}$ = 7.2 Hz, 2 H, 5-H], 2.26 [dddd, $^3J_{(6b,7)}$ = 6.7 Hz, $^3J_{(6b,5a)}$ = 7.2 Hz, $^3J_{(6b,5b)}$ = 7.2 Hz, $^2J_{(6b,6a)}$ = 13.6 Hz, 1 H, 6-H_b], 2.44 (s, 3 H, arom.-CH_3), 4.12 [dd, $^3J_{(7,6a)}$ = 6.8 Hz, $^3J_{(7,6b)}$ = 6.7 Hz, 1 H, 7-H], 5.14 [t, $^3J_{(4,5)}$ = 7.2 Hz, 1 H, 4-H], 7.15- (m_c, 2 H, arom.-C*H*), 7.18–7.25 (m, 3 H, arom.-C*H*), 7.35 (m_c, 1 H, arom.-C*H*), 8.22 (m_c, 2 H, arom.-C*H*). ^{13}C-NMR (151 MHz, $CDCl_3$): δ_C [ppm] = 17.7 (C-1), 19.8 (arom.-CCH_3), 25.7 (C-5), 25.8 (C-2), 32.8 (C-6), 46.3 (C-7), 122.3 (arom.-*C*H), 122.3 (arom.-*C*H), 123.1 (C-4), 125.1 (arom.-*C*H), 125.1 (arom.-*C*H), 126.6 (arom.-*C*H), 127.5 (arom.-*C*H), 130.8 (arom.-*C*H), 130.8 (arom.-*C*H), 133.2 (C-3), 136.6 (arom.-C_{ipso}), 136.6 (arom.-C_{ipso}), 145.3 (*C*O*p*NP), 155.6 (arom.-C_{ipso}), 171.9 (arom.-*C*NO_2). HR-MS (ESI, cation): calculated [$C_{21}H_{23}NO_4$+Na^+]: *m/z* = 376.15193; found [$C_{21}H_{23}NO_4$+Na^+]: *m/z* = 376.15211.

Results

Generation of Fosmid Libraries and Phylogenetic Analysis of Hot Spring and Compost Samples

Environmental samples were obtained from strong acidic and neutral hot spring water sediments and biofilms (pH ranging between 2.0 and 7.0, temperature from 60 to 62°C) from the São Miguel island, Azores, Portugal. In addition, a naturally heated lumber waste compost heap (63.3°C after 4 days) sample was obtained from a compost facility in Göttingen (Germany) (**Table 1**). High molecular weight DNA from both samples could be obtained and enabled the generation of a large-insert metagenomic library in the pCT3FK shuttle fosmid. The library comprised around 6048 single fosmid clones for the Azores samples and 1920 clones for the compost sample with each clone carrying approximately 35–40 kbp metagenomic inserts (**Table 1**). Phylogenetic analysis of the metagenomic DNA inserts

TABLE 1 | Overview of the constructed metagenomic libraries and the esterase screening results in *E. coli* EPI300 and *T. thermophilus* BL03.

Origin of metagenomic DNA	Sample information	Number of fosmid clones	Number of lipolytic clones (60°C)	
			E. coli EPI300	*T. thermophilus* BL03
Hot springs (Azores Islands, Portugal)	AZ2 sediment, 60°C pH 6.0	384	1	1
	AZ3 sediment, 62°C, pH 2.0	5472	0	4
	AZ4 biofilms, 60°C, pH 7.0	192	0	0
Compost from lumber waste (Göttingen, Germany)	M12, heap II, 4th day, 63.3°C	1920	1	1
Summary		7968	2	6

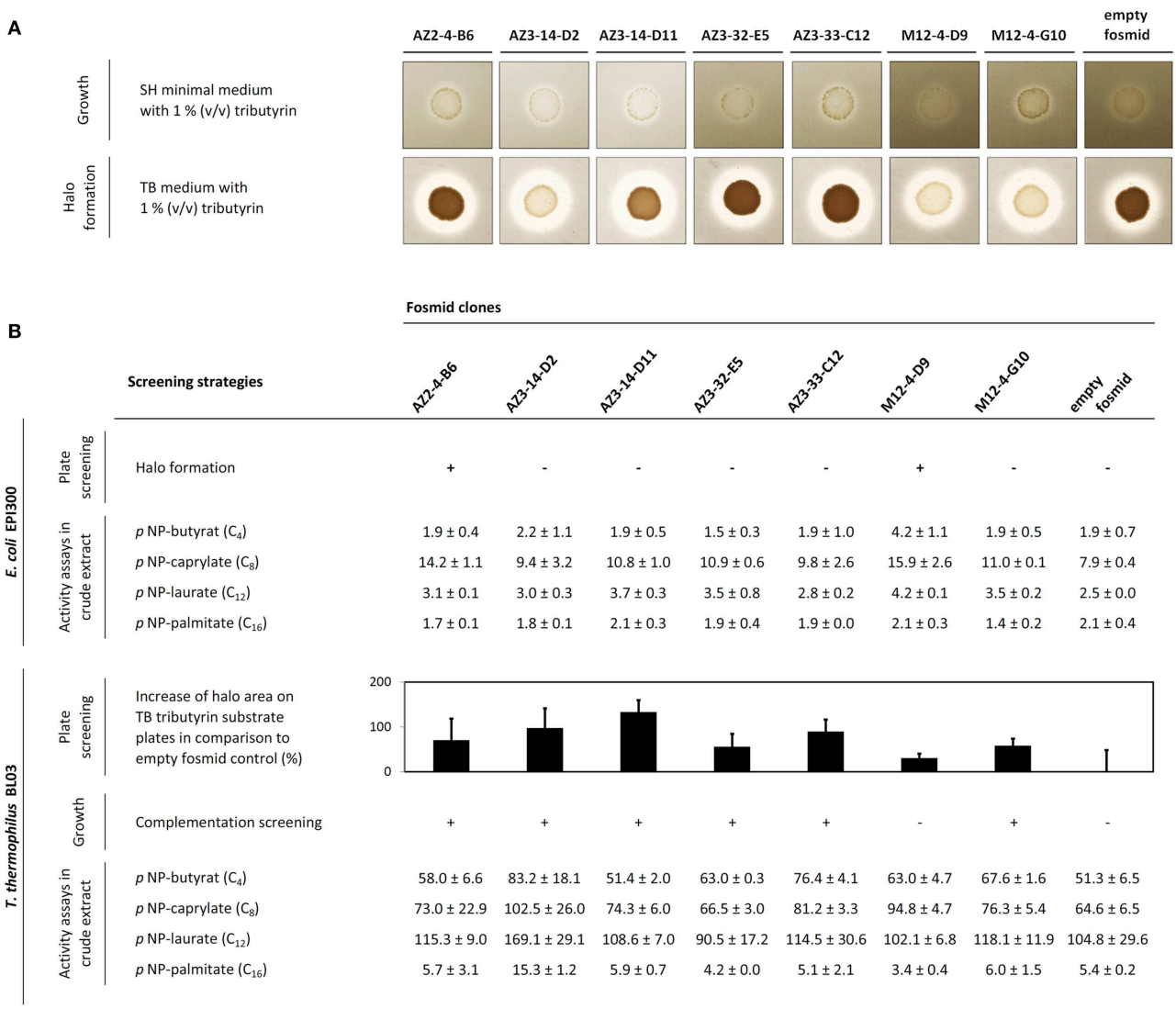
TABLE 2 | Frequency of operational taxonomic units (OTUs) identified by 16S rDNA-analysis of the metagenomic samples.

Metagenomic sample	Kingdom	OTUs with highest similarity to phylum (e.g., most predominant families)	Number of sequences (frequency)
Hot springs (water/sediments)	Archaea	*Crenarchaeota* (*Sulfolobaceae, Stygiolobus azoricus* DSM 6296 only)	5 (100.0%)
	Bacteria	*Proteobacteria* (*Enterobacteriaceae, Methylobacteriaceae, Acetobacteraceae, Sphingomonadaceae, Burkholderiaceae, Acidithiobacillaceae*)	19 (82.6%)
		Bacteriodetes (*Flavobacteriaceae*)	2 (8.7%)
		Others (*Cyanobacteria*)	2 (8.7%)
Compost (lumber waste)	Bacteria	*Firmicutes* (*Bacillaceae, Thermoanaerobacteriaceae, Alicyclobacillaceae, Paenibacillaceae, Clostridiaceae*)	208 (88.1%)
		Actinobacteria (*Streptosporangiaceae*)	17 (7.2%)
		Proteobacteria (*Enterobacteriaceae, Rhodobacteraceae Pseudomonadaceae, Myxococcaceae*)	8 (3.4%)
		Others (*Chloroflexi*, *Actinobacteria*)	3 (1.3%)

was done by sequencing of PCR products obtained with degenerate primers which amplify bacterial and archaeal 16S rDNA sequences (Supplementary Table S1). This analysis showed a very low archaeal diversity in the hot spring water, with only representatives of the genera *Stygiolobus azoricus* DSM 6296 (99% sequence identity) and *Sulfolobus* species (e.g., *S. acidocaldarius* DSM 639 with 95% sequence identity) found. In the compost sample a more diverse representation of bacteria was observed with *Firmicutes* being the most highly represented phylum in which the majority of sequences were assigned to the orders *Bacillales* and *Clostridiales* (**Table 2**).

Functional Screening of Fosmid Libraries in *E. coli* and *T. thermophilus*

After screening of the generated libraries in *E. coli* using a plate assay at 60°C, only two halo-forming colonies were identified on tributyrin agar plates, one from the Azores sample (termed AZ2-4-B6) and one from the compost sample (M12-4-D9). In order to perform the screening in *T. thermophilus,* the BL03 strain was transformed with 83 fosmid pools, each prepared from 96 fosmid clones. The transformant pools obtained in this way were examined for their ability to grow on minimal medium plates supplemented with tributyrin. A total of 13 pools showed



B

Fosmid clones

	Screening strategies	AZ2-4-B6	AZ3-14-D2	AZ3-14-D11	AZ3-32-E5	AZ3-33-C12	M12-4-D9	M12-4-G10	empty fosmid
E. coli EPI300	Plate screening	Halo formation	+	-	-	-	-	+	-	-
	Activity assays in crude extract	*p* NP-butyrat (C_4)	1.9 ± 0.4	2.2 ± 1.1	1.9 ± 0.5	1.5 ± 0.3	1.9 ± 1.0	4.2 ± 1.1	1.9 ± 0.5	1.9 ± 0.7
		p NP-caprylate (C_8)	14.2 ± 1.1	9.4 ± 3.2	10.8 ± 1.0	10.9 ± 0.6	9.8 ± 2.6	15.9 ± 2.6	11.0 ± 0.1	7.9 ± 0.4
		p NP-laurate (C_{12})	3.1 ± 0.1	3.0 ± 0.3	3.7 ± 0.3	3.5 ± 0.8	2.8 ± 0.2	4.2 ± 0.1	3.5 ± 0.2	2.5 ± 0.0
		p NP-palmitate (C_{16})	1.7 ± 0.1	1.8 ± 0.1	2.1 ± 0.3	1.9 ± 0.4	1.9 ± 0.0	2.1 ± 0.3	1.4 ± 0.2	2.1 ± 0.4
T. thermophilus BL03	Plate screening	Increase of halo area on TB tributyrin substrate plates in comparison to empty fosmid control (%)	200 / 100 / 0							
	Growth	Complementation screening	+	+	+	+	+	-	+	-
	Activity assays in crude extract	*p* NP-butyrat (C_4)	58.0 ± 6.6	83.2 ± 18.1	51.4 ± 2.0	63.0 ± 0.3	76.4 ± 4.1	63.0 ± 4.7	67.6 ± 1.6	51.3 ± 6.5
		p NP-caprylate (C_8)	73.0 ± 22.9	102.5 ± 26.0	74.3 ± 6.0	66.5 ± 3.0	81.2 ± 3.3	94.8 ± 4.7	76.3 ± 5.4	64.6 ± 6.5
		p NP-laurate (C_{12})	115.3 ± 9.0	169.1 ± 29.1	108.6 ± 7.0	90.5 ± 17.2	114.5 ± 30.6	102.1 ± 6.8	118.1 ± 11.9	104.8 ± 29.6
		p NP-palmitate (C_{16})	5.7 ± 3.1	15.3 ± 1.2	5.9 ± 0.7	4.2 ± 0.0	5.1 ± 2.1	3.4 ± 0.4	6.0 ± 1.5	5.4 ± 0.2

FIGURE 1 | (A) Growth complementation screening results of *T. thermophilus* BL03 transformed with metagenomic fosmid DNA (upper scan picture) and corresponding tributyrase halo formation capabilities on substrate plates (lower scan picture). **(B)** Tributyrase activity of 8 single fosmids in *E. coli* EPI300 (upper part of the table) and *T. thermophilus* BL03 (below). In this representation, the increase of halo formation (black bars, halo area was calculated as described in the Materials and Methods section) on 1% (v/v) tributyrin substrate plates after 3 days of incubation at 60°C is shown (from triplicate measurements, $n = 3$; error bars indicate the standard deviation). In *T. thermophilus*, heterologous growth complementation results after 3 days of growth at 60°C on minimal medium are depicted. Comparative *p*NP-activity data from crude extracts (specific activity mU × mg^{-1}) is shown for each single fosmid clone as average values from duplicate measurements (± standard deviation).

substantial growth on these plates (namely fosmid pools AZ2-4; AZ3-14; AZ3-25; AZ3-30; AZ3-32; AZ3-33; AZ3-37; AZ3-38; AZ3-47; AZ4-2; M12-3; M12-4; and M12-6). From these candidate pools, single fosmid transformations were performed in 96 well plate format. Again, growth on minimal medium was monitored, and positive clones were selected. After re-streaking them two more times to confirm the stability of their phenotype, six fosmid-containing *T. thermophilus* clones could be isolated from five different fosmid pools. They stably grew on minimal medium agar supplemented with tributyrin, and hydrolysis halos on tributyrin substrate plates were monitored in comparison with the BL03 strain carrying an empty fosmid (**Figure 1A**). In addition, the esterase activity of crude cell extracts obtained from the respective strains was measured with various *p*NP-substrates (**Figure 1B**). Almost all fosmid clones showed preferences for medium to short acyl chain fatty acid *p*NP-esters. Crude extract from the strain AZ3-14-D2 exhibited the highest activities of all crude extracts tested. This extract was most active over a broad range of *p*NP-substrates tested and its long-chain palmitate fatty acid hydrolysis was approximately three-fold higher than in the BL03 empty fosmid control. The two fosmids that conferred tributyrase activity in *E. coli*, AZ2-4-B6 and M12-4-D9, were also found to confer activity in *T. thermophilus* when tested in *p*NP-assays. Fosmid M12-4-D9 could not be identified by heterologous growth complementation screenings in *T. thermophilus* BL03, but showed higher activity on *p*NP-C_8 compared to *E. coli*. In general, the *p*NP-substrate preference of the fosmid-encoded esterases for short-chain acyl esters was in agreement with the observed capabilities of halo formation on tributyrin agar plates.

TABLE 3 | Overview of esterase-positive fosmids.

Fosmid clone	Functional screening positive in	Assembled sequence (kb) (no. of contigs)	Candidate ORF (contig)	Similarity search by blastp (closest *species*), additional information, characterized proteins	Predicted protein families and domains (Pfam)
AZ2-4-B6†	*E. coli* and *T. thermophilus*	10.46 (2)	8c (contig 1)	Meta-fission product hydrolase (*Dyella ginsengisoli* LA-4) accession ACH87186.1 with 71% amino acid identity, a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (Li et al., 2009), α/β-hydrolase (*Alicycliphilus denitrificans* BC)	α/β-hydrolase 6
AZ3-14-D2	*T. thermophilus*	34.71 (4)	2 (contig 2)	Acetyl-CoA acetyltransferase (*Metallosphaera sedula*) WP_012020361.1 with 84% identity	Thiolase, N- and C-terminus
AZ3-14-D11	*T. thermophilus*	32.68 (2)	n.d.	*Sulfolobus* species	n.d.
AZ3-32-E5	*T. thermophilus*	28.97 (3)	12 (contig 1)	Dipeptidyl aminopeptidase/acylaminoacyl-peptidase (*Sulfolobus islandicus* LAL14/1), YP_007865031.1. with 60% identity; shares similarities to α/β hydrolases, COesterases and putative esterases, predicted active sites: Ser^{412}, Asp^{491} and His^{523}	Peptidase_S9
AZ3-33-C12	*T. thermophilus*	39.46 (1)	2	Hypothetical protein (*Acidianus hospitalis* W1) YP_004458132.1 with 66% similarity, phospholipase A2/esterase (*Desulfurococcus kamchatkensis* 1221n) YP_002428819.1 with 40% sequence identity, APE_2325 (*Aeropyrum pernix* K1) NP_148539.1 with 48% identity	Archaeal PaREP1/PaREP8 family, not significant
M12-4-D9‡	*E. coli*	shotgun clone	1	Hypothetical protein (*Caldibacillus debilis*) and carboxylesterase Est30 (*Geobacillus stearothermophilus*), crystal structure available at PDB: 1TQH, accession Q06174.2 with 71% similarity (Liu et al., 2004)	α/β-hydrolase 6
M12-4-G10	*T. thermophilus*	50.87 (1)	18c	Hypothetical protein (*Sphingobium quisquiliarum* P25), EQB08083.1 with 40% sequence identity	Metallo-β-lactamase B superfamily, alkyl sulfatase, N- and C-terminus

†*Sequenced separately at Göttingen Genomics Laboratory (G_2L), no assembly statistics available, fosmid backbone pCT3FK sequence not included.*
‡*Identified from shotgun library in E. coli only.*
n.d., no detectable candidate ORF.

Sequencing and *in silico* Analysis of the Esterase Activity-Conferring Fosmid Inserts

The candidate fosmids were subjected to 454-sequencing and plasmids containing shotgun libraries of positive fosmids were sequenced with Sanger technology. The bioinformatic analysis of the fosmids is summarized in **Table 3**. Annotated α/β hydrolase 6 family proteins sharing high similarity to already characterized proteins were predicted from the sequence of two fosmids, namely M12-4-D9 and AZ2-4-B6. Not all positive fosmids from high-throughput *T. thermophilus* screenings could be completely assembled and sequenced (in average, 32.86 kb sequence information was obtained from each fosmid). The assembly data is summarized in Supplementary Table S2. Only in the case of two fosmids, AZ3-33-C12 (GenBank accession number KP892656) and M12-4-G10 (GenBank accession number KP892657) complete sequence assemblies where available for bioinformatic analysis. By motif search, the conserved GXSXG pentapeptide signature was identified in two of the predicted protein-encoding ORFs (EstA2 from fosmid AZ3-33-C12 and EstB1 from M12-4-G10). Both candidates showed no or only weak sequence similarity to lipolytic enzymes. Pfam prediction did not reveal any esterase/lipase-encoding function. For fosmid clones AZ3-32-E5, AZ3-14-D2, and AZ3-14-D11, sequence information was incomplete, therefore hampering further *in silico* analysis.

Heterologous Expression and Preliminary Characterization of Two Novel Metagenomic Esterases

From all candidate esterase-encoding ORFs, we selected EstA2 and EstB1 for expression, purification and characterization using *T. thermophilus* as an expression host. Expression of these ORFs in pMK18 (de Grado et al., 1999) led to significant *p*NP-butyrate-cleaving activity (measured at 60°C). Further investigation revealed that short-chain acyl esters were the preferred substrates, such as *p*NP-butyrate, that was hydrolyzed most optimally around 80–85°C and pH 8.0. EstA2 (encoded on fosmid AZ3-33-C12) resulted in a significant activity increase in the extracellular fraction, resulting in a 1.54-fold induction of activity, while EstB1 showed a 1.53-fold increased activity level compared to 338.4 ± 4.8 mU/mg protein in BL03 with the empty pMK18 vector. However, no recombinant protein band could be detected by SDS-PAGE analysis. We then attempted to produce C-terminally tagged versions of both proteins in *E. coli* by using the pET21a(+) expression vector. For both proteins, purification

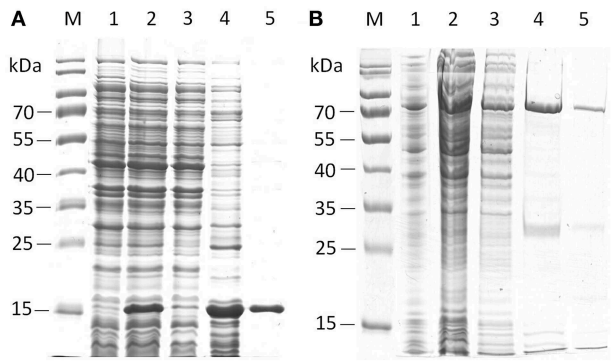


FIGURE 2 | SDS-PAGE analysis of the purification steps of his-tagged EstA2 (A) and EstB1 (B). Lanes M, protein ladder; Lanes 1, empty vector control; lanes 2, crude lysate; lanes 3, flow-through; Lanes 4–5, eluate fractions of target proteins. Predicted sizes of the proteins with C-terminal his-tag fusions are 18.7 kDa for EstA2 and 64.6 kDa for EstB1, respectively.

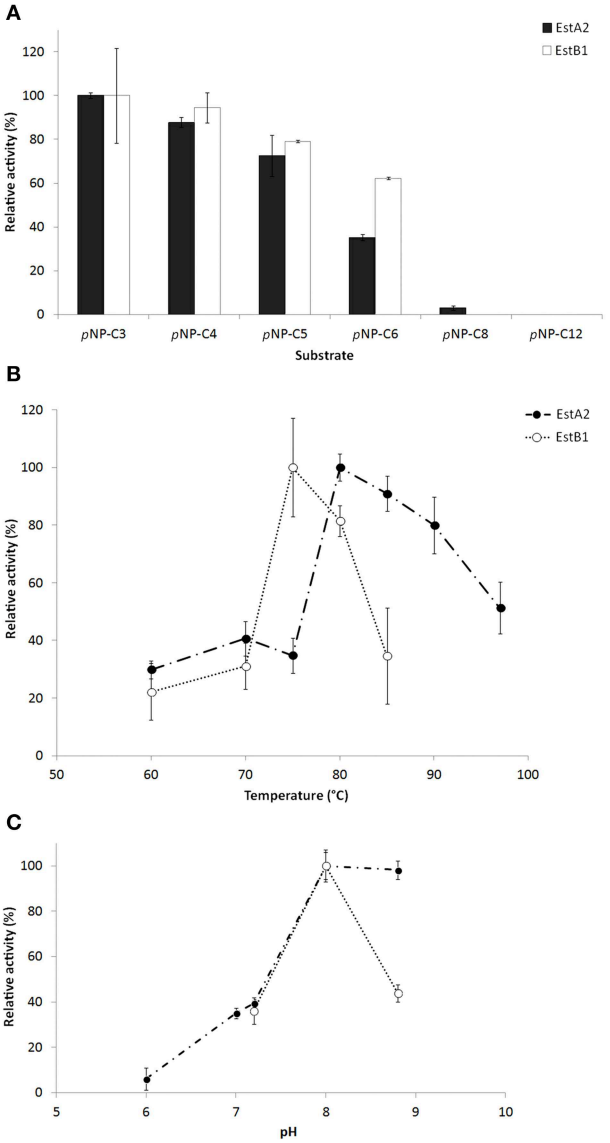


FIGURE 3 | Characterization of the purified esterase-active proteins EstA2 and EstB1. (A) Substrate specificity of various acyl chain length *p*NP-esters. **(B)** Temperature and **(C)** pH optimum. The assays were performed in 50 mM phosphate buffer (pH 6–7) or 50 mM Tris-HCl buffer (pH 7.2–8.8) under optimal activity parameters (80°C for EstA2 and 75°C for EstB1, respectively) against 1.25 mM *p*NP-butyrate. 0.065–1.3 μg of purified enzyme was used for the assays. Data represents average values and standard deviations (error bars).

using heat treatment followed by IMAC gravity flow columns resulted in functional protein preparations (**Figure 2**).

The characterization of the recombinant enzymes revealed similar substrate preferences for short acyl chain substrates (*p*NP-$C_3 > C_4 > C_5 > C_6 > C_8$) as the ones observed in *T. thermophilus*, while activities on long-chain substrates (over C_{10}) were comparably low (**Figure 3**). Under the assay conditions used here, optimum activity of EstA2 was observed around 80°C and pH 8.0, while EstB1 was most active at 75°C and pH 8.0. The specific activities of both enzymes on *para*-nitrophenyl butyrate were 44.05 ± 2.06 U/mg protein for EstA2 and 4.13 ± 0.71 U/mg for EstB1. The influence of several additives, NaCl, KCl, $CaCl_2$, $MgCl_2$, EDTA, and PMSF at 1–10 mM final concentration was negligible. Our data suggests both proteins to be esterases rather than lipases due to their characteristic substrate specificities.

EstA2 and EstB1 were tested toward various racemic mixtures of a selection of *p*NP-esters (**Table 4**). Activity was measured over a wide range of temperatures (60–80°C) to overcome constraints by autohydrolysis of the substrates at elevated temperatures (Chow et al., 2012; López et al., 2014). In general, the activities measured were comparably low (mU range). The highest activities under the conditions tested were found with the 4-nitrophenolates of-Naproxen and Ibuprofen. No activity could be detected with the *p*NP-esters of indancarboxylic acid while 2-methyldecanoate and 6-methyl-2-(*ortho*-tolyl)hept-5-enoate were only hydrolyzed weakly in the presence of EstB1 at the highest temperature tested so far.

Classification of Lipolytic Enzymes

In order to classify the gene products identified in our metagenomic screenings, we performed multiple sequence alignments with known esterases and lipases from families I to VIII (Arpigny and Jaeger, 1999). Recently, the number of known lipolytic enzyme families has been extended by two new families, termed LipS and LipT (Chow et al., 2012). From the positive fosmids identified in *E. coli* (AZ2-4-B6 and M12-4-D9), both encoding α/β-hydrolase ORFs were identified as esterase class V and LipS family protein, respectively. The lipolytic enzymes discovered by using *T. thermophilus* for functional library screening, EstA2 and EstB1, could not be assigned to any known esterase/lipase family (**Figure 4**).

Discussion

In the past, *E. coli* has been shown to be a good provider of interesting biocatalysts and enzymes from metagenomic screenings. Although it is clear that *E. coli* has restricted expression capabilities, it is the best developed and most commonly used host until today. Here, we show that using an additional host in functional screenings of metagenomic libraries can lead to the discovery of enzymes that would have remained hidden if only *E. coli* had been used.

How heterologous sequences are recognized and expressed into functional proteins by the transcriptional and translational machinery of an expression host is poorly understood. Factors influencing these processes, amongst other unknown factors, are: (i) toxicity of the foreign DNA and encoded proteins (Sorek et al., 2007), (ii) recognition of transcriptional signals (promoters) (Warren et al., 2008), (iii) ribosomal binding and translation initiation (Sørensen and Mortensen, 2005; Villegas and Kropinski, 2008), (iv) mRNA stability (Kudla et al., 2009), (v) codon usage (Sørensen and Mortensen, 2005), (vi) (post-translational) protein modification and secretion (Mergulhão et al., 2005). To overcome these restrictions, other screening hosts have been implemented in function-based metagenomic screening approaches in order to increase the detection frequency of active clones. First attempts using additional screening hosts involved *Streptomyces* and *Pseudomonas* species (Courtois et al., 2003; Martinez et al., 2004; Lussier et al., 2011). By the use of broad-host range vectors, the expression could recently be extended to other members of the *Proteobacteria*, i.e., the same taxon as *E. coli* (Aakvik et al., 2009; Craig et al., 2010; Kakirde et al., 2011), and to eukaryotic expression hosts (Damon et al., 2011; Kellner et al., 2011; Parachin and Gorwa-Grauslund, 2011). Our approach is distinguished from these "broad-host vectors" strategies by performing the library construction steps in the well-established *E. coli* system, followed by transfer of the metagenomic fosmids in the alternative host (Angelov et al., 2009). In order to facilitate the detection of esterase-encoding fosmid inserts, we used a selection-based strategy which relies on the complementation of growth of a custom, esterase-deficient strain of *T. thermophilus* (strain BL03, Leis et al., 2014). The BL03 strain is a multiple esterase deletion mutant which exhibits significantly reduced extracellular lipolytic activities compared with the parent strain HB27 and was shown

TABLE 4 | Specific activity (mU/mg) of EstA2 and EstB1 on *p*NP-esters at various temperatures.

Tested substrates (*p*NP-derivatives)	60°C		70°C		80°C	
	EstA2	EstB1	EstA2	EstB1	EstA2	EstB1
2-Methyldecanoate	–	–	–	–	–	0.15 ± 0.04
Ibuprofen	–	1.11 ± 0.31	0.68 ± 0.42	0.63 ± 0.02	2.55 ± 1.89	0.76 ± 0.02
Naproxen	1.01 ± 0.16	1.54 ± 0.03	2.94 ± 0.34	1.29 ± 0.72	3.09 ± 0.07	0.27 ± 0.06
4-Nitrophenyl 6-methyl-2-(*ortho*-tolyl)hept-5-enoate	–	–	–	–	–	0.63 ± 0.00
Indancarboxylic acid	–	–	–	–	–	–

Activity tests were performed in 850 μL of 50 mM Sørensen buffer containing 0.1% (w/v) gum arabic and 5 mM sodium deoxycholate (pH 8.0 at 60, 70, and 80°C, respectively), mixed with 120 μL of DMSO and 20 μL of 10 mM of the pNP-ester substrates. Within 10 μL residual volume, 0.4 μg of the enzymes were added to the assay, which was performed for 30 min. Absorbance at 400 nm was measured in cuvettes, enzyme-free samples were measured as reference (the molar extinction coefficient was determined as $\varepsilon = 14{,}000\ M^{-1} \times cm^{-1}$).

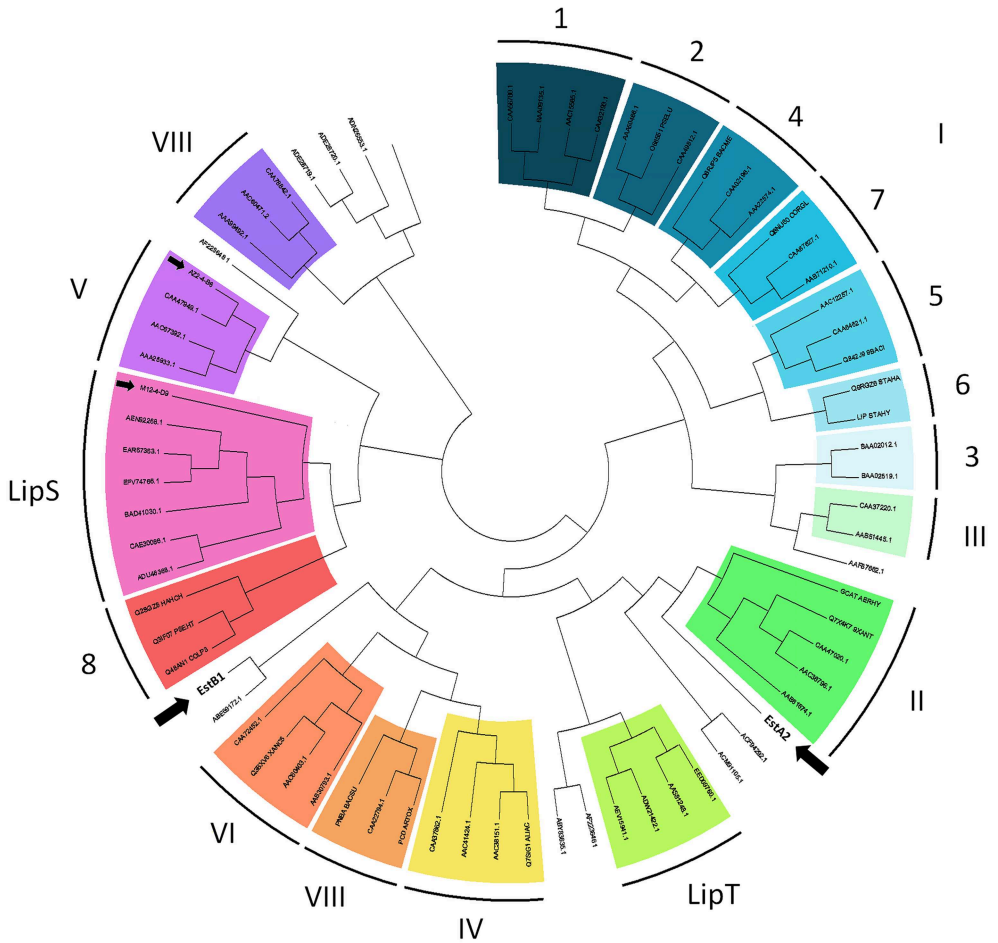


FIGURE 4 | Topologic view of a similarity tree of all lipolytic esterase and lipase protein families I to VIII (according to Arpigny and Jaeger, 1999), including the recently defined families LipS and LipT as well as classified, but still unknown protein families (Chow et al., 2012) and the new esterases from our functional metagenomic screenings (AZ2-4-B6, M12-4-D9, EstA2, EstB1). The data was generated from multiple sequence alignments of selected representative protein sequences (according to Chow et al., 2012) using the ClustalW algorithm (Boc et al., 2012). Tree reconstruction and visualization was performed with MEGA Version 5.2 (Tamura et al., 2011). The metagenomic proteins identified in this study are marked with black arrows. The length of the tree branches does not represent the phylogenetic distance between the protein sequences.

to be severely impaired in growth on minimal medium supplemented with tributyrin (Leis et al., 2014). The growth could be regained via heterologous complementation when metagenomic DNA was expressed by the host. Low detection limits of classical phenotypic plate screenings (clearing zones around the colonies) could thus be overcome, as even weak gene expression levels yield enough metabolites to reconstitute growth. By implementing a high-throughput pooling strategy, approximately 8000 single fosmid clones yielded six single candidate fosmid clones, which represents a detection frequency that is comparable with screening results obtained from other metagenomics studies aimed at lipolytic activities from hot environments (Rhee et al., 2005; Chow et al., 2012). To our knowledge, this is the first report of identifying novel metagenome-borne esterases by the use of a screening organism other than traditional *E. coli*.

Metagenomic esterases having similarity to β-lactamase family proteins have been reported from various environmental or anthropogenic sources, e.g., leachate samples (Rashamuse et al., 2009), arctic soil (Yu et al., 2011), alkaline compost (Kim et al., 2010), drinking water and soil metagenomes (Elend et al., 2006). They share the conserved sequence motif common for class C β-lactamases (Ser-Xaa-Xaa-Lys, whereas Xaa is any amino acid) that is important for catalytic activity of esterases with similarity to penicillin-binding proteins (Petersen et al., 2001), and have been assigned to the less characterized family VIII carboxylesterases (Arpigny and Jaeger, 1999). EstB1 lacks this conserved sequence motif and does not belong to the class C β-lactamase protein family. Instead, it could be assigned to class B metallo-β-lactamase proteins (PF00753). This group comprises hydrolases acting on thioester (thioesterase) and sulfate ester (sulfatase) bonds. Furthermore, thioesterases have also been shown to convert *para*-nitrophenol esters (Shahi et al., 2006; Kotowska et al., 2009). EstB1 shared 30% sequence identity with SdsA1 from *Pseudomonas aeruginosa*, which is a

characterized alkyl-sulfatase (Hagelueken et al., 2006). The other esterase-acting enzyme identified in our metagenomic studies was EstA2. It is most similar to hypothetical proteins from diverse archaeal origins. One characterized enzyme, to which EstA2 shows very weak similarity, is a phospholipase from the hyperthermophilic archaeon *Aeropyrum pernix* K1 encoded by ORF APE2325 (Wang et al., 2004). Also being a small protein with 18 kDa in size, its reported substrate spectrum and activity pattern is comparable to EstA2, as it also preferred short acyl chain *p*NP-substrates and was most active at elevated temperatures (above 80°C).

In this study we have demonstrated that *T. thermophilus* is an excellent host bacterium for the detection of novel metagenome-borne enzymes that could not readily have been detected by the use of *E. coli* or by *in silico* analysis. To our knowledge, this is the first report that metagenome-derived esterases could be identified in an expression host other than *E. coli*. The functional screening implementing *T. thermophilus* BL03 could uncover several lipolytic enzymes from underrepresented species and archaeal origin, and most strikingly, some of them could not be assigned to classical esterase and lipase enzyme families and therefore represent truly novel esterase-active proteins. The confirmation of predicted ORFs responsible for the lipolytic activity of the remaining fosmids will be addressed in the future. The use of complementation screens in *T. thermophilus* with shotgun subclones will help to identify more active genes that do not share any known sequence signatures at all.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00275/abstract

References

Aakvik, T., Degnes, K. F., Dahlsrud, R., Schmidt, F., Dam, R., Yu, L., et al. (2009). A plasmid RK2-based broad-host-range cloning vector useful for transfer of metagenomic libraries to a variety of bacterial species. *FEMS Microbiol. Lett.* 296, 149–158. doi: 10.1111/j.1574-6968.2009.01639.x

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Angelov, A., Mientus, M., Liebl, S., and Liebl, W. (2009). A two-host fosmid system for functional screening of (meta)genomic libraries from extreme thermophiles. *Syst. Appl. Microbiol.* 32, 177–185. doi: 10.1016/j.syapm.2008.01.003

Arpigny, J. L., and Jaeger, K. E. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochem. J.* 343, 177–183. doi: 10.1042/0264-6021:3430177

Boc, A., Diallo, A. B., and Makarenkov, V. (2012). T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks. *Nucleic Acid Res.* 40, W573–W579. doi: 10.1093/nar/gks485

Chow, J., Kovacic, F., Dall Antonia, Y., Krauss, U., Fersini, F., Schmeisser, C., et al. (2012). The metagenome-derived enzymes LipS and LipT increase the diversity of known lipases. *PLoS ONE* 7:e47665. doi: 10.1371/journal.pone.0047665

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acid. Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

Courtois, S., Cappellano, C. M., Ball, M., Francou, F.-X., Normand, P., Helynck, G., et al. (2003). Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Appl. Environ. Microbiol.* 69, 49–55. doi: 10.1128/AEM.69.1.49-55.2003

Craig, J. W., Chang, F.-Y., Kim, J. H., Obiajulu, S. C., and Brady, S. F. (2010). Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. *Appl. Environ. Microbiol.* 76, 1633–1641. doi: 10.1128/AEM.02169-09

Damon, C., Vallon, L., Zimmermann, S., Haider, M. Z., Galeote, V., Dequin, S., et al. (2011). A novel fungal family of oligopeptide transporters identified by functional metatranscriptomics of soil eukaryotes. *ISME J.* 5, 1871–1880. doi: 10.1038/ismej.2011.67

de Grado, M., Castán, P., and Berenguer, J. (1999). A high-transformation-efficiency cloning vector for *Thermus thermophilus*. *Plasmid* 42, 241–245. doi: 10.1006/plas.1999.1427

Elend, C., Schmeisser, C., Leggewie, C., Babiak, P., Carballeira, J. D., Steele, H. L., et al. (2006). Isolation and biochemical characterization of two novel metagenome-derived esterases. *Appl. Environ. Microbiol.* 72, 3637–3645. doi: 10.1128/AEM.72.5.3637-3645.2006

Franken, B., Jaeger, K.-E., and Pietruszka, J. (2010). "Protocols to screen for enantioselective lipases," in *Handbook of Hydrocarbon and Lipid Microbiology,* ed K. N. Timmes (Berlin-Heidelberg: Springer), 4581–4586.

Gabor, E. M., Alkema, W. B. L., and Janssen, D. B. (2004). Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environ. Microbiol.* 6, 879–886. doi: 10.1111/j.1462-2920.2004.00640.x

Gaich, T., and Mulzer, J. (2009). Total synthesis of (-)-Penifulvin A, an insecticide with a dioxafenestrane skeleton. *J. Am. Chem. Soc.* 131, 452–453. doi: 10.1021/ja8083048

Hagelueken, G., Adams, T. M., Wiehlmann, L., Widow, U., Kolmar, H., Tümmler, B., et al. (2006). The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7631–7636. doi: 10.1073/pnas.0510501103

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., and Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* 5, R245–R249. doi: 10.1016/S1074-5521(98)90108-9

Jenney, F. E., and Adams, M. W. W. (2008). The impact of extremophiles on structural genomics (and vice versa). *Extremophiles* 12, 39–50. doi: 10.1007/s00792-007-0087-9

Kakirde, K. S., Wild, J., Godiska, R., Mead, D. A., Wiggins, A. G., Goodman, R. M., et al. (2011). Gram negative shuttle BAC vector for heterologous expression of metagenomic libraries. *Gene* 475, 57–62. doi: 10.1016/j.gene.2010.11.004

Kellner, H., Luis, P., Portetelle, D., and Vandenbol, M. (2011). Screening of a soil metatranscriptomic library by functional complementation

of *Saccharomyces cerevisiae* mutants. *Microbiol. Res.* 166, 360–368. doi: 10.1016/j.micres.2010.07.006

Kim, Y. H., Kwon, E. J., Kim, S. K., Jeong, Y. S., Kim, J., Yun, H. D., et al. (2010). Molecular cloning and characterization of a novel family VIII alkaline esterase from a compost metagenomic library. *Biochem. Biophys. Res. Comm.* 393, 45–49. doi: 10.1016/j.bbrc.2010.01.070

Kotowska, M., Pawlik, K., Smulczyk-Krawczyszyn, A., Bartosz-Bechowski, H., and Kuczek, K. (2009). Type II Thioesterase ScoT, Associated with *Streptomyces coelicolor* A3(2) modular polyketide synthase Cpk, hydrolyzes acyl residues and has a preference for propionate. *Appl. Environ. Microbiol.* 75, 887–896. doi: 10.1128/AEM.01371-08

Kudla, G., Murray, A. W., Tollervey, D., and Plotkin, J. B. (2009). Coding-sequence determinants of gene expression in *Escherichia coli*. *Science* 324, 255–258. doi: 10.1126/science.1170160

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics,* eds E. Stackebrandt and M. Goodfellow (Chichester, NY: Wiley), 115–175.

Leis, B., Angelov, A., and Liebl, W. (2013). Screening and expression of genes from metagenomes. *Adv. Appl. Microbiol.* 83, 1–68. doi: 10.1016/B978-0-12-407678-5.00001-5

Leis, B., Angelov, A., Li, H., and Liebl, W. (2014). Genetic analysis of lipolytic activities in *Thermus thermophilus* HB27. *J. Biotechnol.* 191, 150–157. doi: 10.1016/j.jbiotec.2014.07.448

Li, A., Qu, Y., Zhou, J., and Ma, F. (2009). Enzyme-substrate interaction and characterization of a 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Dyella ginsengisoli* LA-4. *FEMS Microbiol. Lett.* 292, 231–239. doi: 10.1111/j.1574-6968.2009.01487.x

Liebl, W. (2011). "Metagenomics," in *Encyclopedia of Geobiology*, *Encyclopedia of Earth Sciences Series,* eds J. Reitner and V. Thiel (Dordrecht, NL: Springer), 553–558.

Liebl, W., Angelov, A., Juergensen, J., Chow, J., Loeschcke, A., Drepper, T., et al. (2014). Alternative hosts for functional (meta)genome analysis. *Appl. Microbiol. Biotechnol.* 98, 8099–8109. doi: 10.1007/s00253-014-5961-7

Liu, P., Wang, Y.-F., Ewis, H. E., Abdelal, A. T., Lu, C.-D., Harrison, R. W., et al. (2004). Covalent reaction intermediate revealed in crystal structure of the *Geobacillus stearothermophilus* carboxylesterase Est30. *J. Mol. Biol.* 342, 551–561. doi: 10.1016/j.jmb.2004.06.069

López, G., Chow, J., Bongen, P., Lauinger, B., Pietruszka, J., Streit, W. R., et al. (2014). A novel thermostable esterase from *Acidicaldus* sp. strain USBA-GBX-499 with enantioselectivity isolated from an acidic hot spring of Colombian Andes. *Appl. Microbiol. Biotechnol.* 98, 8603–8616. doi: 10.1007/s00253-014-5775-7

Ludwig, W., Strunk, O., Westram, R., Richter, L., and Meier, H., Yadhukumar, et al. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res.* 32, 1363–1371. doi: 10.1093/nar/gkh293

Lussier, F.-X., Chambenoit, O., Côté, A., Hupé, J.-F., Denis, F., Juteau, P., et al. (2011). Construction and functional screening of a metagenomic library using a T7 RNA polymerase-based expression cosmid vector. *J. Ind. Microbiol. Biotechnol.* 38, 1321–1328. doi: 10.1007/s10295-010-0915-2

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., et al. (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* 39, D225–D229. doi: 10.1093/nar/gkq1189

Martinez, A., Kolvek, S. J., Yip, C. L. T., Hopke, J., Brown, K. A., MacNeil, I. A., et al. (2004). Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. *Appl. Environ. Microbiol.* 70, 2452–2463. doi: 10.1128/AEM.70.4.2452-2463.2004

Mergulhão, F. J. M., Summers, D. K., and Monteiro, G. A. (2005). Recombinant protein secretion in *Escherichia coli*. *Biotechnol. Adv.* 23, 177–202. doi: 10.1016/j.biotechadv.2004.11.003

Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H.-Y., and Cohoon, M. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res.* 33, 5691–5702. doi: 10.1093/nar/gki866

Parachin, N. S., and Gorwa-Grauslund, M. F. (2011). Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenomic library. *Biotechnol. Biofuels* 4:9. doi: 10.1186/1754-6834-4-9

Petersen, E. I., Valinger, G., Sölkner, B., Stubenrauch, G., and Schwab, H. (2001). A novel esterase from *Burkholderia gladioli* which shows high deacetylation activity on cephalosporins is related to beta-lactamases and DD-peptidases. *J. Biotechnol.* 89, 11–25. doi: 10.1016/S0168-1656(01)00284-X

Petersen, T. N., Brunak, S., Heijne, G., von and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Pietruszka, J., Simon, R. C., Kruska, F., and Braun, M. (2009). Dynamic enzymatic kinetic resolution of methyl 2,3-dihydro-1H-indene-1-carboxylate. *Eur. J. Org. Chem.* 2009, 6217–6224. doi: 10.1002/ejoc.200901025

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., et al. (2012). The Pfam protein families database. *Nucleic Acids Res.* 40, D290–D301. doi: 10.1093/nar/gkr1065

Ramírez-Arcos, S., Fernández-Herrero, L. A., Marín, I., and Berenguer, J. (1998). Anaerobic growth, a property horizontally transferred by an Hfr-like mechanism among extreme thermophiles. *J. Bacteriol.* 180, 3137–3143.

Rashamuse, K., Magomani, V., Ronneburg, T., and Brady, D. (2009). A novel family VIII carboxylesterase derived from a leachate metagenome library exhibits promiscuous beta-lactamase activity on nitrocefin. *Appl. Microbiol. Biotechnol.* 83, 491–500. doi: 10.1007/s00253-009-1895-x

Reetz, M. T., Prasad, S., Carballeira, J. D., Gumulya, Y., and Bocola, M. (2010). Iterative saturation mutagenesis accelerates laboratory evolution of enzyme stereoselectivity: rigorous comparasion with traditional methods. *J. Am. Chem. Soc.* 132, 9144–9152. doi: 10.1021/ja1030479

Reetz, M. T., Zonta, A., Schimossek, K., Jaeger, K.-E., and Liebeton, K. (1997). Creation of enantioselective biocatalysts for organic chemistry by *in vitro* evolution. *Angew. Chem. Int. Ed. Engl.* 36, 2830–2832. doi: 10.1002/anie.199728301

Rhee, J. K., Ahn, D. G., Kim, Y. G., and Oh, J. W. (2005). New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl. Environ. Microbiol.* 71, 817–825. doi: 10.1128/AEM.71.2.817-825.2005

Sandström, A. G., Wikmark, Y., Engström, K., Nyhlén, J., and Bäckvall, J.-E. (2012). Combinatorial reshaping of the *Candida Antarctica* lipase A substrate pocket for enantioselectivity using an extremely condensed library. *Proc. Natl. Acad. Sci. U.S.A.* 109, 78–83. doi: 10.1073/pnas.1111537108

Satokari, R. M., Vaughan, E. E., Akkermans, A. D., Saarela, M., and de Vos, W. M. (2001). Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67, 504–513. doi: 10.1128/AEM.67.2.504-513.2001

Shahi, P., Kumar, I., Sharma, R., Sanger, S., and Jolly, R. S. (2006). Characterization of a novel long-chain acyl-CoA thioesterase from *Alcaligenes faecalis*. *FEBS J.* 273, 2374–2387. doi: 10.1111/j.1742-4658.2006.05244.x

Sorek, R., Zhu, Y., Creevey, C. J., Francino, M. P., Bork, P., and Rubin, E. M. (2007). Genome-wide experimental determination of barriers to horizontal gene transfer. *Science* 318, 1449–1452. doi: 10.1126/science.1147112

Sørensen, H. P., and Mortensen, K. K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 115, 113–128. doi: 10.1016/j.jbiotec.2004.08.004

Steele, H. L., Jaeger, K.-E., Daniel, R., and Streit, W. R. (2009). Advances in recovery of novel biocatalysts from metagenomes. *J. Mol. Microbiol. Biotechnol.* 16, 25–37. doi: 10.1159/000142892

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Torsvik, V., Goksøyr, J., and Daae, F. L. (1990). High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* 56, 782–787.

Villegas, A., and Kropinski, A. M. (2008). An analysis of initiation codon utilization in the Domain Bacteria—concerns about the quality of bacterial genome annotation. *Microbiology* 154, 2559–2661. doi: 10.1099/mic.0.2008/021360-0

Wang, B., Lu, D., Gao, R., Yang, Z., Cao, S., and Feng, Y. (2004). A novel phospholipase A2/esterase from hyperthermophilic archaeon *Aeropyrum pernix* K1. *Protein Expr. Purif.* 35, 199–205. doi: 10.1016/j.pep.2004.01.010

Warren, R. L., Freeman, J. D., Levesque, R. C., Smailus, D. E., Flibotte, S., and Holt, R. A. (2008). Transcription of foreign DNA in *Escherichia coli*. *Genome Res.* 18, 1798–1805. doi: 10.1101/gr.080358.108

Wu, Q., Soni, P., and Reetz, M. T. (2013). Laboratory evolution of enantiocomplementary *Candida antarctica* lipase B mutants with broad substrate scope. *J. Am. Chem. Soc.* 135, 1872–1881. doi: 10.1021/ja3 10455t

Yu, E. Y., Kwon, M.-A., Lee, M., Oh, J. Y., Choi, J.-E., Lee, J. Y., et al. (2011). Isolation and characterization of cold-active family VIII esterases from an arctic soil metagenome. *Appl. Microbiol. Biotechnol.* 90, 573–581. doi: 10.1007/s00253-011-3132-7

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Bioactive compounds produced by gut microbial tannase: implications for colorectal cancer development

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The microorganisms in the human gastrointestinal tract have a profound influence on the transformation of food into metabolites which can impact human health. Gallic acid (GA) and pyrogallol (PG) are bioactive compounds displaying diverse biological properties, including carcinogenic inhibiting activities. However, its concentration in fruits and vegetables is generally low. These metabolites can be also generated as final products of tannin metabolism by microbes endowed with tannase, which opens up the possibility of their anti-cancer potential being increased. Patients with colorectal cancer (CRC) display an imbalanced gut microbiota respect to healthy population. The recent use of next generation sequencing technologies has greatly improved knowledge of the identity of bacterial species that colonize non-tumorous and tumorous tissues of CRC patients. This information provides a unique opportunity to shed light on the role played by gut microorganisms in the different stages of this disease. We here review the recently published gut microbiome associated to CRC patients and highlight tannase as an underlying gene function of bacterial species that selectively colonize tumorous tissues, but not adjacent non-malignant tissues. Given the anti-carcinogenic roles of GA and PG produced by gut tannin-degrading bacteria, we provide an overview of the possible consequences of this intriguing coincidence for CRC development.

Keywords: microbial tannase, bioactive compounds, colorectal cancer, gallic acid, pyrogallol

INTRODUCTION

An imbalance in intestinal bacteria potentially leading to diseases termed colonic dysbiosis is associated to CRC, however, the possible role of intestinal microorganisms in the different CRC-stages is currently under debate.

Host diet is a crucial variable to shape the gut microbiota and a risk factor for CRC. Increased fruit and vegetable consumption is a dietary habit modification aimed to prevent gastrointestinal cancer (WHO Technical Report Series, No. 916 [TRS 916], 2003). Plant foods are main sources of tannins, dietary phytonutrients that display pro-apoptotic and antimetastasic properties in animal and *in vitro* studies (Serrano et al., 2009). Despite its promising chemopreventive potential, results from human studies to evaluate the association between the intake of foods rich in tannins and cancer risk are more dissuading. For example, inverse association (Theodoratou et al., 2007) but also no association (Kyle et al., 2010) between dietary proanthocyanidin intake and CRC risk have been reported. These apparently conflicting results may reflect interindividual differences in gut microbiota and reveal the difficulty to infer biological effects from dietary intake records without considering tannin-microbiota interactions. The microorganisms in the human gastrointestinal tract have a profound influence on the transformation of food into metabolites which can impact human health (Nicholson et al., 2012). Thus, the highly individual gut microbial activity, in particular the tannin-metabolizing activity of CRC-associated microbiota, will probably determine the bioavailability and physiological effects of tannins and their degradation products on tumorous tissues.

TANNASE: A COMMON FUNCTION IN THE MICROBIOTA COLONIZING COLORECTAL TUMOROUS TISSUES

Gut microbial tannase activity is likely crucial to determine the tannin-metabolizing phenotype of CRC-associated microbiota. Tannase (tannin acylhydrolase) transforms the gallate esters of tannins and other phenolic compounds, such as epigallocatechin gallate, into GA. GA can, subsequently, be decarboxylated by gallate decarboxylase to yield PG as final product of tannin metabolism. The capacity for tannin degradation is a survival advantage over other gut microorganisms because tannins display antimicrobial activity (Chung et al., 1998), reduce the species richness in the gut (Tan et al., 2013) and probably improve the adaptation of tannin-degrading bacteria to the intestinal environment (Reverón et al., 2013).

Recently the CRC microbiome encompassing all DNA within the microbiota of late stage CRC patients has been disclosed (Tjalsma et al., 2012). This information enabled these authors to flesh out the microbiota that colonizes tumorous or adjacent non-malignant sites. If one focuses on these intestinal microorganisms and perform a search for homologous genes to the known *Lactobacillus plantarum* tannase gene (KEGG database http://www.genome.jp/kegg/), only bacterial spp. specifically overrepresented in tumorous tissues, but not in adjacent non-malignant tissues, display tannase-homologous genes (**Table 1**).

Abbreviations: CRC, colorectal cancer; GA, gallic acid; PG, pyrogallol; ROS, reactive oxygen species.

Table 1 | Gut microbial tannase in late stage CRC-patients.

On tumor[a]	Organism	Tannase	Gene[b]
Roseburia	*R. intestinalis* XB6B4	+	rix:RO1_26850
Streptococcaceae	*Streptococcus gallolyticus* ATCC 3143	+	sgt:SGGB_1624
	S. gallolyticus ATCC BAA-2069	+	sgg:SGGBAA2069_c16370
	S. gallolyticus UCN34	+	sga:GALLO_1609
	S. gallolyticus UCN34	+	sga:GALLO_0933
	S. gallolyticus ATCC 3143	+	sgt:SGGB_0917
	S. gallolyticus ATCC BAA-2069	+	sgg:SGGBAA2069_c09070
	S. gallolyticus ATCC BAA-2069	+	sgg:SGGBAA2069_c09080
Fusobacterium	*F. nucleatum* subsp. *vincentii*	+	fnc:HMPREF0946_00654
	F. nucleatum subsp. *nucleatum* ATCC 25586	+	fnu:FN0616
	F. nucleatum subsp. *animalis*	+	fus:HMPREF0409_00517
Aggregatibacter	*A. actinomycetemcomitans* D7S-1	+	aan:D7S_02361
	A. actinomycetemcomitans D11S-1	+	aat:D11S_1302
	A. aphrophilus	+	aap:NT05HA_0741
	A. actinomycetemcomitans ANH9381	+	aao:ANH9381_1645
Coriobacteriaceae	*Slackia heliotrinireducens*	+	shi:Shel_22280
	Collinsella	NA	
	Coriobacterium glomerans	–	
Peptostreptococcaceae		NA	
Off tumor[a]			
Enterobacteriaceae	*Citrobacter*	–	
	Shigella	–	
	Cronobacter	–	
	Kluyveria	NA	
	Serratia	–	
	Salmonella	–	
Ruminococcus		–	
Clostridium[c]		+	
Anaerovorax		NA	
Eubacterium		–	
On/Off tumor[a]			
Bacteroides		–	
Faecalibacterium		–	

[a] *On tumor: bacteria inhabiting tumorous tissues; Off tumor: bacteria inhabiting adjacent healthy tissues; On/Off tumor: bacteria inhabiting indistinctly tumorous and adjacent healthy tissues.*
[b] *Homologous genes to L. plantarum tannase lp_2956.*
[c] *Two Clostridium spp. (C. botulinum E3 and C. strain SY8519) displayed genes with homology to L. plantarum tannase. However, this spp. are not reportedly associated to colorectal malignancies.*
NA. Sequence not available.

This intriguing coincidence could be consistent with the selectivity of plant phenolic compounds to target cancer cells (Azmi et al., 2013) and suggest that the presence of gut microbial tannase activity may have potential implications on the colonic epithelial health.

IMPORTANCE OF TANNASE-GENERATED METABOLITES IN CRC DEVELOPMENT

Recent findings have highlighted the potent DNA-damaging activities of tannins and tannase-derived metabolites, including PG, GA, and epigallocatechin gallate (Hossain et al., 2013). The

genotoxic behavior of these metabolites is determined by its pro-oxidant activity which is in line with the pro-oxidant activity displayed by a number of further anti-cancer phenolic compounds in cellular/biological systems (Azmi et al., 2013).

Although the antioxidant capacity of tannins, PG and GA, is generally accepted, its pro-oxidant behavior should not be surprising. Thus, plants elaborate these natural phenolics to have, among other important functions, antimicrobial activity and antimicrobials share a common mechanism to kill bacteria: through the overproduction of ROS (Kohanski et al., 2007).

It is to be noted that the pro-oxidant behavior of tannase-derived metabolites in CRC-tissues can be boosted in the colon compared to the small intestine due to a more pronounced pro-oxidant environment in the former (Sanders et al., 2004). The presence of cooper, a transition metal participating in the Fenton-like chemistry, is another factor for inducing the chemopreventive, pro-oxidant behavior of certain plant phenolic compounds (Azmi et al., 2013), including GA (Hossain et al., 2013), especially when it has been found in particularly augmented concentrations in a number of malignancies (Linder, 2012). Nevertheless some phenolics, such as PG, do not necessarily require a metal ion catalyst to produce OH radicals (Hossain et al., 2013).

Given the pro-oxidant activity of plant phenolics has been proposed as one of its anti-cancer properties (Azmi et al., 2013), the question arises as to whether the production of GA and PG by tannase-producing CRC-associated bacteria affects CRC development. There are several hints that the answer is yes. The potent ROS generation by GA and particularly PG strongly activates the tumor suppressor p53 in RKO CRC cells (Hossain et al., 2013). In addition GA and PG are known glutathione depletors (Park et al., 2007; You and Park, 2010) and could directly exert a cancer cell killing action via potent ROS generation. Hence, the selective production of GA/PG by the CRC-associated microbiota could promote pro-oxidative stress in the tumorous tissues to increase the direct or apoptotic killing (via p53 activation) of cancerous cells. Consistent with this hypothesis recent findings have suggested that tannase-mediated biotransformation of green tea extracts or epigallocatechin gallate *in vitro* modulates the expression of several pro and anti-apoptotic genes to enhance the pro-apoptotic behavior of these natural compounds (Macedo et al., 2012). Although details on these regulatory effects were not reported, an increased bioavailability of GA produced by tannase could account for the observed differences. Besides influencing apoptotic processes, GA modulates the expression of genes involved in cell cycle, angiogenesis, and metastasis inducing death in various cancer cell lines (Verma et al., 2013). Among other processes GA significantly modulates NF-κB, Akt, and ATM kinase signaling pathways to prevent the processes of carcinogenesis and it is a competitive inhibitor of the pro-inflammatory mediator COX-2.

ENHANCING THE ANTI-CARCINOGENIC POTENTIAL OF GALLIC ACID AND PYROGALLOL BY THE GUT TANNIN-DEGRADING BACTERIA COLONIZING COLORECTAL TUMOROUS TISSUES

Gallic acid and PG display anticancer properties, however, it must be noted that its concentration in fruits and vegetables is generally low (Tomas-Barberán and Clifford, 2000). Hence the tannic acid transformation performance by gut tannin-degrading bacteria will likely play a key role to increase the concentration and bioactivity of GA and PG in the colon. The bioaccumulation of GA and PG in microbial biofilms may be another factor contributing to increase the anti-carcinogenic effects of these bioactive compounds. In this respect, biofilm formation on the collagen-rich surfaces accessible in the displaced mucosal epithelium of carcinomas is the most outstanding characteristic of *Streptococcus gallolyticus*, a tannin-degrading bacterium unquestionably associated to CRC (Tjalsma et al., 2012). This bacterium has been proposed to increase, via adhesion, colonization of tumorous tissues by members of *Fusobacterium* spp. [another bacterium with capacity to produce tannase (**Table 1**)] which in turn own capacity to adhere to other bacterial ssp. and attract them to the tumor (Tjalsma et al., 2012). According to this colonization pattern and given bacterial taxa of the microbiome colonizing colon tumor tissues generally own the capacity to produce tannase (**Table 1**), it is conceivable to speculate on the formation of multispecies biofilms on cancerous tissues settled by bacteria able to process dietary tannins. Microbe-generated GA and PG accumulated in these biofilms may thus selectively increase its bioavailability and bioactivity in the tumorous tissues. While this scenario is probably correct for tumorous tissues of late stage CRC patients, it is doubtful to anticipate the same in early and adenomatous stages as the occurrence of tannase-producing opportunistic pathogens in the healthy human gut, such as *S. gallolyticus* or *Fusobacterium* spp., is very rare (Tjalsma et al., 2012). In this regard, unraveling the microbiomes associated to the early and adenomatous stages of CRC will be crucial to define the specific role of tannase-producing bacteria in CRC development. In addition, the identification of the gut microbiota in CRC patients in epidemiological studies addressing the association between dietary tannin intake records and tumor recurrence or regression, may be critical in understanding the role of gut bacteria on the anti-cancer effects of dietary polyphenols.

CONCLUSION

Tannase is an underlying gene function of gut microorganisms that selectively colonize tumorous tissues of late stage CRC patients. Based on the anti-carcinogenic inducing roles reported for GA and PG, the observed colonization pattern suggests that these gut tannin-degrading bacteria are involved in the prevention rather than promotion of tumor progression. Tannase then could be submitted as a link in the yet elusive relationship between dietary factors, CRC-associated gut microbiota and CRC progression. A drawback to this hypothesis is that the ROS-mediated anti-cancer effects of the GA and PG produced by the tannase-producing bacteria naturally colonizing tumorous tissues could be blocked by the high levels of ROS-destroying antioxidants usually possessed by the cancerous cells. Nevertheless, therapeutic microbiology strategies considering the affinity of tannase-producing bacteria for colonic tumorous tissues could be a powerful weapon to efficiently produce ROS-generating drugs that selectively kill CRC cells.

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REFERENCES

Azmi, A. S., Sarkar, F. H., and Hadi, S. M. (2013). Pro-oxidant activity of dietary chemopreventive agents: an under-appreciated anti-cancer property. *F1000Res.* 2:135. doi: 10.12688/f1000research.2-135.v1

Chung, K. T., Lu, Z., and Chou, M. W. (1998). Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food Chem. Toxicol.* 36, 1053–1060. doi: 10.1016/S0278-6915(98)00086-6

Hossain, M. Z., Gilbert, S. F., Patel, K., Ghosh, S., Bhunia, A. K., and Kern, S. E. (2013). Biological clues to potent DNA-damaging activities in food and flavoring. *Food Chem. Toxicol.* 55, 557–567. doi: 10.1016/j.fct.2013.01.058

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130, 781–783. doi: 10.1016/j.cell.2007.06.049

Kyle, J. A., Sharp, L., Little, J., Duthie, G. G., and McNeill, G. (2010). Dietary flavonoid intake and colorectal cancer: a case-control study. *Br. J. Nutr.* 103, 429–436. doi: 10.1017/S0007114509991784

Linder, M. C. (2012). The relationship of copper to DNA damage and damage prevention in humans. *Mutat. Res.* 733, 83–91. doi: 10.1016/j.mrfmmm.2012.03.010

Macedo, J. A., Ferreira, L. R., Camara, L. E., Santos, J. C., Gambero, A., Macedo, G. A., et al. (2012). Chemopreventive potential of the tannase-mediated biotransformation of green tea. *Food Chem.* 133, 358–365. doi: 10.1016/j.foodchem.2012.01.041

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Park, W. H., Han, Y. W., Kim, S. H., and Kim, S. Z. (2007). A superoxide anion generator, pyrogallol induces apoptosis in As4.1 cells through the depletion of intracellular GSH content. *Mutat. Res.* 619, 81–92. doi: 10.1016/j.mrfmmm.2007.02.004

Reverón, I., Rodríguez, H., Campos, H., Curiel, J. A., Ascaso, C., Carrascosa, A. V., et al. (2013). Tannic acid-dependent modulation of selected *Lactobacillus plantarum* traits linked to gastrointestinal survival. *PLoS ONE* 8:e66473. doi: 10.1371/journal.pone.0066473

Sanders, L. M., Henderson, C. E., Hong, M. Y., Barhoumi, R., Burghardt, R. C., Carrol, R. J., et al. (2004). Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility. *Cancer Lett.* 208, 155–161. doi: 10.1016/j.canlet.2003.12.007

Serrano, J., Puupponen-Pimia, R., Dauer, A., Aura, A. M., and Saura-Calixto, F. (2009). Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* 53, 310–329. doi: 10.1002/mnfr.200900039

Tan, H. Y., Sieo, C. C., Abdullah, N., Liang, J. B., Huang, X. D., and Ho, Y. W. (2013). Effect of condensed tannins on bovine rumen protist diversity based on 18S rRNA gene sequences. *J. Eukaryot. Microbiol.* 60, 98–100. doi: 10.1111/jeu.12011

Theodoratou, E., Kyle, J., Cetnarskyj, R., Farrington, S. M., Tenesa, A., Barnetson, R., et al. (2007). Dietary flavonoids and the risk of colorectal cancer. *Cancer Epidemiol. Biomarkers Prev.* 16, 684–693. doi: 10.1158/1055-9965.EPI-06-0785

Tjalsma, H., Boleij, A., Marchesi, J. R., and Dutilh, B. E. (2012). A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat. Rev. Microbiol.* 10, 575–582. doi: 10.1038/nrmicro2819

Tomas-Barberán, F. A., and Clifford, M. N. (2000). Dietary hydroxybenzoic acid derivatives – nature, occurrence and dietary burden. *J. Sci. Food Agric.* 80, 1024–1032. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1024::AID-JSFA567>3.0.CO;2-S

Verma, S., Singh, A., and Mishra, A. (2013). Gallic acid: molecular rival of cancer. *Environ. Toxicol. Pharmacol.* 35, 473–485. doi: 10.1016/j.etap.2013.02.011

WHO Technical Report Series, No. 916 [TRS 916]. (2003). *Diet, Nutrition and the Prevention of Chronic Diseases.* Report of the joint FAO/WHO expert consultation. Geneva: WHO.

You, B. R., and Park, W. H. (2010). Gallic acid-induced lung cancer cell death is related to glutathione depletion as well as reactive oxygen species increase. *Toxicol. In Vitro* 24, 1356–1362. doi: 10.1016/j.tiv.2010.04.009

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REVIEW
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Gut Microbiota Profiling: Metabolomics Based Approach to Unravel Compounds Affecting Human Health

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The gut microbiota is composed of a huge number of different bacteria, that produce a large amount of compounds playing a key role in microbe selection and in the construction of a metabolic signaling network. The microbial activities are affected by environmental *stimuli* leading to the generation of a wide number of compounds, that influence the host metabolome and human health. Indeed, metabolite profiles related to the gut microbiota can offer deep insights on the impact of lifestyle and dietary factors on chronic and acute diseases. Metagenomics, metaproteomics and metabolomics are some of the meta-omics approaches to study the modulation of the gut microbiota. Metabolomic research applied to biofluids allows to: define the metabolic profile; identify and quantify classes and compounds of interest; characterize small molecules produced by intestinal microbes; and define the biochemical pathways of metabolites. Mass spectrometry and nuclear magnetic resonance spectroscopy are the principal technologies applied to metabolomics in terms of coverage, sensitivity and quantification. Moreover, the use of biostatistics and mathematical approaches coupled with metabolomics play a key role in the extraction of biologically meaningful information from wide datasets. Metabolomic studies in gut microbiota-related research have increased, focusing on the generation of novel biomarkers, which could lead to the development of mechanistic hypotheses potentially applicable to the development of nutritional and personalized therapies.

Keywords: gut microbiota, metabolome, state of health, diseases, dietary habits, omic approach

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INTRODUCTION

The gut microbiota is exclusively responsible for several metabolic important functions, including vitamin and short chain fatty acid (SCFAs) production, amino acid (AAs) synthesis, bile acid biotransformation, hydrolysis and fermentation of non-digestible substrates (Putignani et al., 2015). The beneficial effects of gut microbiota include: (*i*) immune-cell homeostasis and development (Th1 vs. Th2 and Th17), (*ii*) epithelial homeostasis, (*iii*) enteric nerve regulation, (*iv*) support of angiogenesis, food digestion, and fat metabolism (Holmes et al., 2011).

The gut microbiota, through metabolite production/fermentation, modulates signaling pathways involved in the homeostasis of intestinal mucosa. When a balanced interaction between the gastrointestinal (GI) tract and the resident microbiota is disrupted, intestinal and extraintestinal

diseases may develop (Putignani et al., 2015), such as allergy, inflammatory bowel disease (IBD), obesity, cancer and diabetes, metabolic disorders, cardiovascular dyslipidemia, and neuropathology (Holmes et al., 2011).

The advent of the omics-based systems biology era has opened a new scenario in the comprehension of the gut ecosystem by shedding light on its shape, modulation and interplay with microorganisms, food functionality, and the role of nutrients in health (Moco et al., 2013; Putignani et al., 2015).

The "omics" technologies are presently applied to: (*i*) determine specific disease markers and novel diagnostic targets; (*ii*) discover functional alterations in the physiopathology of several diseases; (*iii*) discover the relationship between the gut microbiota and the host metabolisms (De Preter and Verbeke, 2013). In particular, the use of metabolomics, being a well-established and powerful top–down systems biology approach, is crucial to unravel the genetics–environment–health relation, as well as the typical clinical biomarkers of the different diseases (Moco et al., 2013). In fact, metabolomics changed the concept that the cellular metabolism profile is complete (Dettmer et al., 2007). Therefore, metabolomics is useful to elucidate the complex interactions of components, to understand the whole system, and to discover new metabolites in order to provide both a different perspective on cellular homeostasis (Liu et al., 2010), and new, unexpected pathways which may have a key physiological role (Zamboni et al., 2015). The metabolomics experiment (sampling, sample preparation, instrumental analysis, data processing, and data interpretation) fulfill the goal of improving the current status of biological information associated to the metabolome and, more generally, to functional genomics (Harrigan and Goodacre, 2003). Nowadays, metabolomics is used to: (*i*) identify biomarkers that could indicate the presence of a diseases, or a response to drug intervention (Dunn and Ellis, 2005); (*ii*) determine biochemical or environmental stresses (Le Gall et al., 2003); (*iii*) characterize microbial metabolism (Vaidyanathan et al., 2002); and (*iv*) characterize human health or disease (Holmes et al., 2011).

Indeed, the metabolomics approach has been applied to several studies on the gut microbiota, mostly focused on the exploration of disease-related metabolites in order to obtain detailed information on the gut metabolic pathways. In fact, the gut microbiota is involved in several biochemical functions directly associated to the perturbation of specific gut microbial populations, which may lead to the development of diseases (De Preter, 2015; De Preter et al., 2015). In other words, as the gut microbiota interacts with the host metabolism and affects physiological or pathological conditions, (**Figure 1**; **Table 1**; Del Chierico et al., 2012) the study of its composition helps discriminate between unhealthy and healthy subjects.

Moreover, the identification of metabolites may highlight how lifestyle and dietary habits affect specific disease conditions (Vernocchi et al., 2012).

Finally, metabolomics represents an unprecedented approach to collect the complex metabolic interactions between the host and its commensal microbial partners, offering the opportunity to define individual and population phenotypes (Moco et al., 2013). In fact, several cellular metabolites are associated with the phenotypes of living organisms (i.e., human, mice, bacteria), and they represent the substrates and products of different biochemical pathways reflecting genetic and environmental factors (Kim et al., 2016).

Furthermore, these data will serve as a basis to comprehend, at the cellular and molecular levels, the relationships between nutritional status and disease risk predisposition, thus allowing to formulate nutritional recommendations.

This review is focused on the application of MS- and NMR-based metabolomic techniques to describe the gut microbiota metabolome and human physiology in relation to nutritional programs and therapies.

VOLATILE AND NON-VOLATILE COMPOUNDS: DETECTION METHODS AND DATA ANALYSIS

Metabolomics uses high throughput techniques to characterize and quantify small molecules in several biofluids (urine, serum, plasma, feces, saliva), revealing a unique metabolic signature (Nicholson and Lindon, 2008).

However, due to the chemical diversity, the different properties of metabolites, and the large dynamic range of metabolite concentrations in samples, it is almost impossible to measure the complete metabolome with only one technique (De Preter and Verbeke, 2013; Smirnov et al., 2016). Considering that the amount of predictable metabolites and derivatives in mammals, plants and bacteria is unknown (Weckwerth and Morgenthal, 2005), there is the need of different analytical platforms and complex integrated computational pipelines, adjusted by analytical and chemical parameters, to cover complete metabolome pathways in the different biofluids (Savorani et al., 2013). Moreover, the collection and preparation of samples, and the selection of the appropriate analytical platforms are fundamental requisites for reproducibility of sample manipulation (Dunn and Ellis, 2005). Besides, the storage and the continuous sample freeze/thawing may alter the composition and stability of the samples and consequently the precision and accuracy of results (Roessner et al., 2000).

Finally, the fundamental requirements of metabolomics studies are: accurate study design; sample treatment and platform set up, corroborated by data analysis; integration of results; and biological interpretation (Smirnov et al., 2016).

Metabolomics can be divided into two different groups: targeted analysis and non-targeted discovery analysis (Dettmer et al., 2007). In particular, the targeted approach is related to the analysis of the different classes of molecules (i.e., carbohydrates, lipids, aminoacids), while non-targeted analysis gives a rapid snapshot of the metabolic profile of samples by using technologies able to detect a wide number of metabolites (Smirnov et al., 2016).

Detection Methods

At present, we are able to separate, detect, characterize, and quantify metabolites and their relevant metabolic pathways thanks to the rapid development of a range of

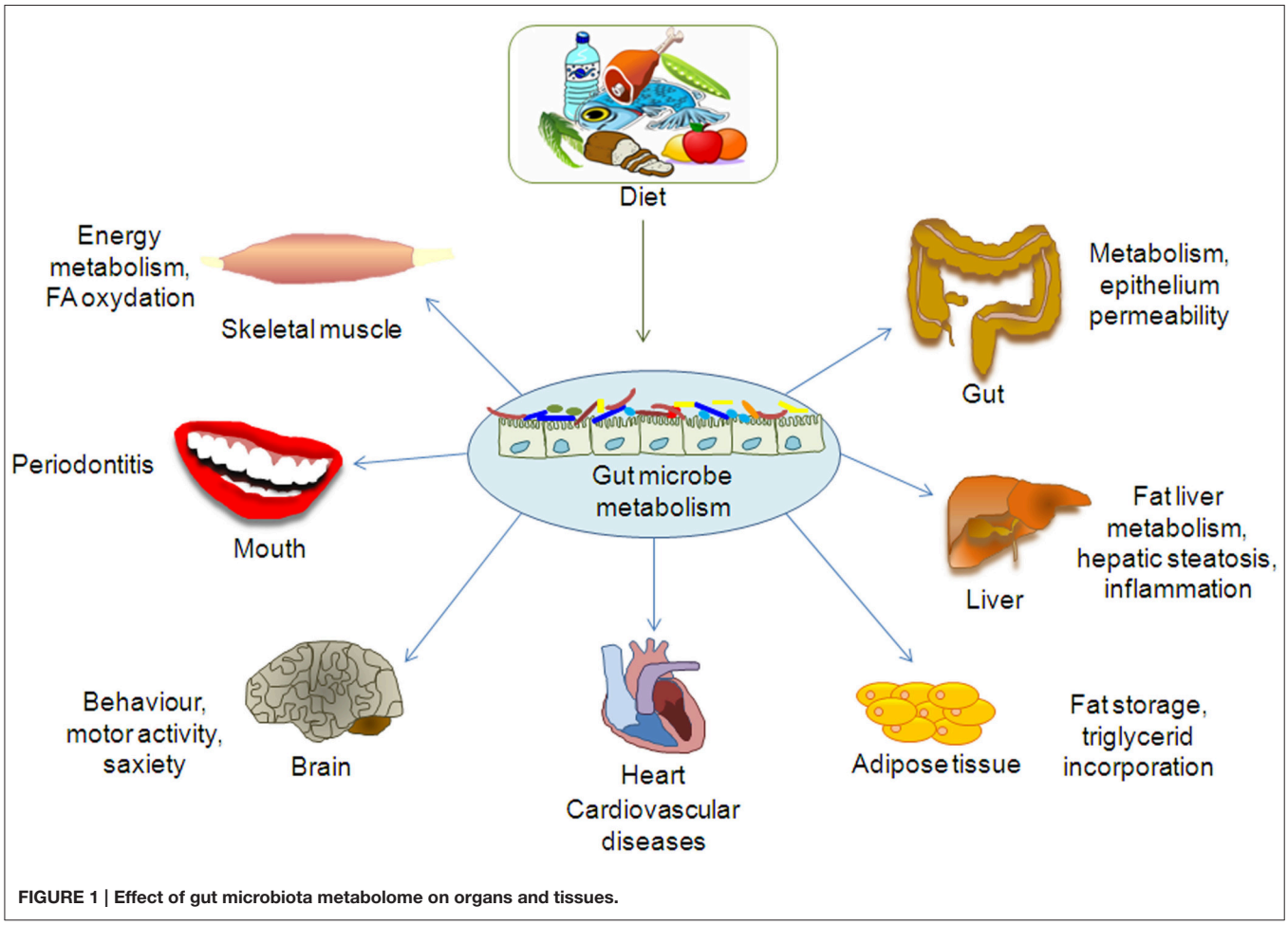

FIGURE 1 | Effect of gut microbiota metabolome on organs and tissues.

TABLE 1 | Role of gut microbiota metabolites on health and disease.

Beneficial microbial activities	Benefits	References	Harmful microbial activities	Drawbacks	References
SCFAs and vitamin production, recovery of energy	Nutrients and energy providing	Putignani et al., 2015	Lipopolysaccharide supply, inflammation	Obesity and metabolic syndrome	Krajmalnik-Brown et al., 2012
Butyrate production, fermentation of non-digestible fibers	Cancer prevention	Louis et al., 2014	Toxins production, inflammation	Cancer promotion	Louis et al., 2014
Antimicrobials production(e.g., bacteriocins, H_2O_2, acids etc.), intestinal pH regulation, competition for ecological niche	Inhibition of pathogens	Kamada et al., 2013	Tissue invasion, inflammation, disruption of the gut barrier/homeostasis	Infectious diseases, leaky gut	Kamada et al., 2013; Michielan and D'Incà, 2015
Anti-inflammatory vs. pro-inflammatory signals development	Normal gastrointestinal immune function	Belkaid and Hand, 2014	Pro-inflammatory vs. anti-inflammatory signals development	IBD, immune disorders	Putignani et al., 2015
Non-digestible carbohydrates metabolism	Normal gut motility	Flint et al., 2012	Metabolism imbalance	IBS, metabolic disease aggravation	Putignani et al., 2015
Propionate production	Gluconeogenesis, cholesterol synthesis inhibition	De Vadder et al., 2014	Acetate production	Cholesterol synthesis, cardiovascular diseases	Krajmalnik-Brown et al., 2012

analytical platforms, including gas chromatography (GC), liquid chromatography (LC), high pressure LC (HPLC), ultra pressure LC (UPLC), Fourier transform infrared spectroscopy (FTIR), ion cyclotrone resonance-FT (ICR-FT), capillary electrophoresis (CE) coupled to mass spectrometry (MS), and nuclear and proton nuclear magnetic resonance spectroscopy (NMR-^{1}H-NMR) (Zheng et al., 2011; Vernocchi et al., 2012).

MS-based metabolomics allows targeted and untargeted metabolome analysis and it has become an indispensable tool in metabolome analysis (Milne et al., 2013); moreover, MS has a broader dynamic range, exhibits a high sensitivity and selectivity (Zhao and Hartung, 2015), and allows determining metabolite fingerprints for establishing metabolome libraries, which facilitate the identification of metabolites (Martins-de-Souza, 2014).

Gas Chromatography Mass Spectrometry

GC-MS is a combined system, with which thermally stable and volatile compounds are separated by GC and then eluting metabolites are detected by electron-impact (EI) mass spectrometers. GC-MS is considered as the gold standard in metabolomics (Harrigan and Goodacre, 2003). Even if GC has several advantages, such as high efficiency, reproducibility and sensitivity, it shows also some drawbacks. In fact, it can only be performed for volatile compounds, or those that can be made volatile, or made stable by derivatization (Roessner et al., 2000; Vernocchi et al., 2012).

Volatile organic compounds (VOCs) are important components of the metabolome (i.e., alcohols, esters, aldehydes, ketones, SCFAs) and are found in biological samples (Mills and Walker, 2001). The sample preparation methods consist of liquid or solid phase extraction (SPE) (Dettmer et al., 2007). Another rapid and solvent-free sample preparation technique is headspace-solid phase microextraction (HS-SPME) (Pawliszyn, 1997), for which the different types of stationary phases (polar and non-polar) used as fiber coatings are commercially available. On the other side, to stabilize the metabolites, two stages of derivatization with different kinds of reagents need to be performed (Roessner et al., 2000). During these processes, small aliquots of samples are analyzed by split or splitless mode on GC columns of various polarities, thus obtaining high chromatographic compound resolution and sensitivity, even if the resulted chromatograms are complex (i.e., multiple derivatization products), contain many metabolite peaks, and need a long run time (longer than 60 min) (Roessner et al., 2000). Therefore, coupling GC with time-of-flight (TOF)-MS, which has high scan rates and produces accurate peak deconvolution of complex samples in faster times, allows improving conventional GC–MS techniques in the analysis of ultra-complex samples (Dunn and Ellis, 2005; Dettmer et al., 2007).

Finally, metabolite quantification is obtained by external calibration or response ratio (peak area of metabolite/peak area of internal standard), while metabolite identification is obtained by matching retention time and mass spectrum of the sample peak with a pure compound previously analyzed, under identical instrumental conditions, with the same or different instruments (Fiehn et al., 2000), or by matching the metabolite against commercial databases (i.e., NIST, WILEY, EPA, NIH).

GC-MS can be used in several fields, such as plant metabolomics, as reported by Stashenko et al. (2004), who used SPME-GC-MS for sampling the volatile plant metabolites, or for example, by Akhatou et al. (2016), who combined GC-MS with multivariate statistical techniques to characterize the primary metabolome of different strawberry cultivars, and to study the influence of multiple agronomic conditions. Moreover, as reported by Currie et al. (2016), GC-MS has proved useful in microbial metabolomics related to pharmaceutical studies to analyze the endogenous metabolite levels produced by *Pseudomonas putida* in response to six pharmaceuticals; or in food studies, when used to characterize the microbial metabolite production in: cheese (Vannini et al., 2008; Pisano et al., 2016), probiotic food (Patrignani et al., 2009; Tabanelli et al., 2015b), sourdough (Guerzoni et al., 2007), wine (Vernocchi et al., 2011; Patrignani et al., 2016), sausages (Tabanelli et al., 2015a), and ready to eat products (Siroli et al., 2015). Moreover, GC-MS is used in clinical applications, for example to analyze volatile compounds (SPME-GC-MS) in urine, blood, feces, hair, breath and saliva (Mills and Walker, 2001), or to evaluate biomarkers in several diseases, such as asthma (Gahleitner et al., 2013; Chang et al., 2015), schizophrenia (Liu et al., 2010), depressive disorders (Ding et al., 2014), ulcerative colitis (Kohashi et al., 2014), and neonatal sepsis (Fanos et al., 2014). In the last years, GC-MS has become one of the most used techniques to study the modulation of gut microbiota as a result of nutrition (i.e., diet, nutraceutical food consumption), diseases, drug, and probiotic administration. Garner et al. (2007) qualitatively and quantitatively analyzed the fecal metabolome to identify potential biomarkers in GI diseases; Di Cagno et al. (2011) characterized the fecal metabolome of celiac children subjected to gluten-free diet, compared to healthy children; Francavilla et al. (2012) evaluated the gut metabolome of allergic children; Vitali et al. analyzed the effects of symbiotic or prebiotic foods and probiotic foods on the human gut metabolome profile (Vitali et al., 2010, 2012); De Filippis et al. evaluated the effects of the Mediterranean diet on the gut microbiota metabolome (De Filippis et al., 2015).

Moreover, De Preter (2015) and De Preter et al. (2015) applied this technique to make a clinical diagnosis of IBD, and to determine the impact of prebiotics on Chron's disease. Del Chierico et al. characterized the gut microbiota of non-alcoholic fatty liver disease (NAFLD), and obese pediatric patients to unravel disease signatures (Del Chierico et al., 2016). Finally, De Angelis et al. analyzed the fecal metabolome of children with autism and pervasive developmental disorders (De Angelis et al., 2013).

Liquid Chromatography Mass Spectrometry

HPLC separation may cover a wide range of compounds' determination, even though its resolution is low. LC is probably the most flexible separation method, as it allows to separate compounds with little effort in few pre-analytical steps (compared to GC-MS) (Moco et al., 2007). Usually,

the metabolite separation obtained with LC is followed by electrospray ionization (ESI) or, to a lesser extent, by atmospheric pressure chemical ionization (APCI) (Bakhtiar et al., 2004). The combination of LC with MS allows to analyze polar, non-polar and neutral compounds separately in a complex matrix (Smirnov et al., 2016). This technique diverges from GC-MS for the lower temperatures of analysis, and it does not require sample volatility, thus entailing an easier sample preparation (Dunn and Ellis, 2005).

LC/MS is an excellent technique showing sensitivity, specificity, resolving power, and capability to extract additional information about metabolites from their retention time (RT) domain (Forcisi et al., 2015).

Sample derivatization is commonly not necessary, although it can be helpful to improve the chromatographic sensitivity and resolution (Leavens et al., 2002), or to produce ionisable groups of metabolites otherwise not detectable by electrospray ionization (ESI) MS. Metabolite quantification is obtained by external calibration or response ratio, and metabolite identification is more time intensive. Moreover, ESI does not produce molecular ion fragmentation as it occurs by electron impact MS, so it does not provide direct metabolite identification by ESI mass spectra comparison, as ESI mass spectral libraries are not generally available. Nevertheless, accurate mass measurements can be obtained by coupling MS/MS using metabolite identification (Lenz et al., 2004). The advent of HPLC and UPLC allowed to shorten the analyzing time, provided higher resolution, sensitivity and efficiency, and permitted to reduce the quantity of samples and solvent necessary for the analysis (Smirnov et al., 2016).

The application of LC/MS allows the identification of target metabolites within a complex sample, not only with the information about monoisotopic mass, but also providing advice on the metabolite structure (Villas-Bôas et al., 2005).

LC-MS applications mainly concern the clinical and pharmaceutical fields (Bakhtiar et al., 2004). Nardotto et al. (2016) used LC/MS/MS systems to investigate patients with type 2 diabetes mellitus treated with an oral dose of racemic carvedilol, who showed accumulation in plasma. Mueller et al. applied this technique to measure plasma concentrations of trimethylamine-N-oxide, betaine and choline in the evaluation of patients with suspected coronary artery disease (Mueller et al., 2015).

An example of LC and GC technologies' combination, is also given by Chow et al. who studied the fecal metabolome, including the application of non-targeted metabolomics to separate breast-fed from formula-fed infants by using GC/MS and LC/MS/MS analysis to identify the various metabolites undergoing change (Chow et al., 2014).

Capillary Electrophoresis Mass Spectrometry

CE may offer high-analyte resolution and detect a wider spectrum of (polar) compounds compared to HPLC, but it is properly applicable only to charged analytes (Ramautar et al., 2013). However, only a few studies on this subject have been published to date, such as Soga et al. (2003), who separated cationic, anionic nucleotidis, and CoA metabolites to describe the coverage of the metabolome. These authors analyzed 1692 metabolites in bacterial extract (Chow et al., 2014).

Fourier Transform Infrared Spectroscopy

FT-IR spectroscopy allows rapid, non-destructive and high-throughput determination of different sample types. In particular, it can simultaneously detect different molecules, such as lipids and fatty acids (FAs), proteins, peptides, carbohydrates, polysaccharides, nucleic acids, (Harrigan and Goodacre, 2003; Dole et al., 2011), but sensitivity and selectivity of this technique are not high. On the contrary, ICR-FT/MS offers an ultrahigh mass resolution able to distinguish slight variations in a wide number of mass signals (Rosselló-Mora et al., 2008), and allowing to obtain the structural identification of new biomarkers (Jansson et al., 2009). In fact, Jansson et al. (2009) used ICR-FT/MS to distinguish between the masses of fecal metabolites in Chron's disease patients and healthy subjects.

Furthermore, FT-IR is principally useful for the identification of functional groups (Vernocchi et al., 2012). In fact, FT-IR has been used to assist infrared imaging in the diagnosis of many diseases, such as Parkinson, cancer, Alzheimer, kidney stone, arthritis (Dole et al., 2011), diabetes, and early stage insulin resistance (Chen et al., 2008).

Moreover, it is possible to combine the LC and FT-IR techniques, as performed by Walker et al. (2014), who identified taurine and sulfate conjugated fatty acids in feces of diabetic mice by coupling ICR-FT/MS and UPLC-MS.

Finally, MS-based metabolomic techniques offer high selectivity and sensitivity for metabolites' identification and quantification. In fact, they are considered as the most appropriate techniques for the detection of large numbers of metabolites, and, in combination with advanced and high-throughput platforms, they may help decrease the complexity of metabolite separation (Zheng et al., 2011; Zhao and Hartung, 2015). In particular, for the total screening of the small molecules in a biological system, MS non-targeted metabolomics is a powerful tool for the identification of metabolite signals present in spectra (Naz et al., 2014). The identification can be partly completed by matching against metabolite and spectral databases, such as METLIN (Smith et al., 2005), HMDB (Wishart et al., 2013), or ChemSpider (Pence and Williams, 2010). To describe the metabolic pathway of biological systems, it is also possible to refer to databases such as KEGG (Kanehisa et al., 2007), or Meta-Cyc (Caspi et al., 2007).

Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy, instead, uses the intramolecular magnetic field around atoms in molecules to change the resonance frequency, thus allowing access to details of molecules' electronic structure and obtaining information about their dynamics, reaction state, and chemical environment. Moreover, a minimal sample preparation is necessary for biofluids, except for feces or gut luminal content, which require the removal of undigested material, dead microbes and other particles (Smirnov et al., 2016). For feces in particular, samples are prepared using methanol (for lipophilic compounds as lipids, cholate, small

phenolic acids) or water (for aminoacids, glucose, glycerol) (Jacobs et al., 2008; Lamichhane et al., 2015).

NMR spectroscopy is mainly useful to determine metabolic fingerprints leading to the identification and quantification of compounds in a non-targeted large-scale, in a non-destructive way, and with a high reproducibility (Lenz et al., 2004; Smolinska et al., 2012).

However, it is a relatively insensitive technique, and can only detect metabolites in high concentrations. The annotation is restricted to a limited number of low molecular weight molecules, and this is the major pitfall of this application (Jansson et al., 2009; Zhao and Hartung, 2015). Indeed, sensitivity depends on the natural concentration of the atoms in the matrix and, to improve sensitivity, long times of analysis, higher magnetic fields, and cryogenic probes are needed (Keun et al., 2002).

Another type of NMR spectroscopy is ^{1}H (proton) NMR, which is unbiased to particular metabolites (Dunn and Ellis, 2005), unlike the other techniques discussed above. The NMR spectrum (chemical shift) depends on the shielding from electrons orbiting the nucleus, whereas for ^{1}H-NMR the chemical shift is arranged as the difference between the resonance frequency of the observed proton and that of a reference proton in a reference metabolite (tetramethylsilane in solution, set at 0 ppm) (Dunn and Ellis, 2005).

The obtained spectra are complex and contain a wide number of signals, and frequently pure metabolites can be added to give a more in-depth clarification. This technique is frequently employed in clinical and pharmaceutical research and applications, in particular in the analysis of biofluids and tissues, where ^{1}H-NMR is used to detect the modulation of metabolites in response to cellular stresses (Lindon et al., 2003). This has also been reported by Bro et al. (2015), who used plasma to determine breast cancer biomarkers, or by Villaseñor et al. (2014), who described the global metabolic phenotyping of acute pancreatitis, and by Dumas et al. (2016), who used this technique to study metabolic syndrome and fatty liver disease.

Moreover, when there is the need to understand how the diet or other external *stimuli* or diseases affect the microbiome composition (i.e., gut, urine, salive), metabolite detection is performed using this functional technique. In fact, several research studies have been conducted using NMR and ^{1}H-NMR, for instance: Ndagijimana et al. (2009) described the effects of symbiotic food on human gut metabolic profile; Martin et al. (2012) studied the influence of gut metabolome on health and diseases; Bjerrum et al. (2015) investigated the gut metabolic biomarkers characterizing Chron's disease, ulcerative colitis and healthy subjects; Zhang et al. (2015) studied how the gut microbiota metabolome could alleviate obesity in children; Laghi et al. (2014) studied the antibiotic effect on vaginal microbiome; and Holmes et al. (2008) analyzed urine samples to discriminate metabolites across populations in order to identify major risk factors for coronary heart disease and stroke.

Hence, MS and ^{1}H-NMR are by far the most frequently applied and the most powerful techniques in metabolomics (Collino et al., 2012). Recent advances in NMR and MS have allowed to evaluate at the same time thousands of metabolites related to the "metabolome," and to define the end-points of metabolic processes in living systems (Nicholson et al., 2005).

In particular, ^{1}H-NMR is currently the most used analytical technique for metabolite profiling compared to MS, while the combination of ^{1}H-NMR and MS technologies would result in a better coverage of the complete metabolome (Bjerrum et al., 2015; De Preter, 2015; Wissenbach et al., 2016). In fact, by coupling metabolite separation technologies with spectrometry and spectroscopy, it is possible to reach a multidimensional approach leading to the structural identification of new metabolites (Chen et al., 2008).

However, the challenge for metabolomics is not only to discover unknown chemical structures, but also to generate meta-information, (i.e., sample origin, tissue, experimental conditions) in an accessible format (Weckwerth and Morgenthal, 2005). Thus, the structural identification of metabolites as potential biomarkers associated with diseases will be a major task of biological interpretation (Nassar and Talaat, 2004). In fact, small molecule metabolites are able to provide new mechanistic information on novel disease biomarkers, which is extremely important, given the paucity of existing markers. Moreover, metabolomics can induce significant progress in the identification of metabolomic fingerprints (which could be used as crucial diagnostic biomarkers) by producing a comprehensive map of metabolic pathway regulations, which represent the downstream expression of genome, transcriptome, and proteome. This comprehensive map may help define the phenotype of an organism at a specific time (Zhang et al., 2012). Therefore, the analysis of metabolic differences between unperturbed and perturbed pathways could provide insights on the underlying disease prognosis and diagnosis (Zhang et al., 2012; **Figure 2**).

Data Analysis

Hence, statistic and bioinformatic techniques are used for data mining complex metabolic profiles containing information related to genetics, environmental factors, gut microbiota activity, lifestyle, and eating habits. These strategies support the complicated process of identifying new biomarkers, which could indicate the individual response to specific physiological factors and/or nutritional interventions, and manage the relevant biological outcomes (Moco et al., 2013).

The application of biostatistics and mathematical approach has a key role in the extraction of biologically meaningful information from wide datasets. In computational analysis, the problems derive from a small batch of samples in contrast with the high number of detected metabolites, and in the consequent high dimensionality of the data matrix (Weckwerth and Morgenthal, 2005). Therefore, different statistical tools can be employed to discriminate among the samples and within the sample set (Worley and Powers, 2012).

In particular, diverse univariate and multivariate methods can be used as parametric (i.e., Student *t*-test, multivariate linear regression) or non-parametric (i.e., Mann-Whitney, Kruskal-Wallis) tests. Moreover, these methods can be divided into unsupervised techniques (i.e., principal component analysis (PCA), hierarchical cluster analysis), and supervised techniques

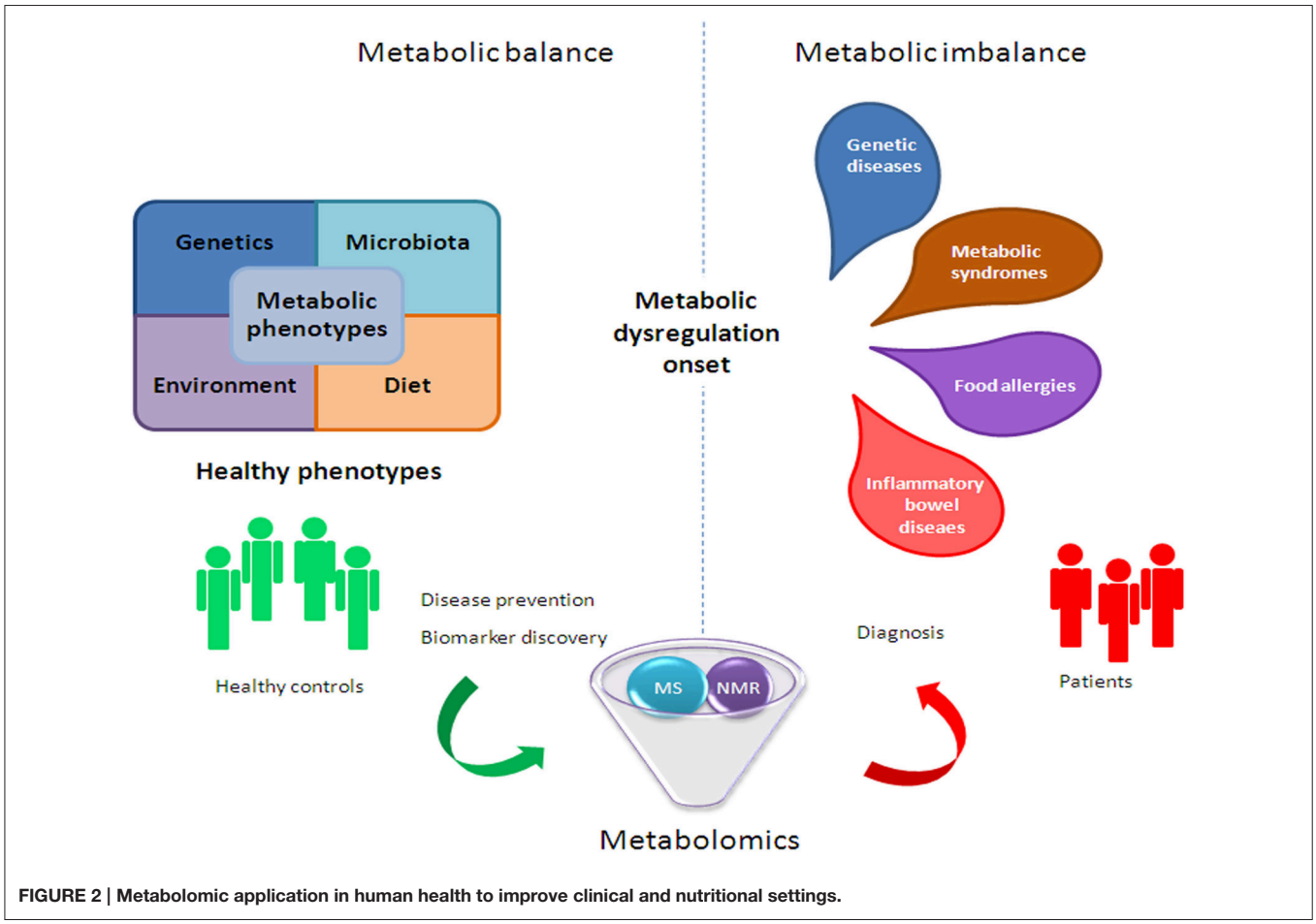

FIGURE 2 | Metabolomic application in human health to improve clinical and nutritional settings.

(i.e., linear discriminant analysis, k-nearest neighbor). Among the supervised multivariate techniques, the partial least squares discriminant analysis (PLS-DA) has proved to be a particularly useful tool, in the presence of an irresolute sample separation obtained from PCA as it offers the possibility to use *a priori* information based on replicates (Raamsdonk et al., 2001; Gromski et al., 2015). PLS-DA is also used for its ability to identify potential biomarkers.

Moreover, statistical analyses of multivariate datasets allow to visualize the biological and molecular consistency. This consistency is based on the correlated functioning of metabolites in response to external conditions. Finally, correlation networks represent fingerprints of biochemical interactions, like the regulation of enzyme activity, and the interplay of anabolism and catabolism between the host and gut microbes (Weckwerth and Morgenthal, 2005).

METABOLITES ASSOCIATED WITH MICROBIAL METABOLISM OR MICROBIAL–HOST COMETABOLISM

The gut microbiota operates in a combined way with the host through the metabolic communication enacted by the different bacterial genera and species responsible for metabolite production (**Table 2**).

Bacterial Metabolism

SCFAs

Several substances present in the large intestine, including indigestible oligosaccharides, dietary plant polysaccharides or fibers, non-digested proteins and intestinal mucin are fermented by the microbiota populations to produce SCFAs (Arora and Sharma, 2011). In particular, clostridial clusters IV, XIVa (e.g., *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus* spp.) and *Lactobacillus* belonging to the phylum Firmicutes and the groups of Actinobacteria (*Bifidobacterium* spp.) are the main bacteria playing a central role in SCFAs metabolism (Nicholson et al., 2012; van Zanten et al., 2014).

Acetate is an important SCFA present in the colon, which could have a trophic effect on the colonic epithelium not only by local action, but also by raising the mucosal blood flux. Moreover, after transport to the portal circulation across the colonic mucosa, acetate passes through the liver and is regained in peripheral blood. Acetate's effect at the ileal level exceeds that of mixed SCFA (Scheppach, 1994). However, acetate is adsorbed by tissues involved in the rise of cholesterol synthesis (Scheppach et al., 1991). On the other side, propionate inhibits cholesterol

TABLE 2 | Metabolites associated with microbial metabolism or microbial–host cometabolism.

Metabolites	Bacteria	Biological functions	References
BACTERIAL METABOLISM			
SCFAs: acetate, propionate, butyrate; branched CFAs: iso-butyrate, valerate and iso-valerate	Clostridial clusters IV and XIVa *Lactobacillus*, *Eubacterium*, *Roseburia*, *Faecalibacterium*, *Coprococcus*	Increasing cholesterol synthesis (acetate); gluconeogenesis (propionate); energy source for colonocytes (butyrate); cholesterol synthesis inhibition; linked to: cardiovascular disease, ulcerative colitis, Crohn's disease, antibiotic-associated diarrhea, obesity, metabolic syndrome, bowel disorders and cancer	Harig et al., 1989; Scheppach et al., 1991; Scheppach, 1994; Sabatino et al., 2005; Binder, 2010; Donohoe et al., 2011; Fukuda et al., 2011; Nicholson et al., 2012; Chambers et al., 2015
Organic acids: benzoate, hippurate, phenylacetate, phenylpropionate, hydroxybenzoate, hydroxyphenylacetate, hydroxyphenylpropionate 3,4-dihydroxyphenylpropionat and D-lactate	*Clostridium difficile*, *Faecalibacterium prausnitzii*, *Bifidobacterium*, *Subdoligranulum*, *Lactobacillus*	Related to hypertension and obesity, colorectal cancer, autism in children in humans and diabetes in a rat model	Lord and Bralley, 2008; Calvani et al., 2010; Qiu et al., 2010; Zhao et al., 2010; Zheng et al., 2011; Nicholson et al., 2012
Vitamins: vitamin B9, vitamin B2, vitamin B12, niacin, pyridoxine, vitamin K, vitamin B1, vitamin B5, vitamin B8	*Bifidobacterium bifidum, Bifidobacterium longum* subsp. *infantis, Bifidobacterium breve*, *B. longum* subsp. *longum Bifidobacterium adolescentis*, commensal Lactobacilli, *Bacillus subtilis Escherichia coli* and anaerobes, Bacteroidetes, Fusobacteria, Proteobacteria, Actinobacteria	Cellular metabolism	Deguchi et al., 1985; Noda et al., 1994; Roth et al., 1996; Bacher et al., 2000; Perkins and Pero, 2002; Stanton et al., 2005; Pompei et al., 2007; Smith et al., 2007; Rossi and Amaretti, 2010; Magnúúsdóóttir, et al., 2015
BACTERIAL TRANSFORMED COMPOUNDS			
Bile salts: cholate, hyocholate, deoxycholate, chenodeoxycholate, α-muricholate, β-muricholate, ω-muricholate, taurocholate, glycocholate, taurochenoxycholate, glycochenodeoxycholate, taurocholate, tauro–α–muricholate, tauro–β–muricholate, lithocholate, ursodeoxycholate, hyodeoxycholate, glycodeoxylcholate, taurohyocholate, taurodeoxylcholate	*Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Eubacterium*, *Escherichia*	Absorption of dietary fats and lipid-soluble vitamins, facilitate lipid assimilation, maintain gut barrier function, regulate triglycerides, cholesterol and glucose by endocrine functions and energy homeostasis. Secondary bile salts linked to colon cancer.	Lis et al., 1976; Russell and Setchell, 1992; Groh et al., 1993; Ridlon et al., 2006; Dawson et al., 2009; Suhre et al., 2010; Nicholson et al., 2012
Polyphenol: Hydroxycinnamic acids and flavonoids	*Lactobacillus*, *Bifidobacterium*	Secondary metabolites production	Couteau et al., 2001; Clifford, 2004; Manach et al., 2004; Taverniti and Guglielmetti, 2012; Amaretti et al., 2015; Marín et al., 2015; Raimondi et al., 2015
Lipids: glycerol	*Bifidobacterium*, *Roseburia*, *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Clostridium*	Intestinal permeability, glucose homeostasis, promotion of chronic systemic inflammation by LPS; hyperinsulinemia improvement by conjugated FAs, immune system enhancement and lipoprotein profiles alteration.	Holmes et al., 2011; Nicholson et al., 2012
Amino Acids	Colonic bacteria*, Clostridium, Peptostreptococcus anaerobius*	ammonia production by deamination, amines production by decarboxylation	Moss et al., 1970; Clinton et al., 1988; Macfarlane and Macfarlane, 1995

synthesis (Scheppach, 1994; Wong et al., 2006). In fact, substrates that can decrease the acetate/propionate ratio may diminish serum lipids and consequently decrease the risk of cardiovascular disease (Wong et al., 2006). Butyrate represents the major energy source for colonocytes and has been studied for its role in nourishing the colonic mucosa and preventing colon cancer by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes (Scheppach, 1994; Velázquez et al., 1997; Walton et al., 2013).

Furthermore, butyrate improves insulin sensitivity and raises energy consumption in obese mice submitted to dietary regimen (Gao et al., 2009), and butyrate irrigation (enema) improves inflammation in diversion colitis (Scheppach et al., 1992; Wong et al., 2006). Butyrate and propionate, but not acetate, induce the production of gut hormones and reduce food intake (Lin et al., 2012). The treatment with acetate induces a marked reduction in lipid accumulation in the adipose tissue, protects against accumulation of fat in the liver, and improves glucose tolerance (Yamashita et al., 2007). In obese subjects, propionate significantly increases the release of post-prandial plasma peptide YY and glucagon-like peptide-1 from colonic cells, and reduces energy intake (Chambers et al., 2015). Chambers et al. (2015) found that inulin-propionate ester administrated to overweight adults significantly reduced weight gain, intra-abdominal adipose tissue distribution, and intrahepatocellular lipid content, and improved insulin resistance in the inulin control group.

Furthermore, other clinical studies demonstrated that the administration of SCFAs has a positive effect on the treatment of ulcerative colitis, Crohn's disease, and antibiotic-associated diarrhea and obesity, metabolic syndrome, bowel disorders, and cancer (Harig et al., 1989; Sabatino et al., 2005; Binder, 2010; Donohoe et al., 2011; Fukuda et al., 2011; Chambers et al., 2015). The degradation of proteins and amino acids by gut microbes also forms small amounts of branched chain FAs (iso-butyrate, valerate and iso-valerate) (Macfarlane and Gibson, 2004). SCFAs can be detected by using both the GC-MS and ^{1}H-NMR spectroscopy techniques.

Organic Acids

Several organic acids result from bacterial metabolism of dietary polyphenols or unassimilated AAs or carbohydrates (Lord and Bralley, 2008). High levels of organic acids in urines are associated with microbial overgrowth (Lord and Bralley, 2008). In particular, the hyperproduction of organic acids is associated with the overgrowth of *Clostridium difficile*, *Faecalibacterium prausnitzii*, *Bifidobacterium* spp., *Subdoligranulum* spp., *Lactobacillus* (Lord and Bralley, 2008; Nicholson et al., 2012).

Amongst organic acids, urinary hippuric acid may be a biomarker of hypertension and obesity in humans, while urinary 4-hydroxyphenylacetate and phenylacetate are potential biomarkers of colorectal cancer (Nicholson et al., 2012).

Lactic acid is the main product in the lactic acid bacteria (LAB) fermentation process. LAB are Gram+ and constitute a heterogeneous group of microorganisms that can also produce proteinaceous antimicrobial molecules, known as bacteriocins, that can help the producer microorganism to outcompete other bacterial species (Alvarez-Sieiro et al., 2016). Moreover, lactic acid represents a secondary metabolite that can be converted by clostridial cluster XIVa species into butyrate, or by clostridial cluster IX into propionate (Louis et al., 2007), thus inducing benefits by inhibiting both the propagation of harmful bacteria, and the production of putrefactive intestinal products. Lactic acid also participates in the intestinal peristalsis regulation (Sugawara et al., 2016). Furthermore, lactic acid is correlated to healthy vaginal microbiota, in fact it decreases in bacterial vaginosis, and it's produced by microbial species such as *Lactobacillus crispatus* and *Lactobacillus jensenii* (Vitali et al., 2007, 2015; Cruciani et al., 2015; Srinivasan et al., 2015). The detection of organic acids is most commonly obtained using LC-MS and ^{1}H-NMR spectroscopy platforms.

Vitamins

Vitamins are indispensable micronutrients, essential for biochemical reactions in all organisms. Humans are unable to synthesize most vitamins, hence, most of them need to be obtained exogenously, and some are produced by the gut microbiota (Stanton et al., 2005; Rossi and Amaretti, 2010).

Recently, Magnúsdóttir et al. (2015), using the PubSEED platform, assessed the genomes of 256 human gut bacteria involved in the biosynthesis of eight B-vitamins: biotin, folate, cobalamin, niacin, pantothenate, riboflavin, pyridoxine and thiamin. In particular, the authors demonstrated that each of the reported vitamins was produced by 40–65% of the 256 human gut microbes (Magnúsdóttir et al., 2015). Moreover, the absorption of some vitamins occurs in the small intestine after conjugation of vitamins with molecules (intrinsic factors) which are produced in the stomach. Since some vitamins are synthetized by the colonic microbiota, they are not adsorbed by the colon but are excreted in feces (Wilson, 2005).

Bifidobacteria strains have been recognized to be the strongest vitamin producers (Deguchi et al., 1985; Noda et al., 1994; Pompei et al., 2007), and in particular Bifidobacteria and Lactobacilli have been proposed as possible folate producers (Pompei et al., 2007; Kleerebezem and Vaughan, 2009). Folate (vitamin B9) is involved in various essential metabolic functions, such as DNA replication, repair and methylation, and synthesis of nucleotides, vitamins and certain AAs (LeBlanc et al., 2013). Folate is contained in leaf vegetables, cereals and liver.

The biosynthesis of thiamin (vitamin B1) consists of two pathways that unite in the final step of thiamin monophosphate production. Although thiamin diphosphate is the functional version of thiamin, all phyla (in particular Bacteroidetes and Fusobacteria), except Firmicutes are producers of thiamin monophosphate (Magnúsdóttir et al., 2015). Vitamin B1 is contained in pork meat, oatmeal, brown rice, vegetables, potatoes, liver, and eggs.

Biotin (vitamin B8) can be synthesized *de novo* from two pimeloyl precursors, namely malonyl-ACP and pimelate. Fusobacteria, Bacteroidetes and Proteobacteria synthetize biotin by different biochemical pathways, while Actinobacteria genomes lack the essential role of biotin biosynthesis (Magnúsdóttir et al., 2015). Vitamin B8 is contained in raw egg yolk, liver, peanuts and green leafy vegetables.

Riboflavin (vitamin B2) plays an essential role in cellular metabolism, being the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Li et al., 2014). Microbial riboflavin biosynthesis has been extensively described in *Bacillus subtilis* (Perkins and Pero, 2002) and *Escherichia coli* (Bacher et al., 1996, 2000). Vitamin B2 is contained in dairy products, bananas, popcorn, green beans and asparagus.

Cobalamin (vitamin B12) is the only vitamin that is exclusively produced by microorganisms, particularly by anaerobes (Roth et al., 1996; Martens et al., 2002; Smith et al., 2007).

Besides, the production of niacin and pyridoxine appears to be generated by Lactobacilli used in yogurt, cheese, and fermented foods (Shahani and Chandan, 1979; Alm, 1982).

Pantothenate (vitamin B5) is a coenzyme A (CoA) precursor and it can be synthesized *de novo* from 2-dihydropantoate and b-alanine. Bacteroidetes and several species of Proteobacteria and Actinobacteria have been demonstrated to be CoA producers (Magnúsdóttir et al., 2015). This vitamin is also contained in meat, broccoli, avocado. Detection of this vitamin is mostly obtained using LC-MS and ^{1}H-NMR spectroscopy platforms.

Furthermore, Vitamin K operates as a co-factor for the enzymatic conversion of specific protein glutamyl to γ-carboxyglutamyl residues. The daily requirement of vitamin K is satisfied by dietary intake of phylloquinone and by the production of polyisoprenyl-containing compounds synthesized by the human gut microbiota (Suttie, 1995; Davidson et al., 1998; Martens et al., 2002). Green leafy vegetables such as spinach, egg yolks, and liver contain vitamin K.

Bacterial-Transformed Compounds

Bile Salts

The metabolism of bile salts is a well-known and basic skill of the gut microbiota metabolism, particularly associated to the genera of *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Eubacterium*, and *Escherichia* (Ridlon et al., 2006; Nicholson et al., 2012). Bacteria also contribute to the recovery of bile salts escaping from active transport in the distal ileum (Begley et al., 2006). The gut microbiota chemically modifies bile acids through a wide range of reactions, resulting in the formation of secondary and tertiary bile acids (Bortolini et al., 1997). Bile salts contribute to the absorption of dietary fats and lipid-soluble vitamins, facilitate lipid assimilation, maintain gut barrier function and regulate triglycerides, cholesterol and glucose by endocrine functions and energy homeostasis (Groh et al., 1993; Ridlon et al., 2006; Dawson et al., 2009).

However, bacterial bile salt hydrolysis has recently been considered as a risk factor for the development of colon cancer because it causes the formation of harmful secondary bile salts after an initial deconjugation step (De Boever et al., 2000). The secondary free bile acids can diffuse through the lipid bilayer of the membrane, thus being much more inhibitory for the cells than the conjugated forms (Mayo and van Sinderen, 2010). De Boever et al. (2000) have speculated a plausible mechanism for the protective properties of probiotic *Lactobacillus reuteri*, which could precipitate the deconjugated bile salts by a physical binding, making the harmful bile salts less bioavailable. Bile salts have antimicrobial activity on gut microbes with inhibitory effects on Bacteroidetes and Actinobacteria microbial population (Islam et al., 2011), but high levels of these biomarkers in serum and urine are correlated with liver diseases (Bathena et al., 2015). The LC-MS and ^{1}H-NMR spectroscopy platforms are the main techniques used to detect bile acids.

Polyphenols

Polyphenols are considerably bioactive components in the diet (Manach et al., 2004). Hydroxycinnamic acids and flavonoids are the two major classes of polyphenols. Fruits commonly contain caffeic acid, representing the most abundant hydroxycinnamic acid (Clifford, 2004). In particular, the chemically derived chlorogenic acid is commonly present in apples, berries and kiwifruit, in vegetables such as potatoes (Manach et al., 2004) and, in high concentrations, in coffee (Clifford, 2004).

Recent studies have demonstrated that gut bacteria, including strains of *Lactobacillus* and *Bifidobacterium*, can metabolize chlorogenic acid to form caffeic acid and quinic acid (Couteau et al., 2001; Tavernitti and Guglielmetti, 2012; Amaretti et al., 2015; Marín et al., 2015; Raimondi et al., 2015), while caffeic acid is further metabolized to form the μ-coumaric acid (3-hydroxycinnamic acid), 3-hydroxylphenylacetic acid and dihydroxyphenylpropionic acid (Konishi and Kobayashi, 2004). The 3,4- dihydroxyphenylacetic acid also derives from the colonic catabolism of rutin (Jaganath et al., 2009). Conversely, phenolic acid metabolites of rutin are not produced in germ-free mice, implying that ring-fission products are generated only by intestinal bacteria (Selma et al., 2009; Parkar et al., 2013). The polyphenol detection is performed using LC-MS, GC-MS, and ^{1}H-NMR spectroscopy platforms.

Lipids

Significant amounts of glycerol derive from daily dietary intake and/or from *in situ* microbial production, or from enterocyte desquamation. Some gut bacteria may anaerobically reduce glycerol to 1,3-propanediol, with the production of the intermediate 3-hydroxypropanal. The accumulation of this metabolite leads to the formation of reuterin, which is known for its antimicrobial properties (De Weirdt et al., 2010). Lipids are also involved in intestinal permeability, in the regulation of glucose homeostasis *via* intestine-brain-liver-neural axis, in the promotion of chronic systemic inflammation by LPS, in the improvement of hyperinsulinemia by conjugated fatty acids (FAs), in the enhancement of the immune system, and in the alteration of lipoprotein profiles. *Bifidobacterium*, *Roseburia*, *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Clostridium* genera have been recognized as the main actors in lipid metabolism (Nicholson et al., 2012). The platform constituted of LC-MS, GC-MS, and ^{1}H-NMR spectroscopy is mainly used to perform lipid detection.

Amino Acids

The bacterial fermentation of proteins, occurring in the distal colon, leads to AAs fermentation products having some relevance for health (Macfarlane and Macfarlane, 1995). For instance, AAs deamination produces ammonia, whereas decarboxylation

produces amines, which may have toxicological effects (Silla Santos, 1996). High ammonia concentrations have been found to act as tumor promoters (Clinton et al., 1988). Bacterial degradation of AAs cysteine and methionine leads to the formation of H_2S, which is toxic and has also been reported to be responsible for inhibition of butyrate oxidation in colonocytes (Roediger et al., 1993). Furthermore, the anaerobic fermentation of the aromatic AAs tyrosine and tryptophan by colonic bacteria produces phenols and indoles respectively, which are eventually excreted in the urine (Macfarlane and Macfarlane, 1997). Phenols, such as *p*-cresol, have been proposed to act as procarcinogens in colon cancer (Bone et al., 1976). Interestingly, gut bacterial production of *p*-cresol is significantly related to autism (Clayton, 2012; De Angelis et al., 2013), and *C. difficile* appears to be a significant *p*-cresol producer (Sivsammye and Sims, 1990).

Finally, certain species of *Clostridium* (Moss et al., 1970) and *Peptostreptococcus anaerobius* (Lambert and Moss, 1980) can convert phenylalanine to benzoic acid in a multistep process with phenylpropionic acid (toxic metabolic product) acting as intermediate (Macfarlane and Macfarlane, 1997; Smith and Macfarlane, 1997). The AAs are mainly detected using LC-MS and ^{1}H-NMR spectroscopy techniques, and also using FT-ICR-MS.

BIOLOGICAL ACTION OF THE GUT MICROBIOTA IN HEALTHY AND DISEASED SUBJECTS

In recent years, growing attention has been targeted to the role of the gut microbiota in the pathogenesis of gastrointestinal (GI) diseases (Lozupone et al., 2012). The alteration of the interplay between host and microbes at the gut level stimulates perturbation of the homeostasis and leads to the development of disorders.

The microbial ecosystem undergoes changes when the equilibrium is broken, which leads to the modification of the bacterial metabolic activity, and/or to transfers in the distribution of local bacterial communities. In fact, the phylotype complexity regulates the equilibrium between pathogenic and commensal taxa at the GI interface (Prakash et al., 2011).

The intestinal gut dysbiosis is associated with a plethora of children and adult diseases, including genetic (i.e., cystic fibrosis [CF]), inflammatory (i.e., inflammatory bowel diseases and syndrome [IBDs, IBS], Chron's [CD], ulcerative colitis [UC], and celiac disease), metabolic (i.e., diabetes, obesity and non-alcoholic fatty liver disease [NAFLD]), and allergic (i.e., atopic dermatitis, food allergies) disorders (Del Chierico et al., 2012), and neuropathologies (i.e., autism) (**Figure 2**).

Indeed, metabolomics is an approach allowing to perform a careful diagnosis of diseases, since metabolite profiles have a high resolution power, which enable to separate the groups based on microbial community profiles (Dicksved et al., 2008). Moreover, metabolites represent the terminal enzymatic process signature occurring in the gut, and the molecules within the pathways range allow to distinguish healthy from diseased subjects, as well as among disease phenotypes (Jansson et al., 2009; **Table 3**).

Inflammatory Bowel Disease/Inflammatory Bowel Syndrome (IBD/IBS)

As concerns inflammatory diseases at GI tract, it is well known that the microbiota results to be abnormal both in IBD and IBS, showing decreased levels of Actinobacteria and Firmicutes, and high levels of Proteobacteria compared to healthy subjects (Kinross et al., 2011; Carroll et al., 2012; Mukhopadhya et al., 2012; De Preter et al., 2013).

Irregular microbial fermentation leads to a high production of hydrogen (in IBS), indole, phenols and others (Kumar et al., 2010). In fact, bacteria release volatile organic compounds (VOCs), determined by SPME-GC-MS, as by-products of metabolism. Hence, the rising acceptance of the gut microbiota involvement in the pathogenesis of IBD has led to the use of fecal matrix as a sample to determine metabolite profiling (Walton et al., 2013). Indeed, specific microbial VOCs profiles can provide specific biomarker candidates for diagnostic purposes (Schöller et al., 1997; Lechner and Rieder, 2007; Bunge et al., 2008).

Walton et al. (2013) observed differences among patient categories (IBD, UC, and CD) based on compounds detected in fecal samples, such as SCFAs and their corresponding alcohols, esters, and molecules, such as indoles and phenols, acetone and sulfur compounds.

The concentrations of propanoic and butanoic acids, revealed by using GC-MS, represent a source of energy affecting colonic mucosal growth, and these concentrations were found to be higher in CD subjects, compared to healthy controls (Best and Laposata, 2003), while acids such as oleic, stearic, palmitic, linoleic and arachidonic were higher in the ileum of CD patients (Jansson et al., 2009). Ahmed et al. observed an increase of esters in diarrhea predominant IBS patients using the SPME-GC-MS technique (Ahmed et al., 2013). On the other side, Walton et al. (2013) detected high levels of indole, phenol and *p*-cresol, generally considered to be toxic for the gut, in CD and UC groups compared to controls.

Moreover, Jansson et al. (2009), using FT-ICR-MS, detected several masses related to metabolites within the tyrosine metabolic pathway, which differentiated CD from healthy controls. In particular, dopaquinone (a dopa oxidation product and intermediate in the melanin formation from tyrosine) was significantly elevated in CD patients compared to healthy subjects. The authors also indicated that tryptophan and phenyalanine were related to the ileum CD phenotype. It was also observed, using ^{1}H-NMR spectroscopy, that AAs in CD patients with active disease showed a different profile (i.e., alanine, isoleucine, leucine, and lysine) compared to CD patients in remission (Marchesi et al., 2007). Furthermore, metabolites related to bile acids pathways (i.e., glycocholate) were found in CD patients in remission (Jansson et al., 2009).

Other studies (Marchesi et al., 2007; Bjerrum et al., 2015; De Preter, 2015; De Preter et al., 2015) using ^{1}H-NMR and GC-MS showed a depletion of bacterial products, such as SCFAs, branched chain FAs, dimethylamine and trimethylamine,

TABLE 3 | Correlated microbes and metabolites to diseases and the relative metabolomic platforms.

Disease	Biofluids	Correlated microbes	Correlated metabolites	Platforms	References
IBD/IBS	Feces Urine	Actinobacteria, Firmicutes (*Faecalibacterium prausnitzii*, *Clostridium* clusters XIVa and IV), Proteobacteria *(Escherichia coli*)	IBS: hydrogen and esters; IBD: alcohols, esters, indoles, phenols, acetone, sulfur compounds, propanoic and butanoic acids, phenol and *p*-cresol, hippurate; CD: tyrosine, dopamine, tryptophan, phenylalanine, isoleucine, leucine, lysisne, bile acid (i.e., glycocholate); UC: cadaverine and taurine	SPME-GC-MS; Breath gas analyzer; ^{1}H-NMR; FT-ICR-MS	Best and Laposata, 2003; Marchesi et al., 2007; Jansson et al., 2009; Kumar et al., 2010; Ahmed et al., 2013; Stephens et al., 2013; Walton et al., 2013; Bjerrum et al., 2015; De Preter, 2015; De Preter et al., 2015
Obesity	Urine Serum	Firmicutes (*Clostridium* spp), Proteobacteria, Bacteroidetes, *Bifidobacterium* spp.	hippurate, 4-hydroxylphenylacetic acid, phenylacetylglycine, FFA, BCAA, primary bile acids (i.e., cholic, chenodeoxycholic acid), secondary bile acids (i.e., lithocholic acid)	^{1}H-NMR; LC-ESI-Q-TOF	Veselkov et al., 2009; Respondek et al., 2013; Zhang et al., 2015; Paul et al., 2016
Cystic fibrosis (CF)	Breath condensate	*Pseudomonas aeruginosa, Clostridium* clusters *XIVa and IV, Clostridium acetobutylicum, F. prausnitzii, Eubacterium limnosum, Eubacterium biforme, E coli, Bifodobacterium* spp.	C5–C16 hydrocarbons and N-methyl-2-methylpropylamine ethanol, methanol, acetate, 2-propanol, lactate, dimethyl sulfide and acetone	^{1}H-NMR; GC-TOF-MS	Wang et al., 2006; Robroeks et al., 2010; Montuschi et al., 2012; Scalan et al., 2012; Schippa et al., 2013
Non-alcoholic Fatty Liver Disease (NAFLD)	Feces	*Oscillospira*, Rickenellaceae, *Parabacteroides*, *Bacteroides fragilis, Sutterella*, Lachanospiraceae	ethanol, esters (i.e., ethyl propionate, methyl pentanoate, methyl acetate), 4-Methyl-2-pentanone, 1-butanol and 2-butanoate	SPME-GC-MS	Raman et al., 2013; Del Chierico et al., 2016
Celiac Disease	Feces Serum Urine	*Lactobacillus, Enterococcus*, Bifidobacteria, *Bacteroides*, *Staphylococcus*, *Salmonella*, *Shighella*, *Klebsiella*	acetoacetate, glucose and 3-hydroxybutyric acid, indoxyl sulfate, meta-[hydroxyphenyl] propionic acid, and phenylacetylglycine, 1-octen-3-ol, ethanol and 1-propanol, AAs (i.e., proline, methionine, histidine, and tryptophan, isoleucine, asparagine, valine, creatinine), choline, lactate, methylamine, non-anal, 4-Methyl-2-hexanone, ethyl acetate and pyruvate	Breath gas analyzer; GC-TOF-MS; NMR	Cani et al., 2008; Di Cagno et al., 2011; Calabrò et al., 2014; Francavilla et al., 2014
Food allergies	Feces	Bifidobacteria, Lactic Acid Bacteria (LAB), Bacteroides, Clostridia	SCFAs (i.e., butyric and acetic acid), lactic acid and threonine	GC-MS; NMR	Francavilla et al., 2012
Neuropathology	Feces Serum	*Faecalibacterium*, *Ruminococcus*, *Clostridium*, Lachnospiraceae, Eubacteriaceae, Bacteroidetes, *Alistipes*, *Akkermansia*, Sutterellaceae, Enterobacteriaceae, *Bifidobacterium*	tryptophan–nicotinic acid, sulfur metabolic pathways, indolepyruvate, *p*-cresol and ethyl sulfate	LC-MS; GC-MS; SPME-GC-MS	Kidd, 2002; Oresic et al., 2008; De Angelis et al., 2013; Hsiao et al., 2013

and high levels of AAs, suggesting a breakdown of the normal bacterial ecology that induces dysbiosis, as a cause or a consequence of the disease in IBD. Le Gall et al. (2011), using Denaturing Gradient Gel Electrophoresis (DGGE), discriminated UC patients from controls, and also found a correlation between the gut microbiota composition and the metabolite composition (high levels of cadaverine and taurine). Besides, UC patients with high levels of AAs showed low fecal concentrations of *Faecalibacterium prausnitzii* and therefore a small amount of butyric acid, since this bacteria is an SCFA producer (De Preter et al., 2015).

The correction of some microbial fermentations by antibiotics or diet could improve symptoms and abnormal fermentation, which, in some IBS cases, is believed to underlie food intolerances (Nanda et al., 1989). Furthermore, using NMR spectroscopy analysis of urine, it is possible to discriminate IBD patients from controls based on metabolite content of hippurate (Stephens et al., 2013). The levels of hippurate were found to be lower in IBD patients compared to controls, suggesting that hippurate is a biomarker of IBD. In particular, hippurate or N-benzoylglycine is a mammalian-microbial co-metabolite deriving from the microbial fermentation of dietary aromatic compounds (polyphenols, purines, or aromatic AAs) to benzoic acid, further conjugated to glycine in the liver (Williams et al., 2010).

Obesity

There is evidence that obese and healthy children (HC) show a different gut microbiota profile. Several mechanisms are involved in the energy metabolism regulation and represent the link between the gut microbiota and the metabolic disease pathophysiology, such as energy harvesting from the diet, fat storage regulation, and energy homeostasis of peptide synthesis (Cani and Delzenne, 2009; Krajmalnik-Brown et al., 2012).

In a study on mouse models, Zhang et al. (2011), using LC-ESI-Q-TOF/NMR, found in urine increased excretion of hippurate, 4-hydroxylphenylacetic acid and phenylacetylglycine, and decreased excretion of acetate and lactate, related to body weight gain and to an alteration of gut microbial changes (Veselkov et al., 2009). Moreover, Paul et al. (2016) identified, with the support of ^{1}H-NMR metabolomics, a maternal metabolic signature that may be related to programming offspring obesity risk in rats. In particular, pregnant rats showed high levels of circulating ketone bodies and free FA (FFA), especially associated with gestational diabetes (Catalano, 2010). The branched chains AA (BCAA) have also found to be related to increased insulin resistance (Scholtens et al., 2014). In humans, FFA are transferred through the placenta from mother to child and are used for lipogenesis. FFA, together with the circulating ketons, may play an important role in the early deposition of excess of body fats in offspring. The production of these molecules in serum metabolome is normalized when it is associated to diet enriched with oligrofructosaccharides (FOS) and to gut microbiota modulation (Paul et al., 2016). Respondek et al. (2013) studied the effects of FOS on the composition of the fecal microbiota and the metabolic parameters in animal models of diet-induced obesity (Respondek et al., 2013). The authors firstly found that the strains particularly stimulated by FOS were *Clostridium coccoides*, *Ruminococcus torques,* and *Dorea longicatena*, and the fecal metabolites modulated by the supplementation, analyzed by using LC-ESI-TOF-MS, were primary bile acids (i.e., cholic and chenodeoxycholic acid) and secondary bile acids (i.e., lithocholic acid).

Cystic Fibrosis (CF)

Regarding CF disease, the majority of studies on the microbiota metabolome concern lung and the upper airways. It was observed that in CF the production of isoprene is associated to either Gram+ and Gram- species (Kuzma et al., 1995), while the production of hydrogen cyanide is prevalently associated to *Pseudomonas aeruginosa*, (Carterson et al., 2004; Cody et al., 2009). Robroeks et al. (2010) analyzed exhaled breath samples by GC-TOF-MS, and they managed to discriminate between CF and healthy controls, mainly based on the presence of C5–C16 hydrocarbons and N-methyl-2-methylpropylamine. Barker et al. (2006) reported a significantly lower level of dimethyl sulfide (probably associated to microbial metabolism), detected by GC-MS in CF patients compared to controls. On the other side, Montuschi et al. (2012), using ^{1}H-NMR forexhaled breath condensate (EBC) analysis, detected significantly higher values of ethanol, acetate, 2-propanol and acetone in CF patients, which differentiated them from controls, whereas acetate, ethanol, 2-propanol and methanol were found to be relevant metabolites for distinguishing between patients with stable CF and patients with unstable CF. Moreover, 2-Propanol, which represents an enzyme-mediated product of acetone reduction, was detected in a breath sample of CF colonized by *P. aeruginosa* (Wang et al., 2006). The high level of ethanol in EBC samples of CF, detected by MS based metabolomics, could also be related to the decreased capacity of *P. aeruginosa* to oxidize ethanol to acetate (Wang et al., 2006). On the contrary, elevated acetate concentrations in healthy subjects may reflect resident bacteria in the oral cavity, such as *Streptococcus mutans*, debasing pyruvate into metabolic end products, such as acetate and lactate (Korithoski et al., 2008).

Non-alcoholic Fatty Liver Disease (NAFLD)

Several VOCs, including ethanol, seem to be produced by colonic bacteria and may have toxic effects on the host after intestinal absorption and delivery to the liver *via* the portal vein (Raman et al., 2013). Moreover, Raman et al. (2013), using SPME-GC-MS, identified esters (i.e., ethyl propanoate, butyl butanoate, methyl pentanoate, methyl acetate) in fecal samples of obese NAFLD patients more frequently than in healthy controls.

The bacterial production of SCFAs and ethanol by several gut microbes is well known, but very little is known about bacteria and biochemical pathways that may be involved in ester production in the intestinal microbiota, even though most of the esters linked to NAFLD are derivatives of short chain aliphatic alcohols and carboxylic acids (Raman et al., 2013). Also Del Chierico et al. (2016) evaluated the gut microbiota profiling of NAFLD and obese patients. The authors evidenced, with the support of multivariate analysis, that OTUs such as *Oscillospira*, Ricknellaceae, *Parabacteroides*, *Bacteroides fragilis*, *Sutterella,* and Lachnospiraceae, and metabolites such as 4-Methyl-2-pentanone, 1-butanol and 2-butanone (detected with

SPME-GC-MS), discriminated NAFLD from healthy subjects (Del Chierico et al., 2016).

Celiac Disease

As regards studies on celiac disease, it was clearly shown that metabolic differences between controls and celiac patients exist (Calabrò et al., 2014).

The main differences detected coupling MS-and NMR- based metabolomics approaches in celiac patients compared to controls were lower levels of several AAs, as asparagine, isoleucine, methionine, proline, and valine, and also methylamine, pyruvate, creatinine, choline, methyl glutarate, lactate, lipids, and glycoproteins, and higher levels of glucose and 3-hydroxybutyric acid in serum and acetoacetate in the urine of celiac patients (Calabrò et al., 2014). The same authors also found higher levels of some metabolites related to the gut microbiota in the urine, such as: indoxyl sulfate, meta-[hydroxyphenyl] propionic acid (m-HPPA), and phenylacetylglycine. In fact, M-HPPA in urine mostly originates from the gut microbiota, being one of the many products of the microbial mediated breakdown of plant phenolic compounds, such as caffeic acid and its conjugate chlorogenic acids (Phipps et al., 1998). Besides, Di Cagno et al. (2011) analyzed, using SPME-GC-MS and ^{1}H-NMR, VOCs, and AAs of fecal and urine samples of treated (gluten free diet) celiac children. The samples showed higher levels of free AAs (proline, methionine, histidine, and tryptophan) and lower levels of SCFAs, as butyric, isocaproic, and propanoic acids compared to controls. In this study, it was also found that the levels of some alcohols, such as 1-octen-3-ol, ethanol and 1-propanol were higher in treated celiac children compared to controls and it was hypothesized that when alcohol production is correlated with intestinal bacteria synthesis this may also induce endotoxemia (Cani et al., 2008).

In another study, saliva samples have been analyzed, using SPME-GC-MS, revealing high levels of non-anal, 4-methyl-2-hexanone, and ethyl-acetate in treated celiac children (Francavilla et al., 2014). These findings suggest the presence of microbial metabolic activities at the oral cavity level (by Firmicutes, Actinobacteria and Bacteroidetes) that may also affect the synthesis of VOCs (Kusano et al., 2013).

Food Allergies

Finally, the gut microbiota is believed to be associated with food allergies. In particular, the prevalence of atopic diseases, including eczema and asthma, suggests that the modulation of the immune response mechanisms in the gut can directly affect the development of allergic diseases and the development of tolerance (Watanabe et al., 2003; Penders et al., 2007). Moreover, the advent of dysbiosis during the early post-natal period may further pre-dispose individuals to later inflammatory, immune, and allergic disorders (Francavilla et al., 2012).

However, there is still little scientific evidence on the relation between the gut microbiota metabolome and food allergy. A study by Francavilla et al. (2012) describing the metabolome of infants with cow's milk allergy, was conducted using the combinate of SPME-GC-MS and ^{1}H-NMR techniques on a group of children fed with hydrolyzed formula with no lactose (CMA-NL), and a group of children fed with lactose-containing (CMA-L) formula compared with controls. The authors found that the addition of lactose to the formula resulted in a significant increase of Bifidobacteria and LAB counts, and a decrease of Bacteroides/Clostridia. Consequently, the levels of SCFAs increased, especially for acetic and butyric acids, in controls and CMA-L compared to CMA-NL infants. The same trend was found for lactic acid and threonine.

Neuropathology

The composition of the intestinal microbiota plays a key role in neuro-gastroenterology, which deals with the interactions between the central nervous system and the gut (gut–brain axis). Numerous neuropathological diseases, such as autism spectrum disorder (ASD), are probably associated with the gut microbiota and thus the possibility to influence this connection is alluring (Holmes et al., 2011), even if to date there are still few studies investigating this field. De Angelis et al. (2013) used SPME-GC-MS and ^{1}H-NMR to study the fecal microbiota and the metabolome of children with Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS), and children with ASD compared to healthy controls. The authors found an altered composition of the microbiota and VOCs, which were partially different between children with PDD-NOS and ASD. The main biological significance of this work was related to the increased levels of *Clostridum* in PDD-NOS and ASD and the decreased levels of some health promoting bacteria (i.e., *Bifidobacterium*) and metabolites, such as free AA and SCFAs in PDD-NOS, in ASD children compared to controls (De Angelis et al., 2013). Furthermore, Kidd (2002) found that subjects with ASD together with their non-ASD siblings, presented with a deep alteration in the tryptophan–nicotinic acid and sulfur metabolic pathways (Kidd, 2002; Oresic et al., 2008). Important metabolic phenotype differences were observed between ASD and controls with perturbations in the relative patterns of urinary metabolites related to the gut microbiota (Kidd, 2002).

Hsiao et al. (2013) used GC- and LC-MS platforms to study the oral treatment with human commensal *Bacteroides fragilis* (that corrects gut permeability) to modulate the microbial composition and the related defects in communicative behaviors in mouse models with maternal immune activation (MIA), showing GI barrier defects and microbiota alterations in displaying features of ASD. The authors detected the presence of indolepyruvate 4-ethylphenylsulfate and *p*-cresol in mice serum metabolome, presumably deriving from microbial metabolism. Furthermore, since *B. fragilis* improves intestinal health, it could also have a role in regulating intestinal permeability and metabolic homeostasis (Nicholson et al., 2012).

CONCLUSIONS AND PERSPECTIVE

The challenge of systems medicine is to interpret the body structure as a whole system and not as a sum of single parts (Moco et al., 2013). To pursue this aim, the wide range of top-down systems biology analyses should be used to interpret the metabolic interactions between the host and its gut microbiota, and to comprehend how these interactions affect

the physiological and pathological conditions. Furthermore, the combination of these techniques with genome analysis may lead to a holistic view of the metabolic pathways, which can also be backed up using mathematical models and statistical assessment of data. In fact, by managing data it is possible to achieve a higher level of biological understanding. Therefore, novel algorithms and statistical analysis need to be improved to integrate the "omics" data, and a stochastic model of metabolic networks needs to be introduced to lead to a novel knowledge of co-regulation in biochemical networks.

The metabolomics approach may identify physiological and clinical biomarkers that are not obtainable using targeted methods (Weckwerth and Morgenthal, 2005).

In conclusion, the generation of new gut microbiota biomarkers will offer the chance to associate complex metabolic pathways with the etiology of different diseases, in order to evaluate the causal relationship between metabolites and pathogenesis. Moreover, these novel biomarkers could lead to the development of mechanistic hypotheses that could be targeted to the development of nutritional and personalized therapy tools in early disease prediction in asymptomatic conditions, and enable a more accurate prognosis of the disease progress.

AUTHOR CONTRIBUTIONS

PV, conceived and wrote the manuscript. FD, participated in the writing and produced tables and figures. LP, supervised and reviewed the manuscript.

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REFERENCES

Ahmed, I., Greenwood, R., Costello Bde, L., Ratcliffe, N. M., and Probert, C. S. (2013). An investigation of fecal volatile organic metabolites in irritable bowel syndrome. *PLoS ONE* 8:e58204. doi: 10.1371/journal.pone.0058204

Akhatou, I., González-Domínguez, R., and Fernández-Recamales, Á. (2016). Investigation of the effect of genotype and agronomic conditions on metabolomic profiles of selected strawberry cultivars with different sensitivity to environmental stress. *Plant Physiol. Biochem.* 101, 14–22. doi: 10.1016/j.plaphy.2016.01.016

Alm, L. (1982). Effect of fermentation on B-vitamin content of milk in Sweden. *J. Dairy Sci.* 65, 353–359. doi: 10.3168/jds.S0022-0302(82)82199-1

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D., and Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl. Microbiol. Biotechnol.* 100, 2939–2951. doi: 10.1007/s00253-016-7343-9

Amaretti, A., Raimondi, S., Leonardi, A., Quartieri, A., and Rossi, M. (2015). Hydrolysis of the rutinose-conjugates flavonoids rutin and hesperidin by the gut microbiota and bifidobacteria. *Nutrients* 7, 2788–2800. doi: 10.3390/nu7042788

Arora, T., and Sharma, R. (2011). Fermentation potential of the gut microbiome: implications for energy homeostasis and weight management. *Nutr. Rev.* 69, 99–106. doi: 10.1111/j.1753-4887.2010.00365.x

Bacher, A., Eberhardt, S., Fischer, M., Kis, K., and Richter, G. (2000). Biosynthesis of vitamin b2 (riboflavin). *Annu. Rev. Nutr.* 20, 153–167. doi: 10.1146/annurev.nutr.20.1.153

Bacher, A., Eberhardt, S., and Richter, G. (1996). "Biosynthesis of riboflavin," in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, ed F. C. Neidhardt (Washington, DC: ASM Press), 657–664.

Bakhtiar, R., Ramos, L., and Tse, F. L. S. (2004). Toxicokinetic assessment of methylphenidate (Ritalin) in a 13-week oral toxicity study in dogs. *Biomed. Chromatogr.* 18, 45–50. doi: 10.1002/bmc.290

Barker, M., Hengst, M., Schmid, J., Buers, H.-J., Mittermaier, B., Klemp, D., et al. (2006). Volatile organic compounds in the exhaled breath of young patients with cystic fibrosis. *Eur. Respir. J.* 27, 929–936. doi: 10.1183/09031936.06.00085105

Bathena, S. P., Thakare, R., Gautam, N., Mukherjee, S., Olivera, M., Meza, J., et al. (2015). Urinary bile acids as biomarkers for liver diseases I. Stability of the baseline profile in healthy subjects. *Toxicol. Sci.* 143, 296–307. doi: 10.1093/toxsci/kfu227

Begley, M., Hill, C., and Gahan, C. G. M. (2006). Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* 72, 1729–1738. doi: 10.1128/AEM.72.3.1729-1738.2006

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Best, C. A., and Laposata, M. (2003). Fatty acid ethyl esters: toxic non-oxidative metabolites of ethanol and markers of ethanol intake. *Front. Biosci.* 8, e202–e217. doi: 10.2741/931

Binder, H. J. (2010). Role of colonic short-chain fatty acid transport in diarrhea. *Annu. Rev. Physiol.* 72, 297–313. doi: 10.1146/annurev-physiol-021909-135817

Bjerrum, J. T., Wang, Y., Hao, F., Coskun, M., Ludwig, C., Günther, U., et al. (2015). Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics* 11, 122–133. doi: 10.1007/s11306-014-0677-3

Bone, E., Tamm, A., and Hill, M. (1976). The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. *Am. J. Clin. Nutr.* 29, 1448–1454.

Bortolini, O., Medici, A., and Poli, S. (1997). Biotransformations on steroid nucleus of bile acids. *Steroids* 62, 564–577. doi: 10.1016/S0039-128X(97)00043-3

Bro, R., Kamstrup-Nielsen, M. H., Engelsen, S. B., Savorani, F., Rasmussen, M. A., Hansen, L., et al. (2015). Forecasting individual breast cancer risk using plasma metabolomics and biocontours. *Metabolomics* 11, 1376–1380. doi: 10.1007/s11306-015-0793-8

Bunge, M., Araghipour, N., Mikoviny, T., Dunkl, J., Schnitzhofer, R., Hansel, A., et al. (2008). On-line monitoring of microbial volatile metabolites by proton transfer reaction-mass spectrometry. *Appl. Environ. Microbiol.* 74, 2179–2186. doi: 10.1128/AEM.02069-07

Calabrò, A., Gralka, E., Luchinat, C., Saccenti, E., and Tenori, L. (2014). A metabolomic perspective on coeliac disease. *Autoimmune Dis.* 2014:756138. doi: 10.1155/2014/756138

Calvani, R., Miccheli, A., Capuani, G., Tomassini Miccheli, A., Puccetti, C., Delfini, M., et al. (2010). Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype. *Int. J. Obes.* 34, 1095–1098. doi: 10.1038/ijo.2010.44

Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M., et al. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 57, 1470–1481. doi: 10.2337/db07-1403

Cani, P. D., and Delzenne, N. M. (2009). The role of the gut microbiota in energy metabolism and metabolic disease. *Curr. Pharm. Des.* 15, 1546–1558. doi: 10.2174/138161209788168164

Carroll, I. M., Ringel-Kulka, T., Siddle, J. P., and Ringel, Y. (2012). Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol. Motil.* 24, 521–530, e248. doi: 10.1111/j.1365-2982.2012.01891.x

Carterson, A. J., Morici, L. A., Jackson, D. W., Frisk, A., Lizewski, S. E., Jupiter, R., et al. (2004). The transcriptional regulator AlgR controls cyanide

production in *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 6837–6844. doi: 10.1128/JB.186.20.6837-6844.2004

Caspi, R., Foerster, H., Fulcher, C. A., Kaipa, P., Krummenacker, M., Latendresse, M., et al. (2007). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res.* 36, D623–D631. doi: 10.1093/nar/gkm900

Catalano, P. M. (2010). Obesity, insulin resistance, and pregnancy outcome. *Reproduction* 140, 365–371. doi: 10.1530/REP-10-0088

Chambers, E. S., Viardot, A., Psichas, A., Morrison, D. J., Murphy, K. G., Zac-Varghese, S. E., et al. (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut* 64, 1744–1754. doi: 10.1136/gutjnl-2014-307913

Chang, C., Guo, Z., He, B., and Yao, W. (2015). Metabolic alterations in the sera of Chinese patients with mild persistent asthma: a GC-MS-based metabolomics analysis. *Acta Pharmacol. Sin.* 36, 1356–1366. doi: 10.1038/aps.2015.102

Chen, J., Zhao, X., Fritsche, J., Yin, P., Schmitt-Kopplin, P., Wang, W., et al. (2008). Practical approach for the identification and isomer elucidation of biomarkers detected in a metabonomic study for the discovery of individuals at risk for diabetes by integrating the chromatographic and mass spectrometric information. *Anal. Chem.* 80, 1280–1289. doi: 10.1021/ac702089h

Chow, J., Panasevich, M. R., Alexander, D., Vester Boler, B. M., Rossoni Serao, M. C., Faber, T. A., et al. (2014). Fecal metabolomics of healthy breast-fed versus formula-fed infants before and during *in vitro* batch culture fermentation. *J. Proteome Res.* 13, 2534–2542. doi: 10.1021/pr500011w

Clayton, T. A. (2012). Metabolic differences underlying two distinct rat urinary phenotypes, a suggested role for gut microbial metabolism of phenylalanine and a possible connection to autism. *FEBS Lett.* 586, 956–961. doi: 10.1016/j.febslet.2012.01.049

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Med.* 70, 1103–1114. doi: 10.1055/s-2004-835835

Clinton, S. K., Bostwick, D. G., Olson, L. M., Mangian, H. J., and Visek, W. J. (1988). Effects of ammonium acetate and sodium cholate on N-methyl-N'-nitro-N-nitrosoguanidine-induced colon carcinogenesis of rats. *Cancer Res.* 48, 3035–3039.

Cody, W. L., Pritchett, C. L., Jones, A. K., Carterson, A. J., Jackson, D., Frisk, A., et al. (2009). *Pseudomonas aeruginosa* AlgR controls cyanide production in an AlgZ-dependent manner. *J. Bacteriol.* 191, 2993–3002. doi: 10.1128/JB.01156-08

Collino, S., Martin, F. P. J., and Rezzi, S. (2012). Clinical Metabolomics paves the way towards future healthcare strategies. *Br. J. Clin. Pharmacol.* 75, 619–629. doi: 10.1111/j.1365-2125.2012.04216.x

Couteau, D., McCartney, A. L., Gibson, G. R., Williamson, G., and Faulds, C. B. (2001). Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *J. Appl. Microbiol.* 90, 873–881. doi: 10.1046/j.1365-2672.2001.01316.x

Cruciani, F., Biagi, E., Severgnini, M., Consolandi, C., Calanni, F., Donders, G., et al. (2015). Development of a microarray-based tool to characterize vaginal bacterial fluctuations and application to a novel antibiotic treatment for bacterial vaginosis. *Antimicrob. Agents Chemother.* 59, 2825–2834. doi: 10.1128/AAC.00225-15

Currie, F., Broadhurst, D. I., Dunn, W. B., Sellick, C. A., and Goodacre, R. (2016). Metabolomics reveals the physiological response of *Pseudomonas putida* KT2440 (UWC1) after pharmaceutical exposure. *Mol. BioSyst.* 12, 1367–1377. doi: 10.1039/C5MB00889A

Davidson, R. T., Foley, A. L., Engelke, J. A., and Suttie, J. W. (1998). Conversion of dietary phylloquinone to tissue menaquinone-4 in rats is not dependent on gut bacteria. *J. Nutr.* 128, 220–223.

Dawson, P. A., Lan, T., and Rao, A. (2009). Bile acid transporters. *J. Lipid Res.* 50, 2340–2357. doi: 10.1194/jlr.R900012-JLR200

De Angelis, M., Piccolo, M., Vannini, L., Siragusa, S., De Giacomo, A., Serrazzanetti, D. I., et al. (2013). Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS ONE* 8:e76993. doi: 10.1371/journal.pone.0076993

De Boever, P., Wouters, R., Verschaeve, L., Berckmans, P., Schoeters, G., and Verstraete, W. (2000). Protective effect of the bile salt hydrolase-active *Lactobacillus reuteri* against bile salt cytotoxicity. *Appl. Microbiol. Biotechnol.* 53, 709–714. doi: 10.1007/s002530000330

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2015). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut*. doi: 10.1136/gutjnl-2015-309957. [Epub ahead of print].

Deguchi, Y., Morishita, T., and Mutai, M. (1985). comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. *Agric. Biol. Chem.* 49, 13–19.

Del Chierico, F., Nobili, V., Vernocchi, P., Russo, A., De Stefanis, C., Gnani, D., et al. (2016). Gut microbiota profiling of pediatric NAFLD and obese patients unveiled by an integrated meta-omics based approach. *Hepatology*. doi: 10.1002/hep.28572. [Epub ahead of print].

Del Chierico, F., Vernocchi, P., Bonizzi, L., Carsetti, R., Castellazzi, A. M., Dallapiccola, B., et al. (2012). Early-life gut microbiota under physiological and pathological conditions: the central role of combined meta-omics-based approaches. *J. Proteomics* 75, 4580–4587. doi: 10.1016/j.jprot.2012.02.018

De Preter, V. (2015). Metabolomics in the clinical diagnosis of inflammatory bowel disease. *Dig. Dis. Basel Switz.* 33(Suppl. 1), 2–10. doi: 10.1159/000437033

De Preter, V., Joossens, M., Ballet, V., Shkedy, Z., Rutgeerts, P., Vermeire, S., et al. (2013). Metabolic profiling of the impact of oligofructose-enriched inulin in Crohn's disease patients: a double-blinded randomized controlled trial. *Clin. Transl. Gastroenterol.* 4, e30. doi: 10.1038/ctg.2012.24

De Preter, V., Machiels, K., Joossens, M., Arijs, I., Matthys, C., Vermeire, S., et al. (2015). Faecal metabolite profiling identifies medium-chain fatty acids as discriminating compounds in IBD. *Gut* 64, 447–458. doi: 10.1136/gutjnl-2013-306423

De Preter, V., and Verbeke, K. (2013). Metabolomics as a diagnostic tool in gastroenterology. *World J. Gastrointest. Pharmacol. Ther.* 4, 97–107. doi: 10.4292/wjgpt.v4.i4.97

Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* 26, 51–78. doi: 10.1002/mas.20108

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., et al. (2014). Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* 156, 84–96. doi: 10.1016/j.cell.2013.12.016

De Weirdt, R., Possemiers, S., Vermeulen, G., Moerdijk-Poortvliet, T. C. W., Boschker, H. T. S., Verstraete, W., et al. (2010). Human faecal microbiota display variable patterns of glycerol metabolism. *FEMS Microbiol. Ecol.* 74, 601–611. doi: 10.1111/j.1574-6941.2010.00974.x

Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., et al. (2011). Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. *BMC Microbiol.* 11:219. doi: 10.1186/1471-2180-11-219

Dicksved, J., Halfvarson, J., Rosenquist, M., Järnerot, G., Tysk, C., Apajalahti, J., et al. (2008). Molecular analysis of the gut microbiota of identical twins with Crohn's disease. *ISME J.* 2, 716–727. doi: 10.1038/ismej.2008.37

Ding, X., Yang, S., Li, W., Liu, Y., Li, Z., Zhang, Y., et al. (2014). The potential biomarker panels for identification of major depressive disorder (MDD) patients with and without early life stress (ELS) by metabonomic analysis. *PLoS ONE* 9:e97479. doi: 10.1371/journal.pone.0097479

Dole, M. N., Patel, P. A., Sawant, S. D., and Shedpure, P. S. (2011). Advance applications of fourier transform infrared spectroscopy. *Int. J. Pharm. Sci. Rev. Res.* 7, 159–166.

Donohoe, D. R., Garge, N., Zhang, X., Sun, W., O'Connell, T. M., Bunger, M. K., et al. (2011). The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. *Cell Metab.* 13, 517–526. doi: 10.1016/j.cmet.2011.02.018

Dumas, M. E., Kinross, J., and Nicholson, J. K. (2016). Metabolic phenotyping and systems biology approaches to understanding metabolic syndrome and fatty liver disease. *Gastroenterology* 146, 46–62. doi: 10.1053/j.gastro.2013.11.001

Dunn, W. B., and Ellis, D. I. (2005). Metabolomics: current analytical platforms and methodologies. *TrAC Trends Anal. Chem.* 24, 285–294. doi: 10.1016/j.trac.2004.11.021

Fanos, V., Caboni, P., Corsello, G., Stronati, M., Gazzolo, D., Noto, A., et al. (2014). Urinary 1H-NMR and GC-MS metabolomics predicts early and late onset neonatal sepsis. *Early Hum. Dev.* 90, S78–S83. doi: 10.1016/S0378-3782(14)70024-6

Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R. N., and Willmitzer, L. (2000). Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* 18, 1157–1161. doi: 10.1038/81137

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3, 289–306. doi: 10.4161/gmic.19897

Forcisi, S., Moritz, F., Lucio, M., Lehmann, R., Stefan, N., and Schmitt-Kopplin, P. (2015). Solutions for low and high accuracy mass spectrometric data matching: a data-driven annotation strategy in nontargeted metabolomics. *Anal. Chem.* 87, 8917–8924. doi: 10.1021/acs.analchem.5b02049

Francavilla, R., Calasso, M., Calace, L., Siragusa, S., Ndagijimana, M., Vernocchi, P., et al. (2012). Effect of lactose on gut microbiota and metabolome of infants with cow's milk allergy. *Pediatr. Allergy Immunol.* 23, 420–427. doi: 10.1111/j.1399-3038.2012.01286.x

Francavilla, R., Ercolini, D., Piccolo, M., Vannini, L., Siragusa, S., De Filippis, F., et al. (2014). Salivary microbiota and metabolome associated with celiac disease. *Appl. Environ. Microbiol.* 80, 3416–3425. doi: 10.1128/AEM.00362-14

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547. doi: 10.1038/nature09646

Gahleitner, F., Guallar-Hoyas, C., Beardsmore, C. S., Pandya, H. C., and Thomas, C. P. (2013). Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath. *Bioanalysis* 5, 2239–2247. doi: 10.4155/bio.13.184

Gao, Z., Yin, J., Zhang, J., Ward, R. E., Martin, R. J., Lefevre, M., et al. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* 58, 1509–1517. doi: 10.2337/db08-1637

Garner, C. E., Smith, S., de Lacy Costello, B., White, P., Spencer, R., Probert, C. S. J., et al. (2007). Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *FASEB J.* 21, 1675–1688. doi: 10.1096/fj.06-6927com

Groh, H., Schade, K., and Hörhold-Schubert, C. (1993). Steroid metabolism with intestinal microorganisms. *J. Basic Microbiol.* 33, 59–72. doi: 10.1002/jobm.3620330115

Gromski, P. S., Muhamadali, H., Ellis, D. I., Xu, Y., Correa, E., Turner, M. L., et al. (2015). A tutorial review: metabolomics and partial least squares-discriminant analysis – a marriage of convenience or a shotgun wedding. *Anal. Chim. Acta* 879, 10–23. doi: 10.1016/j.aca.2015.02.012

Guerzoni, M. E., Vernocchi, P., Ndagijimana, M., Gianotti, A., and Lanciotti, R. (2007). Generation of aroma compounds in sourdough: effects of stress exposure and lactobacilli–yeasts interactions. *Food Microbiol.* 24, 139–148. doi: 10.1016/j.fm.2006.07.007

Harig, J. M., Soergel, K. H., Komorowski, R. A., and Wood, C. M. (1989). Treatment of diversion colitis with short-chain-fatty acid irrigation. *N. Engl. J. Med.* 320, 23–28. doi: 10.1056/NEJM198901053200105

Harrigan, G. G., and Goodacre, R. (2003). *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*. Boston, MA: Springer US; Imprint; Springer.

Holmes, E., Li, J. V., Athanasiou, T., Ashrafian, H., and Nicholson, J. K. (2011). Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. *Trends Microbiol.* 19, 349–359. doi: 10.1016/j.tim.2011.05.006

Holmes, E., Loo, R. L., Stamler, J., Bictash, M., Yap, I. K. S., Chan, Q., et al. (2008). Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* 453, 396–400. doi: 10.1038/nature06882

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463. doi: 10.1016/j.cell.2013.11.024

Islam, K. B., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., et al. (2011). Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 141, 1773–1781. doi: 10.1053/j.gastro.2011.07.046

Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A., Bingham, M., Vaughan, E. E., et al. (2008). (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed.* 21, 615–626. doi: 10.1002/nbm.1233

Jaganath, I. B., Mullen, W., Lean, M. E. J., Edwards, C. A., and Crozier, A. (2009). *In vitro* catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. *Free Radic. Biol. Med.* 47, 1180–1189. doi: 10.1016/j.freeradbiomed.2009.07.031

Jansson, J., Willing, B., Lucio, M., Fekete, A., Dicksved, J., Halfvarson, J., et al. (2009). Metabolomics reveals metabolic biomarkers of Crohn's disease. *PLoS ONE* 4:e6386. doi: 10.1371/journal.pone.0006386

Kamada, N., Chen, G. Y., Inohara, N., and Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nat. Immunol.* 14, 685–690. doi: 10.1038/ni.2608

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2007). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, D480–D484. doi: 10.1093/nar/gkm882

Keun, H. C., Beckonert, O., Griffin, J. L., Richter, C., Moskau, D., Lindon, J. C., et al. (2002). Cryogenic probe 13C NMR spectroscopy of urine for metabonomic studies. *Anal. Chem.* 74, 4588–4593. doi: 10.1021/ac025691r

Kidd, P. (2002). Autism, an extreme challenge to integrative medicine. Part: 1: The knowledge base. *Altern. Med. Rev.* 7, 292–316.

Kim, S. J., Kim, S. H., Kim, J. H., Hwang, S., and Yoo, H. J. (2016). Understanding metabolomics in biomedical research. *Endocrinol. Metab. Seoul Korea* 31, 7–16. doi: 10.3803/EnM.2016.31.1.7

Kinross, J. M., Darzi, A. W., and Nicholson, J. K. (2011). Gut microbiome-host interactions in health and disease. *Genome Med.* 3, 14. doi: 10.1186/gm228

Kleerebezem, M., and Vaughan, E. E. (2009). Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. *Annu. Rev. Microbiol.* 63, 269–290. doi: 10.1146/annurev.micro.091208.073341

Kohashi, M., Nishiumi, S., Ooi, M., Yoshie, T., Matsubara, A., Suzuki, M., et al. (2014). A novel gas chromatography mass spectrometry-based serum diagnostic and assessment approach to ulcerative colitis. *J. Crohns Colitis* 8, 1010–1021. doi: 10.1016/j.crohns.2014.01.024

Konishi, Y., and Kobayashi, S. (2004). Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal caco-2 cell monolayers. *J. Agric. Food Chem.* 52, 2518–2526. doi: 10.1021/jf035407c

Korithoski, B., Lévesque, C. M., and Cvitkovitch, D. G. (2008). The involvement of the pyruvate dehydrogenase E1alpha subunit, in Streptococcus mutans acid tolerance. *FEMS Microbiol. Lett.* 289, 13–19. doi: 10.1111/j.1574-6968.2008.01351.x

Krajmalnik-Brown, R., Ilhan, Z.-E., Kang, D.-W., and DiBaise, J. K. (2012). Effects of gut microbes on nutrient absorption and energy regulation. *Nutr. Clin. Pract.* 27, 201–214. doi: 10.1177/0884533611436116

Kumar, S., Misra, A., and Ghoshal, U. C. (2010). Patients with irritable bowel syndrome exhale more hydrogen than healthy subjects in fasting state. *J. Neurogastroenterol. Motil.* 16, 299–305. doi: 10.5056/jnm.2010.16.3.299

Kusano, M., Mendez, E., and Furton, K. G. (2013). Comparison of the volatile organic compounds from different biological specimens for profiling potential. *J. Forensic Sci.* 58, 29–39. doi: 10.1111/j.1556-4029.2012.02215.x

Kuzma, J., Nemecek-Marshall, M., Pollock, W. H., and Fall, R. (1995). Bacteria produce the volatile hydrocarbon isoprene. *Curr. Microbiol.* 30, 97–103. doi: 10.1007/BF00294190

Laghi, L., Picone, G., Cruciani, F., Brigidi, P., Calanni, F., Donders, G., et al. (2014). Rifaximin modulates the vaginal microbiome and metabolome in women affected by bacterial vaginosis. *Antimicrob. Agents Chemother.* 58, 3411–3420. doi: 10.1128/AAC.02469-14

Lambert, M. A., and Moss, C. W. (1980). Production of p-hydroxyhydrocinnamic acid from tyrosine by *Peptostreptococcus anaerobius*. *J. Clin. Microbiol.* 12, 291–293.

Lamichhane, S., Yde, C. C., Schmedes, M. S., Jensen, H. M., Meier, S., and Bertram, H. C. (2015). Strategy for nuclear-magnetic-resonance-based metabolomics of human feces. *Anal. Chem.* 87, 5930–5937. doi: 10.1021/acs.analchem.5b00977

Leavens, W. J., Lane, S. J., Carr, R. M., Lockie, A. M., and Waterhouse, I. (2002). Derivatization for liquid chromatography/electrospray mass spectrometry: synthesis of tris(trimethoxyphenyl)phosphonium compounds and their derivatives of amine and carboxylic acids. *Rapid Commun. Mass Spectrom.* 16, 433–441. doi: 10.1002/rcm.559

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., and Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr. Opin. Biotechnol.* 24, 160–168. doi: 10.1016/j.copbio.2012.08.005

Lechner, M., and Rieder, J. (2007). Mass spectrometric profiling of low-molecular-weight volatile compounds–diagnostic potential and latest applications. *Curr. Med. Chem.* 14, 987–995. doi: 10.2174/092986707780362916

Le Gall, G., Colquhoun, I. J., Davis, A. L., Collins, G. J., and Verhoeyen, M. E. (2003). Metabolite profiling of tomato (Lycopersicon esculentum) using 1H NMR spectroscopy as a tool to detect potential unintended effects following a genetic modification. *J. Agric. Food Chem.* 51, 2447–2456. doi: 10.1021/jf0259967

Le Gall, G., Noor, S. O., Ridgway, K., Scovell, L., Jamieson, C., Johnson, I. T., et al. (2011). Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *J. Proteome Res.* 10, 4208–4218. doi: 10.1021/pr2003598

Lenz, E. M., Bright, J., Knight, R., Wilson, I. D., and Major, H. (2004). Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabonomic investigation using high field 1H NMR spectroscopy, HPLC-TOF/MS and chemometrics. *J. Pharm. Biomed. Anal.* 35, 599–608. doi: 10.1016/j.jpba.2004.02.013

Li, L., Hu, L., Han, L.-P., Ji, H., Zhu, Y., Wang, X., et al. (2014). Expression of turtle riboflavin-binding protein represses mitochondrial electron transport gene expression and promotes flowering in Arabidopsis. *BMC Plant Biol.* 14:381. doi: 10.1186/s12870-014-0381-5

Lin, H. V., Frassetto, A., Kowalik, E. J. Jr., Nawrocki, A. R., Lu, M. M., Kosinski, J. R., et al. (2012). Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS ONE* 7:e35240. doi: 10.1371/journal.pone.0035240

Lindon, J. C., Holmes, E., and Nicholson, J. K. (2003). So what's the deal with metabonomics? *Anal. Chem.* 75, 384A–391A. doi: 10.1021/ac031386+

Lis, A. W., McLaughlin, I., Mpclaughlin, R. K., Lis, E. W., and Stubbs, E. G. (1976). Profiles of ultraviolet-absorbing components of urine from autistic children, as obtained by high-resolution ion-exchange chromatography. *Clin. Chem.* 22, 1528–1532.

Liu, J.-Y., Li, N., Yang, J., Li, N., Qiu, H., Ai, D., et al. (2010). Metabolic profiling of murine plasma reveals an unexpected biomarker in rofecoxib-mediated cardiovascular events. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17017–17022. doi: 10.1073/pnas.1011278107

Lord, R. S., and Bralley, J. A. (2008). Clinical applications of urinary organic acids. Part 2. Dysbiosis markers. *Altern. Med. Rev. J. Clin. Ther.* 13, 292–306.

Louis, P., Hold, G. L., and Flint, H. J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. *Nat. Rev. Microbiol.* 12, 661–672. doi: 10.1038/nrmicro3344

Louis, P., Scott, K. P., Duncan, S. H., and Flint, H. J. (2007). Understanding the effects of diet on bacterial metabolism in the large intestine. *J. Appl. Microbiol.* 102, 1197–1208. doi: 10.1111/j.1365-2672.2007.03322.x

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Macfarlane, G. T., and Gibson, G. R. (2004). "Microbiological aspects of SCFA production in the large bowel," in *Physiological and Clinical Aspects of Short-Chain Fatty Acids*, eds J. H. Cummings, J. L. Rombeau, and T. Sakata (Cambridge; New York, NY: Cambridge University Press), 87–105.

Macfarlane, G. T., and Macfarlane, S. (1997). Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. *Scand. J. Gastroenterol. Suppl.* 222, 3–9. doi: 10.1080/00365521.1997.11720708

Macfarlane, S., and Macfarlane, G. T. (1995). "Proteolysis and amino acid fermentation.," in *Human Colonic Bacteria: Role in Nutrition, Physiology, and Pathology*, eds G. R. Gibson and G. T. Macfarlane (Boca Raton, FL: CRC press), 75–100.

Magnúsdóttir, S., Ravcheev, D., de Crécy-Lagard, V., and Thiele, I. (2015). Systematic genome assessment of B vitamin biosynthesis suggests co-operation among gut microbes. *Front Genet.* 20:148. doi: 10.3389/fgene.2015.00148

Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* 79, 727–747.

Marchesi, J. R., Holmes, E., Khan, F., Kochhar, S., Scanlan, P., Shanahan, F., et al. (2007). Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *J. Proteome Res.* 6, 546–551. doi: 10.1021/pr060470d

Marín, L., Miguélez, E. M., Villar, C. J., and Lombó, F. (2015). Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. *Biomed Res. Int.* 2015:905215. doi: 10.1155/2015/905215

Martens, J. H., Barg, H., Warren, M. J., and Jahn, D. (2002). Microbial production of vitamin B12. *Appl. Microbiol. Biotechnol.* 58, 275–285. doi: 10.1007/s00253-001-0902-7

Martin, F.-P. J., Collino, S., Rezzi, S., and Kochhar, S. (2012). Metabolomic applications to decipher gut microbial metabolic influence in health and disease. *Front. Physiol.* 3:113. doi: 10.3389/fphys.2012.00113

Martins-de-Souza, D. (2014). Proteomics, metabolomics, and protein interactomics in the characterization of the molecular features of major depressive disorder. *Dialogues Clin. Neurosci.* 16, 63–73.

Mayo, B., and van Sinderen, D. (eds.). (2010). *Bifidobacteria: Genomics and Molecular Aspects*. Norfolk: Caister Academic.

Michielan, A., and D'Incà, R. (2015). Intestinal permeability in inflammatory bowel disease: pathogenesis, clinical evaluation, and therapy of leaky gut. *Med. Inflamm.* 2015:628157. doi: 10.1155/2015/628157

Mills, G. A., and Walker, V. (2001). Headspace solid-phase microextraction profiling of volatile compounds in urine: application to metabolic investigations. *J. Chromatogr. B. Biomed. Sci. App.* 753, 259–268. doi: 10.1016/S0378-4347(00)00554-5

Milne, S. B., Mathews, T. P., Myers, D. S., Ivanova, P. T., and Brown, H. A. (2013). Sum of the parts: mass spectrometry-based metabolomics. *Biochemistry (Mosc.)* 52, 3829–3840. doi: 10.1021/bi400060e

Moco, S., Collino, S., Rezzi, S., and Martin, F.-P. J. (2013). Metabolomics perspectives in pediatric research. *Pediatr. Res.* 73, 570–576. doi: 10.1038/pr.2013.1

Moco, S., Vervoort, J., Moco, S., Bino, R. J., De Vos, R. C. H., and Bino, R. (2007). Metabolomics technologies and metabolite identification. *TrAC Trends Anal. Chem.* 26, 855–866. doi: 10.1016/j.trac.2007.08.003

Montuschi, P., Paris, D., Melck, D., Lucidi, V., Ciabattoni, G., Raia, V., et al. (2012). NMR spectroscopy metabolomic profiling of exhaled breath condensate in patients with stable and unstable cystic fibrosis. *Thorax* 67, 222–228. doi: 10.1136/thoraxjnl-2011-200072

Moss, C. W., Lambert, M. A., and Goldsmith, D. J. (1970). Production of hydrocinnamic acid by clostridia. *Appl. Microbiol.* 19, 375–378.

Mueller, D. M., Allenspach, M., Othman, A., Saely, C. H., Muendlein, A., Vonbank, A., et al. (2015). Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis* 243, 638–644. doi: 10.1016/j.atherosclerosis.2015.10.091

Mukhopadhya, I., Hansen, R., El-Omar, E. M., and Hold, G. L. (2012). IBD-what role do Proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230. doi: 10.1038/nrgastro.2012.14

Nanda, R., James, R., Smith, H., Dudley, C. R., and Jewell, D. P. (1989). Food intolerance and the irritable bowel syndrome. *Gut* 30, 1099–1104. doi: 10.1136/gut.30.8.1099

Nardotto, G. H. B., Coelho, E. B., Marques, M. P., and Lanchote, V. L. (2016). Chiral analysis of carvedilol and its metabolites hydroxyphenyl carvedilol and O-desmethyl carvedilol in human plasma by liquid chromatography-tandem mass spectrometry: application to a clinical pharmacokinetic study. *J. Chromatogr. B* 1015–1016, 173–180. doi: 10.1016/j.jchromb.2016.02.028

Nassar, A.-E. F., and Talaat, R. E. (2004). Strategies for dealing with metabolite elucidation in drug discovery and development. *Drug Discov. Today* 9, 317–327. doi: 10.1016/S1359-6446(03)03018-6

Naz, S., Vallejo, M., García, A., and Barbas, C. (2014). Method validation strategies involved in non-targeted metabolomics. *J. Chromatogr. A* 1353, 99–105. doi: 10.1016/j.chroma.2014.04.071

Ndagijimana, M., Laghi, L., Vitali, B., Placucci, G., Brigidi, P., and Guerzoni, M. E. (2009). Effect of a synbiotic food consumption on human gut metabolic profiles evaluated by (1)H Nuclear Magnetic Resonance spectroscopy. *Int. J. Food Microbiol.* 134, 147–153. doi: 10.1016/j.ijfoodmicro.2009.04.016

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005). Opinion: gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* 3, 431–438. doi: 10.1038/nrmicro1152

Nicholson, J. K., and Lindon, J. C. (2008). Systems biology: metabonomics. *Nature* 455, 1054–1056. doi: 10.1038/4551054a

Noda, H., Akasaka, N., and Ohsugi, M. (1994). Biotin production by bifidobacteria. *J. Nutr. Sci. Vitaminol. (Tokyo)* 40, 181–188. doi: 10.3177/jnsv.40.181

Oresic, M., Simell, S., Sysi-Aho, M., Näntö-Salonen, K., Seppänen-Laakso, T., Parikka, V., et al. (2008). Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. *J. Exp. Med.* 205, 2975–2984. doi: 10.1084/jem.20081800

Parkar, S. G., Trower, T. M., and Stevenson, D. E. (2013). Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe* 23, 12–19. doi: 10.1016/j.anaerobe.2013.07.009

Patrignani, F., Burns, P., Serrazanetti, D., Vinderola, G., Reinheimer, J., Lanciotti, R., et al. (2009). Suitability of high pressure-homogenized milk for the production of probiotic fermented milk containing Lactobacillus paracasei and *Lactobacillus acidophilus*. *J. Dairy Res.* 76, 74. doi: 10.1017/S0022029908003828

Patrignani, F., Chinnici, F., Serrazanetti, D. I., Vernocchi, P., Ndagijimana, M., Riponi, C., et al. (2016). Production of volatile and sulfur compounds by 10 saccharomyces cerevisiae strains inoculated in trebbiano must. *Front. Microbiol.* 7:243. doi: 10.3389/fmicb.2016.00243

Paul, H. A., Bomhof, M. R., Vogel, H. J., and Reimer, R. A. (2016). Diet-induced changes in maternal gut microbiota and metabolomic profiles influence programming of offspring obesity risk in rats. *Sci. Rep.* 6:20683. doi: 10.1038/srep20683

Pawliszyn, J. (1997). *Solid Phase Microextraction: Theory and Practice*. New York, NY: Wiley-VCH.

Pence, H. E., and Williams, A. (2010). ChemSpider: an online chemical information resource. *J. Chem. Educ.* 87, 1123–1124. doi: 10.1021/ed100697w

Penders, J., Thijs, C., van den Brandt, P. A., Kummeling, I., Snijders, B., Stelma, F., et al. (2007). Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. *Gut* 56, 661–667. doi: 10.1136/gut.2006.100164

Perkins, N. J., and Pero, J. (2002). "Vitamin biosynthesis," in *Bacillus subtilis and Its Closest Relatives from Genes to Cells*, eds A. L. Sonenshein, R. Losick, and J. A. Hoch (Washington, DC: ASM Press), 271–286.

Phipps, A. N., Stewart, J., Wright, B., and Wilson, I. D. (1998). Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. *Xenobiotica Fate Foreign Compd. Biol. Syst.* 28, 527–537. doi: 10.1080/004982598239443

Pisano, M. B., Scano, P., Murgia, A., Cosentino, S., and Caboni, P. (2016). Metabolomics and microbiological profile of Italian mozzarella cheese produced with buffalo and cow milk. *Food Chem.* 192, 618–624. doi: 10.1016/j.foodchem.2015.07.061

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Matteuzzi, D., and Rossi, M. (2007). Folate production by bifidobacteria as a potential probiotic property. *Appl. Environ. Microbiol.* 73, 179–185. doi: 10.1128/AEM.01763-06

Prakash, S., Rodes, L., Coussa-Charley, M., and Tomaro-Duchesneau, C. (2011). Gut microbiota: next frontier in understanding human health and development of biotherapeutics. *Biol. Targets Ther.* 5, 71–86. doi: 10.2147/BTT.S19099

Putignani, L., Del Chierico, F., Vernocchi, P., Cicala, M., Cucchiara, S., Dallapiccola, B., et al. (2015). Gut microbiota dysbiosis as risk and premorbid factors of IBD and IBS along the childhood-adulthood transition. *Inflamm. Bowel Dis.* 22, 487–504. doi: 10.1097/MIB.0000000000000602

Qiu, Y., Cai, G., Su, M., Chen, T., Liu, Y., Xu, Y., et al. (2010). Urinary metabonomic study on colorectal cancer. *J. Proteome Res.* 9, 1627–1634. doi: 10.1021/pr901081y

Raamsdonk, L. M., Teusink, B., Broadhurst, D., Zhang, N., Hayes, A., Walsh, M. C., et al. (2001). A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Nat. Biotechnol.* 19, 45–50. doi: 10.1038/83496

Raimondi, S., Anighoro, A., Quartieri, A., Amaretti, A., Tomás-Barberán, F. A., Rastelli, G., et al. (2015). Role of bifidobacteria in the hydrolysis of chlorogenic acid. *Microbiologyopen* 4, 41–52. doi: 10.1002/mbo3.219

Raman, M., Ahmed, I., Gillevet, P. M., Probert, C. S., Ratcliffe, N. M., Smith, S., et al. (2013). Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin. Gastroenterol. Hepatol.* 11, 868–875.e1–3. doi: 10.1016/j.cgh.2013.02.015

Ramautar, R., Somsen, G. W., and de Jong, G. J. (2013). CE-MS for metabolomics: developments and applications in the period 2010-2012. *Electrophoresis* 34, 86–98. doi: 10.1002/elps.201200390

Respondek, F., Gerard, P., Bossis, M., Boschat, L., Bruneau, A., Rabot, S., et al. (2013). Short-chain fructo-oligosaccharides modulate intestinal microbiota and metabolic parameters of humanized gnotobiotic diet induced obesity mice. *PLoS ONE* 8:e71026. doi: 10.1371/journal.pone.0071026

Ridlon, J. M., Kang, D.-J., and Hylemon, P. B. (2006). Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* 47, 241–259. doi: 10.1194/jlr.R500013-JLR200

Robroeks, C. M. H. H. T., van Berkel, J. J. B. N., Dallinga, J. W., Jöbsis, Q., Zimmermann, L. J. I., Hendriks, H. J. E., et al. (2010). Metabolomics of volatile organic compounds in cystic fibrosis patients and controls. *Pediatr. Res.* 68, 75–80. doi: 10.1203/PDR.0b013e3181df4ea0

Roediger, W. E., Duncan, A., Kapaniris, O., and Millard, S. (1993). Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. *Gastroenterology* 104, 802–809. doi: 10.1016/0016-5085(93)91016-B

Roessner, U., Wagner, C., Kopka, J., Trethewey, R. N., and Willmitzer, L. (2000). Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J. Cell Mol. Biol.* 23, 131–142. doi: 10.1046/j.1365-313x.2000.00774.x

Rosselló-Mora, R., Lucio, M., Peña, A., Brito-Echeverría, J., López-López, A., Valens-Vadell, M., et al. (2008). Metabolic evidence for biogeographic isolation of the extremophilic bacterium Salinibacter ruber. *ISME J.* 2, 242–253. doi: 10.1038/ismej.2007.93

Rossi, M., and Amaretti, A. (2010). "Probiotic properties of *Bifidobacteria*," in *Bifidobacteria: Genomics and Molecular Aspects*, eds M. Baltasar and D. van Sinderen (Norfolk: Caister Academic), 97–123.

Roth, J. R., Lawrence, J. G., and Bobik, T. A. (1996). Cobalamin (coenzyme B12): synthesis and biological significance. *Annu. Rev. Microbiol.* 50, 137–181. doi: 10.1146/annurev.micro.50.1.137

Russell, D. W., and Setchell, K. D. (1992). Bile acid biosynthesis. *Biochemistry (Mosc.)* 31, 4737–4749. doi: 10.1021/bi00135a001

Sabatino, A. D., Morera, R., Ciccocioppo, R., Cazzola, P., Gotti, S., Tinozzi, F. P., et al. (2005). Oral butyrate for mildly to moderately active Crohn's disease. *Aliment. Pharmacol. Ther.* 22, 789–794. doi: 10.1111/j.1365-2036.2005.02639.x

Savorani, F., Rasmussen, M. A., Mikkelsen, M. S., and Engelsen, S. B. (2013). A primer to nutritional metabolomics by NMR spectroscopy and chemometrics. *Food Res. Int.* 54, 1131–1145. doi: 10.1016/j.foodres.2012.12.025

Scalan, P. D., Buckling, A., Kong, W., Wild, Y., Lynch, S. W., and Harrison, F. (2012). Gut dysbiosis in cystic fibrosis. *J. Cyst. Fibros.* 11, 454–455. doi: 10.1016/j.jcf.2012.03.007

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. *Gut* 35, S35–S38. doi: 10.1136/gut.35.1_suppl.s35

Scheppach, W., Pomare, E. W., Elia, M., and Cummings, J. H. (1991). The contribution of the large intestine to blood acetate in man. *Clin. Sci.* 80, 177–182. doi: 10.1042/cs0800177

Scheppach, W., Sommer, H., Kirchner, T., Paganelli, G. M., Bartram, P., Christl, S., et al. (1992). Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 103, 51–56. doi: 10.1016/0016-5085(92)91094-K

Schippa, S., Iebba, V., Santangelo, F., Gagliardi, A., De Biase, R. V., Stamato, A., et al. (2013). Cystic fibrosis transmembrane conductance regulator (CFTR) allelic variants relate to shifts in faecal microbiota of cystic fibrosis patients. *PLoS ONE* 8:e61176. doi: 10.1371/journal.pone.0061176

Schöller, C., Molin, S., and Wilkins, K. (1997). Volatile metabolites from some gram-negative bacteria. *Chemosphere* 35, 1487–1495. doi: 10.1016/S0045-6535(97)00209-9

Scholtens, D. M., Muehlbauer, M. J., Daya, N. R., Stevens, R. D., Dyer, A. R., Lowe, L. P., et al. (2014). Metabolomics reveals broad-scale metabolic perturbations in hyperglycemic mothers during pregnancy. *Diabetes Care* 37, 158–166. doi: 10.2337/dc13-0989

Selma, M. V., Espín, J. C., and Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* 57, 6485–6501. doi: 10.1021/jf902107d

Shahani, K. M., and Chandan, R. C. (1979). Nutritional and healthful aspects of cultured and culture-containing dairy foods. *J. Dairy Sci.* 62, 1685–1694. doi: 10.3168/jds.S0022-0302(79)83481-5

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* 29, 213–231. doi: 10.1016/0168-1605(95)00032-1

Siroli, L., Patrignani, F., Serrazanetti, D. I., Tabanelli, G., Montanari, C., Gardini, F., et al. (2015). Lactic acid bacteria and natural antimicrobials to improve the

safety and shelf-life of minimally processed sliced apples and lamb's lettuce. *Food Microbiol.* 47, 74–84. doi: 10.1016/j.fm.2014.11.008

Sivsammye, G., and Sims, H. V. (1990). Presumptive identification of *Clostridium difficile* by detection of p-cresol in prepared peptone yeast glucose broth supplemented with p-hydroxyphenylacetic acid. *J. Clin. Microbiol.* 28, 1851–1853.

Smirnov, K. S., Maier, T. V., Walker, A., Heinzmann, S. S., Forcisi, S., Martinez, I., et al. (2016). Challenges of metabolomics in human gut microbiota research. *Int. J. Med. Microbiol.* doi: 10.1016/j.ijmm.2016.03.006. [Epub ahead of print].

Smith, A. G., Croft, M. T., Moulin, M., and Webb, M. E. (2007). Plants need their vitamins too. *Curr. Opin. Plant Biol.* 10, 266–275. doi: 10.1016/j.pbi.2007.04.009

Smith, C. A., O'Maille, G., Want, E. J., Qin, C., Trauger, S. A., Brandon, T. R., et al. (2005). METLIN: a metabolite mass spectral database. *Ther. Drug Monit.* 27,747–751. doi: 10.1097/01.ftd.0000179845.53213.39

Smith, E. A., and Macfarlane, G. T. (1997). Dissimilatory amino Acid metabolism in human colonic bacteria. *Anaerobe* 3, 327–337. doi: 10.1006/anae.1997.0121

Smolinska, A., Blanchet, L., Buydens, L. M. C., and Wijmenga, S. S. (2012). NMR and pattern recognition methods in metabolomics: from data acquisition to biomarker discovery: a review. *Anal. Chim. Acta* 750, 82–97. doi: 10.1016/j.aca.2012.05.049

Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., and Nishioka, T. (2003). Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *J. Proteome Res.* 2, 488–494. doi: 10.1021/pr034020m

Srinivasan, S., Morgan, M. T., Fiedler, T. L., Djukovic, D., Hoffman, N. G., Raftery, D., et al. (2015). Metabolic signatures of bacterial vaginosis. *MBio* 6, e00204–e00215. doi: 10.1128/mBio.00204-15

Stanton, C., Ross, R. P., Fitzgerald, G. F., and Van Sinderen, D. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Curr. Opin. Biotechnol.* 16, 198–203. doi: 10.1016/j.copbio.2005.02.008

Stashenko, E. E., Jaramillo, B. E., and Martínez, J. R. (2004). Analysis of volatile secondary metabolites from Colombian Xylopia aromatica (Lamarck) by different extraction and headspace methods and gas chromatography. *J. Chromatogr. A* 1025, 105–113. doi: 10.1016/j.chroma.2003.10.059

Stephens, N. S., Siffledeen, J., Su, X., Murdoch, T. B., Fedorak, R. N., and Slupsky, C. M. (2013). Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy. *J. Crohns Colitis* 7, e42–e48. doi: 10.1016/j.crohns.2012.04.019

Sugawara, T., Sawada, D., Ishida, Y., Aihara, K., Aoki, Y., Takehara, I., et al. (2016). Regulatory effect of paraprobiotic *Lactobacillus gasseri* CP2305 on gut environment and function. *Microb. Ecol. Health Dis.* 27:30259. doi: 10.3402/mehd.v27.30259

Suhre, K., Meisinger, C., Döring, A., Altmaier, E., Belcredi, P., Gieger, C., et al. (2010). Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PLoS ONE* 5:e13953. doi: 10.1371/journal.pone.0013953

Suttie, J. W. (1995). The importance of menaquinones in human nutrition. *Annu. Rev. Nutr.* 15, 399–417. doi: 10.1146/annurev.nu.15.070195.002151

Tabanelli, G., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., et al. (2015a). Physico-chemical and microbiological characterisation of Italian fermented sausages in relation to their size: Italian fermented sausages characterisation. *J. Sci. Food Agric.* 96, 2773–2781. doi: 10.1002/jsfa.7442

Tabanelli, G., Vernocchi, P., Patrignani, F., Del Chierico, F., Putignani, L., Vinderola, G., et al. (2015b). Effects of sub-lethal high-pressure homogenization treatment on the outermost cellular structures and the volatile-molecule profiles of two strains of probiotic lactobacilli. *Front. Microbiol.* 6:1006. doi: 10.3389/fmicb.2015.01006

Taverniti, V., and Guglielmetti, S. (2012). Health-promoting properties of *Lactobacillus helveticus*. *Front. Microbiol.* 3:392. doi: 10.3389/fmicb.2012.00392

Vaidyanathan, S., Kell, D. B., and Goodacre, R. (2002). Flow-injection electrospray ionization mass spectrometry of crude cell extracts for high-throughput bacterial identification. *J. Am. Soc. Mass Spectrom.* 13, 118–128. doi: 10.1016/S1044-0305(01)00339-7

Vannini, L., Patrignani, F., Iucci, L., Ndagijimana, M., Vallicelli, M., Lanciotti, R., et al. (2008). Effect of a pre-treatment of milk with high pressure homogenization on yield as well as on microbiological, lipolytic and proteolytic patterns of "Pecorino" cheese. *Int. J. Food Microbiol.* 128, 329–335. doi: 10.1016/j.ijfoodmicro.2008.09.018

van Zanten, G. C., Krych, L., Röytiö, H., Forssten, S., Lahtinen, S. J., Al-Soud, W. A., et al. (2014). Synbiotic *Lactobacillus acidophilus* NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acids: a randomized, double-blinded cross-over trial. *FEMS Microbiol. Ecol.* 90, 225–236. doi: 10.1111/1574-6941.12397

Velázquez, O. C., Lederer, H. M., and Rombeau, J. L. (1997). Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications. *Adv. Exp. Med. Biol.* 427, 123–134. doi: 10.1007/978-1-4615-5967-2_14

Vernocchi, P., Ndagijimana, M., Serrazanetti, D. I., López, C. C., Fabiani, A., Gardini, F., et al. (2011). Use of Saccharomyces cerevisiae strains endowed with β-glucosidase activity for the production of Sangiovese wine. *World J. Microbiol. Biotechnol.* 27, 1423–1433. doi: 10.1007/s11274-010-0594-1

Vernocchi, P., Vannini, L., Gottardi, D., Del Chierico, F., Serrazanetti, D. I., Ndagijimana, M., et al. (2012). Integration of datasets from different analytical techniques to assess the impact of nutrition on human metabolome. *Front. Cell. Infect. Microbiol.* 2:156. doi: 10.3389/fcimb.2012.00156

Veselkov, K. A., Lindon, J. C., Ebbels, T. M. D., Crockford, D., Volynkin, V. V., Holmes, E., et al. (2009). Recursive segment-wise peak alignment of biological (1)h NMR spectra for improved metabolic biomarker recovery. *Anal. Chem.* 81, 56–66. doi: 10.1021/ac8011544

Villas-Bôas, S. G., Mas, S., Akesson, M., Smedsgaard, J., and Nielsen, J. (2005). Mass spectrometry in metabolome analysis. *Mass Spectrom. Rev.* 24, 613–646. doi: 10.1002/mas.20032

Villaseñor, A., Kinross, J. M., Li, J. V., Penney, N., Barton, R. H., Nicholson, J. K., et al. (2014). 1H NMR global metabolic phenotyping of acute pancreatitis in the emergency unit. *J. Proteome Res.* 5, 362–375. doi: 10.1021/pr500161w

Vitali, B., Cruciani, F., Picone, G., Parolin, C., Donders, G., and Laghi, L. (2015). Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 2367–2376. doi: 10.1007/s10096-015-2490-y

Vitali, B., Ndagijimana, M., Cruciani, F., Carnevali, P., Candela, M., Guerzoni, M. E., et al. (2010). Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. *BMC Microbiol.* 10:4. doi: 10.1186/1471-2180-10-4

Vitali, B., Ndagijimana, M., Maccaferri, S., Biagi, E., Guerzoni, M. E., and Brigidi, P. (2012). An *in vitro* evaluation of the effect of probiotics and prebiotics on the metabolic profile of human microbiota. *Anaerobe* 18, 386–391. doi: 10.1016/j.anaerobe.2012.04.014

Vitali, B., Pugliese, C., Biagi, E., Candela, M., Turroni, S., Bellen, G., et al. (2007).Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis, or no infection, analyzed by PCR-denaturing gradient gel electrophoresis and real-time PCR. *Appl. Environ. Microbiol.* 73, 5731–5741. doi: 10.1128/AEM.01251-07

Walker, A., Lucio, M., Pfitzner, B., Scheerer, M. F., Neschen, S., de Angelis, M. H., et al. (2014). Importance of sulfur-containing metabolites in discriminating fecal extracts between normal and type-2 diabetic mice. *J. Proteome Res.* 13, 4220–4231. doi: 10.1021/pr500046b

Walton, C., Fowler, D. P., Turner, C., Jia, W., Whitehead, R. N., Griffiths, L., et al. (2013). Analysis of volatile organic compounds of bacterial origin in chronic gastrointestinal diseases. *Inflamm. Bowel Dis.* 19, 2069–2078. doi: 10.1097/MIB.0b013e31829a91f6

Wang, T., Carroll, W., Lenny, W., Boit, P., and Smith, D. (2006). The analysis of 1-propanol and 2-propanol in humid air samples using selected ion flow tube mass spectrometry. *Rapid Commun. Mass Spectrom.* 20, 125–130. doi: 10.1002/rcm.2285

Watanabe, S., Narisawa, Y., Arase, S., Okamatsu, H., Ikenaga, T., Tajiri, Y., et al. (2003). Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J. Allergy Clin. Immunol.* 111, 587–591. doi: 10.1067/mai.2003.105

Weckwerth, W., and Morgenthal, K. (2005). Metabolomics: from pattern recognition to biological interpretation. *Drug Discov. Today* 10, 1551–1558. doi: 10.1016/S1359-6446(05)03609-3

Williams, H. R. T., Cox, I. J., Walker, D. G., Cobbold, J. F. L., Taylor-Robinson, S. D., Marshall, S. E., et al. (2010). Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Crohn's disease. *BMC Gastroenterol.* 10:108. doi: 10.1186/1471-230X-10-108

Wilson, M. (2005). *Microbial Inhabitants of Human*. Cambridge; New York, NY: Cambridge University Press.

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., et al. (2013). HMDB 3.0–the human metabolome database in 2013. *Nucleic Acids Res.* 41, D801–D807. doi: 10.1093/nar/gks1065

Wissenbach, D. K., Oliphant, K., Rolle-Kampczyk, U., Yen, S., Höke, H., Baumann, S., et al. (2016). Optimization of metabolomics of defined in vitro gut microbial ecosystems. *Int. J. Med. Microbiol.* doi: 10.1016/j.ijmm.2016.03.007. [Epub ahead of print].

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., and Jenkins, D. J. A. (2006). Colonic health: fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* 40, 235–243. doi: 10.1097/00004836-200603000-00015

Worley, B., and Powers, R. (2012). Multivariate analysis in metabolomics. *Curr. Metabolomics* 1, 92–107. doi: 10.2174/2213235x11301010092

Yamashita, H., Fujisawa, K., Ito, E., Idei, S., Kawaguchi, N., Kimoto, M., et al. (2007). Improvement of obesity and glucose tolerance by acetate in type 2 diabetic otsuka long-evans tokushima fatty (OLETF) rats. *Biosci. Biotechnol. Biochem.* 71, 1236–1243. doi: 10.1271/bbb.60668

Zamboni, N., Saghatelian, A., and Patti, G. J. (2015). Defining the metabolome: size, flux, and regulation. *Mol. Cell* 58, 699–706. doi: 10.1016/j.molcel.2015.04.021

Zhang, G., Bai, H., Zhang, H., Dean, C., Wu, Q., Li, J., et al. (2011). Neuropeptide exocytosis involving synaptotagmin-4 and oxytocin in hypothalamic programming of body weight and energy balance. *Neuron* 69, 523–535. doi: 10.1016/j.neuron.2010.12.036

Zhang, A., Sun, H., and Wang, X. (2012). Saliva metabolomics opens door to biomarker discovery, disease diagnosis, and treatment. *Appl. Biochem. Biotechnol.* 168, 1718–1727. doi: 10.1007/s12010-012-9891-5

Zhang, C., Yin, A., Li, H., Wang, R., Wu, G., Shen, J., et al. (2015). Dietary modulation of gut microbiota contributes to alleviation of both genetic and simple obesity in children. *EBioMedicine* 2, 966–982. doi: 10.1016/j.ebiom.2015.07.007

Zhao, L., and Hartung, T. (2015). Metabonomics and toxicology. *Methods Mol. Biol. Clifton NJ* 1277, 209–231. doi: 10.1007/978-1-4939-2377-9_15

Zhao, L., Liu, X., Xie, L., Gao, H., and Lin, D. (2010). 1H NMR-based metabonomic analysis of metabolic changes in streptozotocin-induced diabetic rats. *Anal. Sci. Int. J. Jpn. Soc. Anal. Chem.* 26, 1277–1282. doi: 10.2116/analsci.26.1277

Zheng, X., Xie, G., Zhao, A., Zhao, L., Yao, C., Chiu, N. H. L., et al. (2011). The footprints of gut microbial-mammalian co-metabolism. *J. Proteome Res.* 10, 5512–5522. doi: 10.1021/pr2007945

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Molecular Players Involved in the Interaction Between Beneficial Bacteria and the Immune System

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The human gastrointestinal tract is a very complex ecosystem, in which there is a continuous interaction between nutrients, host cells, and microorganisms. The gut microbiota comprises trillions of microbes that have been selected during evolution on the basis of their functionality and capacity to survive in, and adapt to, the intestinal environment. Host bacteria and our immune system constantly sense and react to one another. In this regard, commensal microbes contribute to gut homeostasis, whereas the necessary responses are triggered against enteropathogens. Some representatives of our gut microbiota have beneficial effects on human health. Some of the most important roles of these microbes are to help to maintain the integrity of the mucosal barrier, to provide nutrients such as vitamins, or to protect against pathogens. In addition, the interaction between commensal microbiota and the mucosal immune system is crucial for proper immune function. This process is mainly performed via the pattern recognition receptors of epithelial cells, such as Toll-like or Nod-like receptors, which are able to recognize the molecular effectors that are produced by intestinal microbes. These effectors mediate processes that can ameliorate certain inflammatory gut disorders, discriminate between beneficial and pathogenic bacteria, or increase the number of immune cells or their pattern recognition receptors (PRRs). This review intends to summarize the molecular players produced by probiotic bacteria, notably *Lactobacillus* and *Bifidobacterium* strains, but also other very promising potential probiotics, which affect the human immune system.

Keywords: *Bifidobacterium*, immunomodulation, *Lactobacillus*, molecular players, probiotic

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THE HUMAN GUT MICROBIOTA AND THE IMMUNE SYSTEM

From the early stages of life, one of the most important roles of the gut microbiota is to contribute to the development of a proper immune system. Normally, humans live in a homeostatic symbiosis with their gastrointestinal microbes, providing them with nutrients and a friendly environment, whereas the microbiota aids in the appropriate development and maintenance of the host's gut mucosa. Epithelial function is influenced by direct host/microbiota interactions and microbial metabolism. The large intestine acts as an anaerobic bioreactor for the enteric bacterial community, which is fueled by host diet components that cannot be processed in the small intestine, as well as by endogenous nutrients, such as host glycans from mucus and cell debris released from epithelial cells. Additionally, the microbiota synthesizes essential amino acids, vitamins, and short chain fatty acids (SCFAs) by degrading a variety of proteins and otherwise non-digestible polysaccharides (Sekirov et al., 2010).

The commensal microbiota ensures the mechanical integrity of the mucosal barrier, thereby offering protection against harmful pathogenic microbes (**Figure 1**). Commensal bacteria can adhere to the intestinal mucus and competitively inhibit the adhesion of enteropathogens; they also produce bacteriocins and SCFAs, compounds that are able to inhibit the growth of other microorganisms. Additionally, some antimicrobial metabolites, such as the defensins secreted in the intestine, contribute to the host's control of these microorganisms (Salzman et al., 2007). Further protection of the host is provided by inducing the mucosal immune system to produce immunoglobulin A, which is released in the intestinal lumen in large amounts and limits local bacterial colonization, thereby preventing bacteria from penetrating the epithelium (Salzman et al., 2007).

A major issue is how the intestine distinguishes between the abundant, normal microbiota and rare pathogens. The immune system fights pathogenic bacteria, but tolerates the presence of commensal species, even though their cellular structures are quite similar and they have common mechanisms of interacting with host immune cell receptors; this phenomenon is called immune tolerance. In this way, our immune cells differentiate between commensals and pathogens. This is carried out by our innate immune system through pattern recognition receptors (PRRs) (**Figure 1**), including Toll-like receptors (TLRs), transmembrane receptors that scan the external milieu of the intestinal lumen, and Nod-like intracellular receptors (NODLR), which guard the cytoplasmic space (Claes et al., 2015; Sellge and Kufer, 2015). Other PRRs have also been described, such as C-type lectin receptors, formylated peptide receptors, retinoic acid-inducible (RIG)-like helicases, and intracellular interleukin-1 (IL-1)-converting enzyme protease-activating factor (Denes et al., 2012; Bufe et al., 2015; Dambuza and Brown, 2015; Yao et al., 2015). PRRs are able to specifically recognize and bind different microbial macromolecular ligands, which are designated as microbial-associated molecular patterns (MAMPs), such as lipopolysaccharide, flagellin and other proteins, bacterial peptidoglycan, viral RNAs, and fungal carbohydrates. As a result, the T cell subset involved in regulating the immune balance is finely tuned by the host and the microbes with which it interacts, and disequilibrium between effector T helper (Th) and regulatory T cells (Treg) leads to impaired immune responses (Noack and Miossec, 2014; Nyirenda et al., 2015; Yousefi et al., 2015). Effector Th cells are derived from progenitor naïve CD4+ T cells via maturational processes

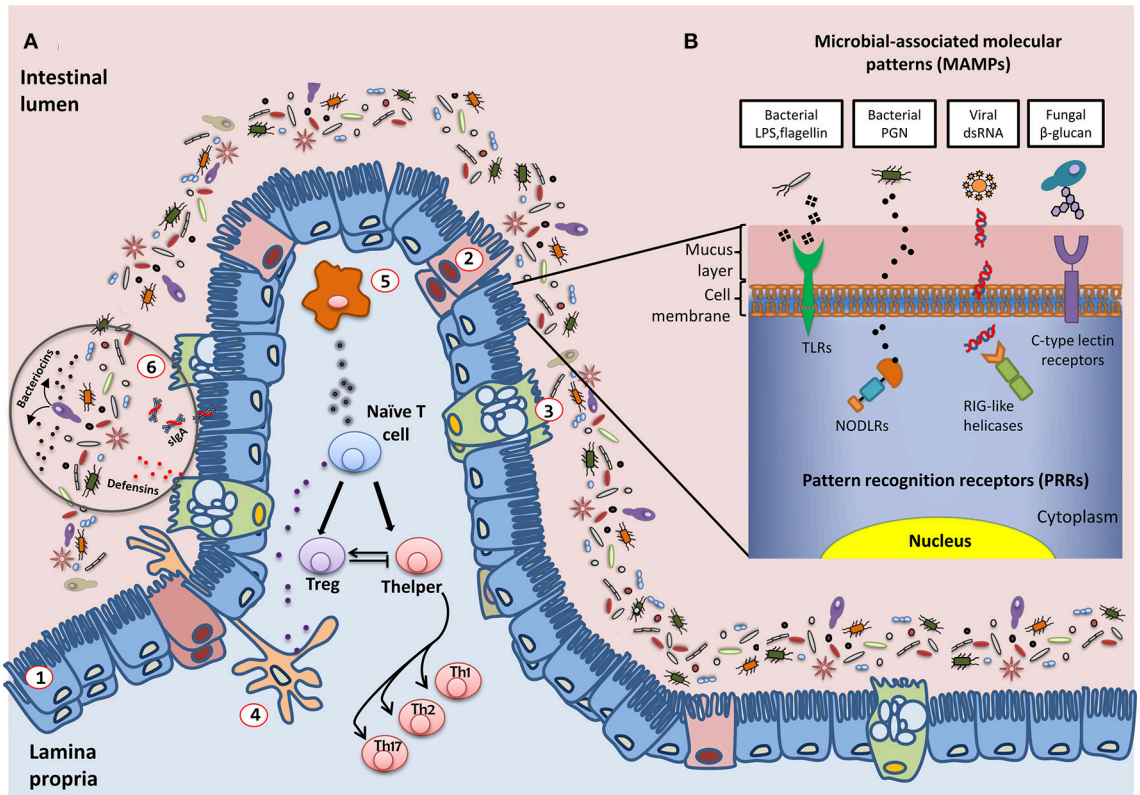

FIGURE 1 | Schematic representation of the interactions established between the intestinal microbiota and the host immune system. (A) General overview of the epithelium in contact with multiple species of microorganisms that constitute the intestinal microbiota: (1) enterocytes; (2) M cells; (3) Goblet cells; antigen presentation cells (APC): (4) dendritic cells and (5) macrophages; (6) defensins, bacteriocins, and secreted IgA (sIgA) also play an important role in controlling the levels of the different populations of microorganisms. A fine-tuned balance of Tcell maturation toward Treg or Thelper cells must be established to assure the tolerogenic response of the host immune system. **(B)** Examples of molecular interactions between microbial antigens and host cells through Pattern Recognition Receptors (PRRs). LPS, lipopolysaccharide; PGN, peptidoglycan; dsRNA, double-strand RNA; TLR, toll-like receptor; NODLR, nucleotide-binding oligomerization domain-like receptors; RIG-like helicases, retinoic acid-inducible gene 1 like helicases.

that are induced by antigenic stimulation. Their function depends on complex interactions with antigen-presenting cells (APCs) in a permissive environment, which is characterized by the antigen type and load, costimulatory molecules, and cytokine signaling. CD4+ T cells may differentiate into different Th phenotypes (mainly Th1, Th2, and Th17) that produce distinct cytokines with different biological functions, or they may evolve into the inducible Treg lineage, which performs immunomodulatory functions (Sakaguchi et al., 2010; Wing and Sakaguchi, 2014). The Th1 subgroup recognizes intracellular pathogens and mainly produces IL-2, interferon (IFN), and tumor necrosis factor alpha (TNFα), thereby supporting typical cellular immunity. Th2 cells, which are essential for eliminating extracellular pathogens such as helminths, express IL-4, IL-5, IL-10, and IL-13, which aid humoral immunity. The Th17 subset, which is involved in fighting Gram-negative bacteria, fungi, and some protozoa, secretes IL-17, which has strong pro-inflammatory effects. Overall, Th responses are accurately balanced to avoid both self-antigen reactivity and excessive reactions to antigens. In fact, dysregulated Th1 responses drive cell-mediated autoimmune disorders, and enhanced Th2 activity is involved in atopy, whereas Th17 cells are probably responsible for chronic tissue inflammation. In contrast, skewing the response away from Treg cells may lead to the onset and/or progression of autoimmune diseases in humans (Eisenstein and Williams, 2009).

PROBIOTICS AND THE IMMUNE SYSTEM

During the last few years, it has been proposed that the intestinal microbiota can be positively modulated by the administration of bacteria or bacterial substrates, and it is likely that, to some extent, this might lead to a significant modulation of the immune system (Dongarrà et al., 2013; Sánchez et al., 2015; Scott et al., 2015). To this end, substantial research efforts are concentrated on using probiotics as potential modulators of gut microbial community. Probiotics are commensal microorganisms that are present in the intestinal tract and in many fermented foods, and they are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The vast majority of probiotic bacteria are Gram-positive strains, mainly species of the *Lactobacillus* and *Bifidobacterium* genera, although some non-pathogenic strains of *Escherichia coli* and certain yeasts are also considered to be probiotics. Currently, there is an increasing interest in considering some common colonizers of the human gut to be novel probiotics, because of their potential health properties; they are called *emerging probiotics* (Hill et al., 2014; Rodríguez et al., 2015). Some examples are *Faecalibacterium prausnitzii* and *Akkermansia muciniphila.*

Probiotics can exert their beneficial properties in a wide range of ways, including direct cell-to-cell contact in the human gut, by secreting diverse molecules that act as the final mediators of probiotic crosstalk, or through cross-feeding mechanisms. The chemical composition of the molecular effectors is very diverse and consists of proteins that are secreted into the extracellular milieu or localized on the surface of the bacteria, low molecular weight peptides, amino acids, cell-wall polysaccharides or components, bacterial DNA, or SCFAs (Macpherson and Harris, 2004; Turroni et al., 2013). Given the different molecular natures of these molecular effectors, their mechanisms of action are very diverse. Therefore, this review includes only a summary of the molecular bases underlying the immunomodulatory properties of probiotic bacteria (**Figure 2**). In addition, we must consider that genetic differences in the expression of host receptors, the variable composition of the autochthonous microbiota in different individuals, and other host factors that contribute to the response to bacterial signals are likely to explain the variability in responses to probiotics in responding and non-responding individuals (Salonen et al., 2014).

IMMUNOMODULATORY EFFECTORS

A significant number of relevant studies have highlighted the immunomodulatory effects that *Lactobacillus* and *Bifidobacterium* strains exert on the host immune system. For instance, there is evidence that *Bifidobacterium bifidum* LMG13195 and *Bifidobacterium breve* IPLA 20004 enhance intestinal barrier function and preferentially elicit Treg cell differentiation, which induces the expression of anti-inflammatory cytokines, when co-cultured with the human colorectal adenocarcinoma cell line HT29 (López et al., 2012). *Lactobacillus rhamnosus* GG interacts with macrophages in such a way that activated macrophages can discriminate between probiotic and pathogenic bacteria by INF-mediated TLR gene regulation (Miettinen et al., 2008), and the interaction between *Lactobacillus casei* CRL 431 and gut-associated immune cells induces an increase in the number of CD-206 and TLR2 receptors (Aragón et al., 2014).

The mediators of these interactions are largely unknown, although surface and cell-envelope molecules have been identified as some of the main players. Among them, we can distinguish between proteins and other components, such as peptidoglycan (PG), exopolysaccharides (EPS), teichoic acids (TA), and lipoteichoic acids (LTA). Known molecular effectors that mediate immunomodulatory mechanisms are listed in **Table 1**.

Surface Proteins

Cell surface proteins include the S-layer proteins (Slp), which constitute the major surface proteins of some lactobacilli. In *Lactobacillus helveticus* fb213, *Lactobacillus acidophilus* fb116, and *L. acidophilus* fb214, Slp are well studied, and it is likely that they are necessary for lactobacilli survival in the gastrointestinal tract, as they can bind to components of the extracellular matrix, such as collagen and fibronectin, of intestinal cells (Meng et al., 2014; Yadav et al., 2015). Konstantinov and colleagues used an *slpA* knockout mutant of *L. acidophilus* to show that the interaction occurs via the recognition of SlpA by a specific receptor of dendritic cells, denominated DC-SIGN (Konstantinov et al., 2008). Additionally, proteins from structures that are included in the PG layer, such as pili, fimbria, and flagella, are recognized by the host immune

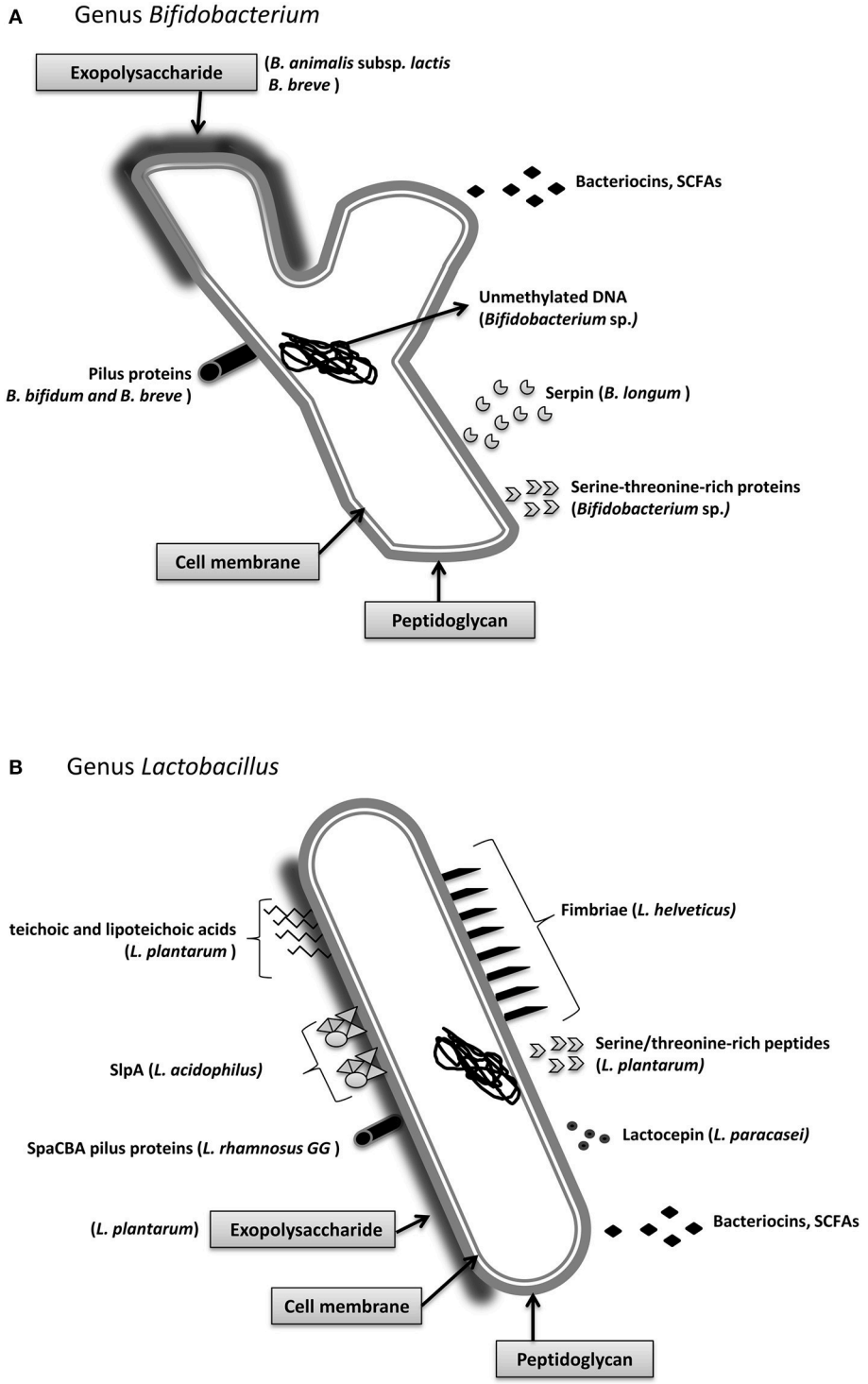

FIGURE 2 | Main molecular effectors that are able to trigger immunomodulatory responses in the host: *Bifidobacterium* (A) and *Lactobacillus* (B). Some of these effectors are species-specific, such as the S-layer protein A from *Lactobacillus acidophilus*, whereas others, such as short chain fatty acids, are secreted by the vast majority of strains. Detailed information about the mechanisms and the molecular effectors is included in Section Immunomodulatory Effectors.

system. Recently, it has been reported that bacterial SpaCBA pilus fibers in *L. rhamnosus* GG may be responsible for its well-known adhesion properties and confer the ability to contact host cells (Reunanen et al., 2012). *B. bifidum* PRL2010 pili have been shown to induce TNF-α production and decrease IL-10 production in the mouse mucosa, as well as to adhere

TABLE 1 | Examples of immunomodulatory effectors produced by classic/emerging probiotics.

Immunomodulatory effector	Species	Probiotic type	Effect on host immune system	References
Surface Layer Protein A (SlpA)	*L. acidophilus*	Classic	Immunomodulation of intestinal dendritic cells	Konstantinov et al., 2008
Pili proteins (SpaCBA)	*L. rhamnosus*	Classic	Contact with mucosal cells	Reunanen et al., 2012
Pili	*B. bifidum*	Classic	Increase TNF-α and decrease IL-10 production	Turroni et al., 2013
	B. breve	Classic	Host colonization	O'Connell Motherway et al., 2011
Fimbriae	*E. coli*	Emerging	Host-colonization	Kleta et al., 2014
	L. plantarum	Classic	Immunomodulation	Murofushi et al., 2015
Serpin	*B. longum*	Classic	Human neutrophil and pancreatic elastase inhibitor	Ivanov et al., 2006
Serine-threonine rich proteins	*Bifidobacterium sp.*	Classic	Intestinal homeostasis	Nezametdinova et al., 2014
	Lactobacillus sp.	Classic	Intestinal homeostasis	Zakharevich et al., 2012
Serine-threonine rich peptide (STp)	*L. plantarum*	Classic	Anti-inflammatory; modulates intestinal dendritic cell function	Bernardo et al., 2012; Al-Hassi et al., 2014
Lactocepin	*L. paracasei*	Classic	Hydrolyzes IP-10	von Schillde et al., 2012
Secreted 15 kDa protein	*F. prausnitzii*	Emerging	Anti-inflammatory	Quévrain et al., 2015
Exopolysaccharides	*B. breve*	Classic	Immunomodulation	Fanning et al., 2012
	*B. lactis**	Classic	Immunomodulation	Hidalgo-Cantabrana et al., 2014
Unmethylated CpG DNA	*Bifidobacterium sp.*	Classic	Induces Th1 response	Ménard et al., 2010
Teichoic/Lipoteichoic acids	*L. plantarum*	Classic	Anti-inflammatory	Grangette et al., 2005
Butyrate	*R. hominis* *F. prausnitzii* *A. muciniphila*	Emerging	Anti-inflammatory	Maslowski et al., 2009

**Synonym of B. animalis subsp. lactis.*

to diverse extracellular matrix proteins (Turroni et al., 2013), while *B. breve* UCC2003 pili are essential for host colonization (O'Connell Motherway et al., 2011). In another recent work, gene complementation studies were used to show that the fimbriae of the probiotic strain *E. coli* Nissle 1917 were involved in the adhesion to porcine intestinal cells, thereby helping to prevent infection with enteropathogenic *E. coli* (EPEC) (Kleta et al., 2014).

Cell Wall Non-proteinaceous Components

Non-proteinaceous cell wall components have different roles in microbe-host crosstalk. It has been shown that the EPS from *Lactobacillus* and *Bifidobacterium* strains can have a modulator role in preventing pathogen invasion, even though the EPS of pathogenic bacteria have been classically viewed as possible virulent factors. Examples of immunomodulatory EPS are those from *B. breve* and *Bifidobacterium animalis* subsp. *lactis* (Fanning et al., 2012; Hidalgo-Cantabrana et al., 2014) or *Lactobacillus plantarum* strains (Murofushi et al., 2015). TAs are linear polymers of ribitol phosphate or glycerol phosphate that are covalently bound to D-alanine, monosaccharides, or amino sugars, and they are attached either to PG (wall TAs) or to the cytoplasmic membrane (membrane TAs or lipoteichoic acids; LTAs). TAs from *L. plantarum* were shown to display anti-inflammatory properties, as shown by the different cytokine production profiles of peripheral blood mononuclear cells (PBMCs) and monocytes exposed to this molecule (Grangette et al., 2005). In addition, mice fed a diet supplemented with *L. plantarum* LTAs or with an LTA-producing strain showed better scores in a colitis model compared with the control group and mice that were fed a *L. plantarum* LTA-deficient strain (Grangette et al., 2005). Although there have been a few promising results, this topic requires further research to clarify the mechanisms of action of the cell wall components of probiotics on the human gut microbiota.

Soluble Compounds

Soluble components that are produced by probiotic bacteria can also affect the bacterial-host interplay. In *Bifidobacterium longum* the secretion of serpin, a serine protease inhibitor, which specifically binds and inactivates human neutrophil and pancreatic elastase, was shown to contribute to gut homeostasis (Ivanov et al., 2006). Additionally, it has been observed that some proteins with characteristic biochemical motifs that are produced by both commensal and pathogenic bacteria can elicit specific functions and affect immune cells of the intestinal lumen. This is the case for a family of serine-threonine rich proteins, which was described in species of *Lactobacillus* and *Bifidobacterium*, with a recently described kinase function (Zakharevich et al., 2012; Nezametdinova et al., 2014). In lactobacilli, a serine-threonine peptide, STp, which is contained in a protein secreted by *L. plantarum*, was shown to be involved in bacterial aggregation (Hevia et al., 2013). Additionally, this peptide can modulate the dendritic cell phenotype of ulcerative colitis (UC) patients (Bernardo et al., 2012; Al-Hassi et al., 2014). It was also demonstrated that the

immunomodulatory effect of *Lactobacillus paracasei* is mediated, at least in part, by the secreted protease lactocepin, which selectively degrades the chemokine IFN-γ-inducible protein 10 (IP-10) that functions in lymphocyte recruitment (von Schillde et al., 2012). There are other examples of non-proteinaceous compounds that can exert certain effects on the host. Some species of *Bifidobacterium* possess unmethylated CpG motifs in their DNA that were able to induce TLR9 activation, which is known to trigger a Th1 orientation of the immune system (Ménard et al., 2010). In contrast, in other studies, it was shown that intragastric and subcutaneous administration of DNA from a probiotic mix ameliorated the severity of colitis in a murine experimental colitis model, whereas a methylated probiotic DNA had no effect (Rachmilewitz et al., 2004).

EMERGING PROBIOTICS, A NOVEL SOURCE OF IMMUNOMODULATORY EFFECTORS

In addition to *Lactobacillus* and *Bifidobacterium*, other microorganisms have received substantial interest among researchers as potentially new, beneficial gut bacteria. Most of them are common colonizers of the human gut under normal conditions. Some of these microbial types are considered to be markers of dysbiosis in intestinal inflammatory diseases, such as UC and Crohn's disease (Manichanh et al., 2006; El Aidy et al., 2013). In these conditions, a loss of microbial diversity and a significant reduction of members of *Clostridium* clusters IV and XIVa have been reported, particularly in bacteria involved in butyrate and propionate metabolism, such as *Ruminococcus*, *Eubacterium*, *Roseburia*, and *Faecalibacterium*. In this section, we will highlight current research on *F. prausnitzii* and *A. muciniphila*, two bacteria that have received much attention during the last few years because of their potential immunomodulatory properties.

F. prausnitzii is a "novel" intestinal bacterium whose immunomodulatory properties have been well characterized *in vitro* and *in vivo*. This anaerobic, Gram-positive bacterium seems to play a role in the maintenance of gut homeostasis, and its population is normally reduced in intestinal inflammatory diseases (Sokol et al., 2008; Cao et al., 2014; Machiels et al., 2014). In 2008, Sokol and colleagues studied the effects of whole bacteria, a cell culture supernatant, bacterial DNA, or membrane-derived fractions *in vitro* using the Caco-2 epithelial colorectal adenocarcinoma cell line and PBMCs, as well as *in vivo* in a mouse model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (Sokol et al., 2008). The results showed that *F. prausnitzii* cells exerted anti-inflammatory effects in PBMCs. Furthermore, its culture supernatant reduced IL-8 secretion and abolished the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in Caco-2 cells. Moreover, although no significant improvement of the disease was detected in mice, partial disease scores significantly improved in colitic mice receiving the cell culture supernatants, compared with the non-receiving group. Accordingly, the authors hypothesized that the beneficial effects of *F. prausnitzii* must be executed by a soluble compound that is secreted by the bacteria. In relation to this, recent work showed that *F. prausnitzii* secreted a 15-kDa protein with anti-inflammatory properties. This protein was able to inhibit the NF-κB pathway in intestinal epithelial cells, and it prevented colitis in an animal model (Quévrain et al., 2015). Additional research showed that this bacterium restored physiological parameters and downregulated cytokine profiles in mice with colitis, as well as increased the Treg population to a greater degree than other commensals such as *B. longum* (Qiu et al., 2013; Martín et al., 2015). UC patients have fewer butyrate-producing *Roseburia hominis* and *F. prausnitzii* (Machiels et al., 2014). It is likely that a significant part of their anti-inflammatory action results from the effect of SCFAs in colonocytes, as acetate, propionate, and butyrate modulate the inflammatory responses of immune cells through receptors such as Gpr43 and Gpr41 (Maslowski et al., 2009). However, despite all the information that has recently been discovered about these bacterial groups in healthy and diseased states, and besides butyrate seeming to be the key homeostasis promoter, additional work is required to elucidate the molecular mechanisms through which *F. prausnitzii* interacts with the host gut environment.

A. muciniphila is another common member of the healthy gut microbiota in humans at all stages of age (Collado et al., 2007; Belzer and de Vos, 2012). *A. muciniphila* is a Gram-negative, strictly anaerobic, mucin-degrading microorganism member of the *Verrucomicrobia* phylum, and it was one of the first bacteria shown to utilize mucin, the glycosylated protein layer that covers the gut epithelium, as its sole carbon, nitrogen, and sulfur source. The products derived from mucin degradation are mainly SCFAs that feed colonocyte metabolism and confer health properties to the host. By degrading the mucin of the external mucus layer, *A. muciniphila* helps with the continuous renovation of the protective cover of the mucosae, and it maintains a healthy protective barrier that prevents the entrance of enteropathogens into the epithelium (Lukovac et al., 2014). In addition, when *A. muciniphila* was administered to mice, there were increased intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion, suggesting an immunomodulatory role for this bacterium (Everard et al., 2013).

CONCLUDING REMARKS

In conclusion, even though much effort has been put into probiotic research during recent decades, the mechanisms underlying the immunomodulatory effects of beneficial intestinal bacteria have scarcely been elucidated. There is compelling evidence that novel bacterial players, other than *Lactobacillus* and *Bifidobacterium*, could play a role in these processes and are much more important than previously thought; however, difficulties in growing some of these bacteria on laboratory- and industrial-scales, and the lack of molecular tools needed to perform functional genomic analyses, seriously hamper the characterization of novel strains. Further research is needed to overcome these culturing and functional characterization difficulties to perform well-designed pre-clinical

and intervention studies that shed new light on the mechanisms responsible for the beneficial effects attributed to these bacteria.

AUTHOR CONTRIBUTIONS

AH, SD, BS, and AM contributed to the conception and design of the work, and to the acquisition, analysis, and interpretation of the data. All authors contributed to the drafting of the manuscript and approved the final version to be published.

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REFERENCES

Al-Hassi, H. O., Mann, E. R., Sanchez, B., English, N. R., Peake, S. T., Landy, J., et al. (2014). Altered human gut dendritic cell properties in ulcerative colitis are reversed by *Lactobacillus plantarum* extracellular encrypted peptide STp. *Mol. Nutr. Food Res.* 58, 1132–1143. doi: 10.1002/mnfr.201300596

Aragón, F., Carino, S., Perdigón, G., and de Moreno de LeBlanc, A. (2014). The administration of milk fermented by the probiotic *Lactobacillus casei* CRL 431 exerts an immunomodulatory effect against a breast tumour in a mouse model. *Immunobiology* 219, 457–464. doi: 10.1016/j.imbio.2014.02.005

Belzer, C., and de Vos, W. M. (2012). Microbes inside-from diversity to function: the case of *Akkermansia*. *ISME J.* 6, 1449–1458. doi: 10.1038/ismej.2012.6

Bernardo, D., Sánchez, B., Al-Hassi, H. O., Mann, E. R., Urdaci, M. C., Knight, S. C., et al. (2012). Microbiota/host crosstalk biomarkers: regulatory response of human intestinal dendritic cells exposed to *Lactobacillus* extracellular encrypted peptide. *PLoS ONE* 7:e36262. doi: 10.1371/journal.pone.0036262

Bufe, B., Schumann, T., Kappl, R., Bogeski, I., Kummerow, C., Podgórska, M., et al. (2015). Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens. *J. Biol. Chem.* 290, 7369–787. doi: 10.1074/jbc.M114.626747

Cao, Y., Shen, J., and Ran, Z. H. (2014). Association between *Faecalibacterium prausnitzii* reduction and inflammatory bowel disease: a meta-analysis and systematic review of the literature. *Gastroenterol. Res. Pract.* 2014, 872725. doi: 10.1155/2014/872725

Claes, A. K., Zhou, J. Y., and Philpott, D. J. (2015). NOD-like receptors: guardians of intestinal mucosal barriers. *Physiology (Bethesda)* 30, 241–250. doi: 10.1152/physiol.00025.2014

Collado, M. C., Derrien, M., Isolauri, E., de Vos, W. M., and Salminen, S. (2007). Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. *Appl. Environ. Microbiol.* 73, 7767–7770. doi: 10.1128/AEM.01477-07

Dambuza, I. M., and Brown, G. D. (2015). C-type lectins in immunity: recent developments. *Curr. Opin. Immunol.* 32, 21–27. doi: 10.1016/j.coi.2014.12.002

Denes, A., Lopez-Castejon, G., and Brough, D. (2012). Caspase-1: is IL-1 just the tip of the ICEberg? *Cell Death Dis.* 3, e338. doi: 10.1038/cddis.2012.86

Dongarrà, M. L., Rizzello, V., Muccio, L., Fries, W., Cascio, A., Bonaccorsi, I., et al. (2013). Mucosal immunology and probiotics. *Curr. Allergy Asthma Rep.* 13, 19–26. doi: 10.1007/s11882-012-0313-0

Eisenstein, E. M., and Williams, C. B. (2009). The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. *Pediatr. Res.* 65, 26R–31R. doi: 10.1203/PDR.0b013e31819e76c7

El Aidy, S., Van den Abbeele, P., Van de Wiele, T., Louis, P., and Kleerebezem, M. (2013). Intestinal colonization: how key microbial players become established in this dynamic process: microbial metabolic activities and the interplay between the host and microbes. *Bioessays* 35, 913–923. doi: 10.1002/bies.201300073

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., et al. (2013). Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9066–9071. doi: 10.1073/pnas.1219451110

Fanning, S., Hall, L. J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., et al. (2012). Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2108–2113. doi: 10.1073/pnas.1115621109

Grangette, C., Nutten, S., Palumbo, E., Morath, S., Hermann, C., Dewulf, J., et al. (2005). Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10321–10326. doi: 10.1073/pnas.0504084102

Hevia, A., Martínez, N., Ladero, V., Alvarez, M. A., Margolles, A., and Sánchez, B. (2013). An extracellular Serine/Threonine-rich protein from *Lactobacillus plantarum* NCIMB 8826 is a novel aggregation-promoting factor with affinity to mucin. *Appl. Environ. Microbiol.* 79, 6059–6066. doi: 10.1128/AEM.01657-13

Hidalgo-Cantabrana, C., Sánchez, B., Milani, C., Ventura, M., Margolles, A., and Ruas-Madiedo, P. (2014). Genomic overview and biological functions of exopolysaccharide biosynthesis in *Bifidobacterium* spp. *Appl. Environ. Microbiol.* 80, 9–18. doi: 10.1128/AEM.02977-13

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Ivanov, D., Emonet, C., Foata, F., Affolter, M., Delley, M., Fisseha, M., et al. (2006). A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. *J. Biol. Chem.* 281, 17246–17252. doi: 10.1074/jbc.M601678200

Kleta, S., Nordhoff, M., Tedin, K., Wieler, L. H., Kolenda, R., Oswald, S., et al. (2014). Role of F1C fimbriae, flagella, and secreted bacterial components in the inhibitory effect of probiotic *Escherichia coli* Nissle 1917 on a typical enteropathogenic *E. coli* infection. *Infect Immunol.* 82, 1801–1812. doi: 10.1128/IAI.01431-13

Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C., Singh, S. K., Valence, F., et al. (2008). S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19474–19479. doi: 10.1073/pnas.0810305105

López, P., González-Rodríguez, I., Sánchez, B., Ruas-Madiedo, P., Suárez, A., Margolles, A., et al. (2012). Interaction of *Bifidobacterium bifidum* LMG13195 with HT29 cells influences regulatory-T-cell-associated chemokine receptor expression. *Appl. Environ. Microbiol.* 78, 2850–2857. doi: 10.1128/AEM.07581-11

Lukovac, S., Belzer, C., Pellis, L., Keijser, B. J., de Vos, W. M., Montijn, R. C., et al. (2014). Differential modulation by *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* of host peripheral lipid metabolism and histone acetylation in mouse gut organoids. *mBio* 5, e01438–e01414. doi: 10.1128/mBio.01438-14

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., et al. (2014). A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 63, 1275–1283. doi: 10.1136/gutjnl-2013-304833

Macpherson, A. J., and Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nat. Rev. Immunol.* 4, 478–485. doi: 10.1038/nri1373

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., et al. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55, 205–211. doi: 10.1136/gut.2005.073817

Martín, R., Miquel, S., Chain, F., Natividad, J. M., Jury, J., Lu, J., et al. (2015). *Faecalibacterium prausnitzii* prevents physiological damages in a chronic low-grade inflammation murine model. *BMC Microbiol.* 15:67. doi: 10.1186/s12866-015-0400-1

Maslowski, K. M., Vieira, A. T., Ng, A., Kranich, J., Sierro, F., Yu, D., et al. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 461, 1282–1286. doi: 10.1038/nature08530

Ménard, O., Gafa, V., Kapel, N., Rodriguez, B., Butel, M. J., and Waligora-Dupriet, A. J. (2010). Characterization of immunostimulatory CpG-rich sequences from different *Bifidobacterium* species. *Appl. Environ. Microbiol.* 76, 2846–255. doi: 10.1128/AEM.01714-09

Meng, J., Zhu, X., Gao, S. M., Zhang, Q. X., Sun, Z., and Lu, R. R. (2014). Characterization of surface layer proteins and its role in probiotic properties of three *Lactobacillus* strains. *Int. J. Biol. Macromol.* 65, 110–114. doi: 10.1016/j.ijbiomac.2014.01.024

Miettinen, M., Veckman, V., Latvala, S., Sareneva, T., Matikainen, S., and Julkunen, I. (2008). Live *Lactobacillus rhamnosus* and *Streptococcus pyogenes* differentially regulate Toll-like receptor (TLR) gene expression in human primary macrophages. *J. Leukoc. Biol.* 84, 1092–1100. doi: 10.1189/jlb.1206737

Murofushi, Y., Villena, J., Morie, K., Kanmani, P., Tohno, M., Shimazu, T., et al. (2015). The toll-like receptor family protein RP105/MD1 complex is involved in the immunoregulatory effect of exopolysaccharides from *Lactobacillus plantarum* N14. *Mol. Immunol.* 64, 63–75. doi: 10.1016/j.molimm.2014.10.027

Nezametdinova, V. Z., Zakharevich, N. V., Alekseeva, M. G., Averina, O. V., Mavletova, D. A., and Danilenko, V. N. (2014). Identification and characterization of the serine/threonine protein kinases in *Bifidobacterium*. *Arch. Microbiol.* 196, 125–136. doi: 10.1007/s00203-013-0949-8

Noack, M., and Miossec, P. (2014). Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun. Rev.* 13, 668–677. doi: 10.1016/j.autrev.2013.12.004

Nyirenda, M. H., Morandi, E., Vinkemeier, U., Constantin-Teodosiu, D., Drinkwater, S., Mee, M., et al. (2015). TLR2 stimulation regulates the balance between regulatory T Cell and Th17 function: a novel mechanism of reduced regulatory T Cell function in multiple sclerosis. *J. Immunol.* 194, 5761–5774. doi: 10.4049/jimmunol.1400472

O'Connell Motherway, M., Zomer, A., Leahy, S. C., Reunanen, J., Bottacini, F., Claesson, M. J., et al. (2011). Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11217–11222. doi: 10.1073/pnas.1105380108

Qiu, X., Zhang, M., Yang, X., Hong, N., and Yu, C. (2013). *Faecalibacterium prausnitzii* upregulates regulatory T cells and anti-inflammatory cytokines in treating TNBS-induced colitis. *J. Crohns. Colitis.* 7, e558–e568. doi: 10.1016/j.crohns.2013.04.002

Quévrain, E., Maubert, M. A., Michon, C., Chain, F., Marquant, R., Tailhades, J., et al. (2015). Identification of an anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient in Crohn's disease. *Gut*. doi: 10.1136/gutjnl-2014-307649. [Epub ahead of print].

Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., et al. (2004). Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 126, 520–528. doi: 10.1053/j.gastro.2003.11.019

Reunanen, J., von Ossowski, I., Hendrickx, A. P., Palva, A., and de Vos, W. M. (2012). Characterization of the SpaCBA pilus fibers in the probiotic *Lactobacillus rhamnosus* GG. *Appl. Environ. Microbiol.* 78, 2337–2344. doi: 10.1128/AEM.07047-11

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., et al. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb. Ecol. Health Dis.* 26:26050. doi: 10.3402/mehd.v26.26050

Sakaguchi, S., Miyara, M., Costantino, C. M., and Hafler, D. A. (2010). FOXP3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10, 490–500. doi: 10.1038/nri2785

Salonen, A., Lahti, L., Salojärvi, J., Holtrop, G., Korpela, K., Duncan, S. H., et al. (2014). Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J.* 8, 2218–2230. doi: 10.1038/ismej.2014.63

Salzman, N. H., Underwood, M. A., and Bevins, C. L. (2007). Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. *Semin. Immunol.* 19, 70–83. doi: 10.1016/j.smim.2007.04.002

Sánchez, B., Gueimonde, M., Peña, A. S., and Bernardo, D. (2015). Intestinal microbiota as modulators of the immune system. *J. Immunol. Res.* 2015:159094. doi: 10.1155/2015/159094

Scott, K. P., Antoine, J. M., Midtvedt, T., and van Hemert, S. (2015). Manipulating the gut microbiota to maintain health and treat disease. *Microb. Ecol. Health Dis.* 26:25877. doi: 10.3402/mehd.v26.25877

Sekirov, I., Russell, S. L., Antunes, L. C., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904. doi: 10.1152/physrev.00045.2009

Sellge, G., and Kufer, T. A. (2015). PRR-signaling pathways-Learning from microbial tactics. *Semin. Immunol.* 27, 75–84. doi: 10.1016/j.smim.2015.03.009

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G., Gratadoux, J. J., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16731–16736. doi: 10.1073/pnas.0804812105

Turroni, F., Serafini, F., Foroni, E., Duranti, S., O'Connell Motherway, M., Taverniti, V., et al. (2013). Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium–host interactions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11151–11156. doi: 10.1073/pnas.1303897110

von Schillde, M. A., Hörmannsperger, G., Weiher, M., Alpert, C. A., Hahne, H., Bäuerl, C., et al. (2012). Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. *Cell Host Microbe* 11, 387–396. doi: 10.1016/j.chom.2012.02.006

Wing, J. B., and Sakaguchi, S. (2014). Foxp3$^+$ T(reg) cells in humoral immunity. *Int. Immunol.* 26, 61–69. doi: 10.1093/intimm/dxt060

Yadav, A. K., Tyagi, A., Kumar, A., Panwar, S., Grover, S., Saklani, A. C., et al. (2015). Adhesion of lactobacilli and their anti-infectivity Potential. *Crit. Rev. Food Sci. Nutr*. doi: 10.1080/10408398.2014.918533. [Epub ahead of print].

Yao, H., Dittmann, M., Peisley, A., Hoffmann, H. H., Gilmore, R. H., Schmidt, T., et al. (2015). ATP-Dependent Effector-like Functions of RIG-I-like Receptors. *Mol. Cell.* 58, 541–548. doi: 10.1016/j.molcel.2015.03.014

Yousefi, M., Movassaghpour, A. A., Shamsasenjan, K., Ghalamfarsa, G., Sadreddini, S., Jadidi-Niaragh, F., et al. (2015). The skewed balance between Tregs and Th17 in chronic lymphocytic leukemia. *Future Oncol.* 11, 1567–182. doi: 10.2217/fon.14.298

Zakharevich, N. V., Osolodkin, D. I., Artamonova, I. I., Palyulin, V. A., Zefirov, N. S., and Danilenko, V. N. (2012). Signatures of the ATP-binding pocket as a basis for structural classification of the serine/threonine protein kinases of gram-positive bacteria. *Proteins* 80, 1363–1376. doi: 10.1002/prot.24032

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REVIEW
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Bioactive Molecules Released From Cells Infected with the Human Cytomegalovirus

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Following primary infection in humans, the human cytomegalovirus (HCMV) persists in a latent state throughout the host's lifetime despite a strong and efficient immune response. If the host experiences some form of immune dysregulation, such as immunosuppression or immunodeficiency, HCMV reactivates, thereby emerging from latency. Thus, in the absence of effective functional immune responses, as occurs in immunocompromised or immunoimmature individuals, both HCMV primary infections and reactivations from latency can cause significant morbidity and mortality. However, even in immunocompetent hosts, HCMV represents a relevant risk factor for the development of several chronic inflammatory diseases and certain forms of neoplasia. HCMV infection may shift between the lytic and latent state, regulated by a delicate and intricate balance between virus-mediated immunomodulation and host immune defenses. Indeed, HCMV is a master in manipulating innate and adaptive host defense pathways, and a large portion of its genome is devoted to encoding immunomodulatory proteins; such proteins may thus represent important virulence determinants. However, the pathogenesis of HCMV-related diseases is strengthened by the activities of bioactive molecules, of both viral and cellular origin, that are secreted from infected cells and collectively named as the secretome. Here, we review the state of knowledge on the composition and functions of HCMV-derived secretomes. In lytic infections of fibroblasts and different types of endothelial cells, the majority of HCMV-induced secreted proteins act in a paracrine fashion to stimulate the generation of an inflammatory microenvironment around infected cells; this may lead to vascular inflammation and angiogenesis that, in turn, foster HCMV replication and its dissemination through host tissues. Conversely, the HCMV secretome derived from latently infected hematopoietic progenitor cells induces an immunosuppressive extracellular environment that interferes with immune recognition and elimination of latently infected cells, thereby promoting viral persistence. Characterization of the composition and biological activities of HCMV secretomes from different types of infected cells will lay the foundation for future advances in our knowledge about the pathogenesis HCMV diseases and may provide targets for the development of novel antiviral intervention strategies.

Keywords: human cytomegalovirus, lytic infection, latency, bioactive molecules, secretome, chronic disease, angiogenesis, immunoevasion

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THE HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV) is an opportunistic beta-Herpesvirus that infects more than 90% of people worldwide with an infection rate that increases with age (Landolfo et al., 2003; Britt, 2008; Mocarski et al., 2014). HCMV infection, as for the other human Herpesviruses, is characterized by two phases: lytic and latent. Following primary infection, in which the virus productively replicates in a broad range of different cell types (Sinzger et al., 2008), HCMV establishes latency in cells of myeloid lineage through a still poorly understood mechanism (Britt, 2008; Crough and Khanna, 2009; Mocarski et al., 2014). In the normal immunocompentent host, primary infection is usually asymptomatic or mildly symptomatic and persistent (Britt, 2008; Mocarski et al., 2014). Nevertheless, HCMV infection is considered, even in immunocompetent hosts, a risk factor for the development of various vascular diseases, immunosenescence, and tumor development (Britt, 2008; Mocarski et al., 2014; Nogalski et al., 2014). In contrast, in individuals in which the ability to develop an appropriate cellular immune response is compromised, such as immunosuppressed patients and the immunoimmature fetus during pregnancy, HCMV infection is one of the major causes of morbidity and mortality. Indeed, primary infection or reactivation from latency causes overt diseases in immunosuppressed hosts, such as transplant recipients taking immunosuppressive drugs and AIDS patients. In these settings, HCMV can be responsible for a wide range of clinical conditions, for example retinitis, pneumonia, colitis, hepatitis, and several chronic inflammatory diseases, such as atherosclerosis, transplant vascular sclerosis (TSV), and chronic allograft rejection (CR; Britt, 2008; Crough and Khanna, 2009; Mocarski et al., 2014). Congenital HCMV infections, on the other hand, represent the most prominent viral cause of birth defects such as malformations, hearing loss and learning disabilities (Kenneson and Cannon, 2007; Britt, 2008; Mocarski et al., 2014), and the incidence of HCMV transmission to the fetus is strictly related to maternal seroprevalence. Primary maternal infection carries a risk of transmission between 14.2 and 52.4% (Kenneson and Cannon, 2007), while a transmission rate of 1.4% has been reported in relation to maternal reactivated infections. However, the severity of congenital HCMV disease is similar for both primary and non-primary infections (Ahlfors et al., 2001; Rahav et al., 2007). Globally, the incidence of HCMV congenital infection is between 0.3 and 2.3% of all live births in developing countries. In congenitally infected fetuses, about 10–15% develops evident symptoms after birth, with an incidence of perinatal mortality of about 3–20 newborns per 100,000 live births, and of 24–160 cases of neurologic diseases (hearing loss and mental retardation) per 100,000 neonates. In the remaining 85–90% of HCMV-infected newborns, no symptoms are displayed at birth, but late signs of infection, such as hearing defects, are evident in 30–200 newborns per 100,000 live births. The impact of congenital HCMV infection on public health is thus significant, given that hearing loss, the most common long-term sequelae of infants with congenital HCMV, it is the leading cause of non-genetic deafness in children.

The broad range of clinical manifestations of HCMV diseases reflects the capacity of the virus to productively infect an extremely wide range of cell types in the host, such as skin and lung fibroblasts, epithelial and endothelial cells (ECs), vascular smooth muscle cells, hepatocytes, monocyte-derived macrophages, neuronal, and glial cells, thus determining its potential to spread to all areas of the body (Sinzger, 2008; Sinzger et al., 2008).

The HCMV genome is the largest among the Herpesviruses; its 235 kb double-stranded DNA is structured into a unique long (UL) and a unique short (US) region, both of which are flanked by terminal and internal inverted repeats (TRL/S and IRL/S, respectively; Landolfo et al., 2003; Mocarski et al., 2014). Even though its annotation remains provisional and its coding capacity has recently been proposed to be much greater than originally thought (Stern-Ginossar et al., 2012), it is generally accepted that the HCMV genome encodes at least 170 canonical proteins (Mocarski et al., 2014). Whole-genome functional profiling of two HCMV laboratory strains revealed that a set of just 50 herpesvirus-common proteins, encoded by genes mainly located in the central region of the U_L domain, is required for productive viral replication in primary fibroblasts (Murphy and Shenk, 2008; Mocarski et al., 2014). The remaining two-thirds of canonical HCMV protein-coding genes, mostly betaherpesvirus- or CMV-specific, are confined within the terminal regions of the genome and are not essential in cultured fibroblasts. Although, specific functions have yet to be assigned to many of these non-essential genes, they are mainly thought to be involved in regulating virus cell tropism, dissemination, and viral persistence and latency within the host, as well as the modulation of intrinsic, innate, and acquired host immune responses, thus contributing to viral pathogenesis in a variety of ways (Mocarski et al., 2014).

During lytic infection, viral gene expression occurs in three phases, named immediate early (IE or α), early (E or β), and late (L or γ) in relation to their temporal kinetic profiles, and leads to genome replication, assembly and release of mature infectious viral particles. In brief, HCMV gene expression begins with *de novo* expression of IE genes, predominantly IE1-72 and IE2-86, that activate the expression of E genes, required for replication of the viral genome and the subsequent transcription of L (primarily structural) genes. IE2-86 protein autoregulates its own expression by negatively acting on the Major IE Promoter (MIEP) of HCMV (**Figure 1**; Stinski and Petrik, 2008); it binds to the *cis*-repressive sequence (CRS) of MIEP, resulting in a decrease in MIEP transcription (Stinski and Petrik, 2008). The activities of IE proteins, in turn, determine the expression of E genes, which are divided in two subgroups, β1 (E) and β2 (E-L), according to their time of expression; their functions are mainly related to HCMV replication machinery, viral DNA replication factors, repair enzymes, and immune evasion (Landolfo et al., 2003; Mocarski et al., 2014). L proteins are then expressed according to two different kinetics profiles (γ1 and γ2), shaped by distinct times of expression and their sensitivity to inhibitors of viral DNA replication. L proteins functions are associated to the assembly, maturation and egress of newly formed viral particles from the host cell.

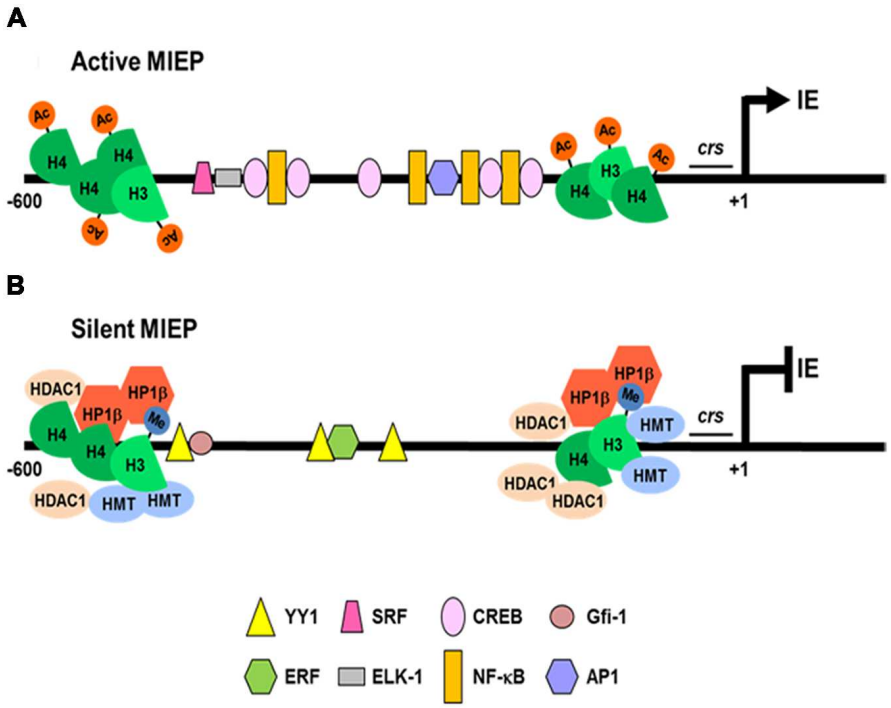

FIGURE 1 | Regulation of HCMV MIEP activity during lytic and latent infections. (A) In cells permissive to lytic infection, the MIEP is associated with acetylated histones (Ac; H4 and H3) and many constitutive and inducible cellular transcription factors (NF-κB, CREB, AP1, SRF, and Elk-1) bind to cognate binding sites to activate IE genes transcription. **(B)** In latently infected cells, cellular transcriptional repressors (YY1, ERF, and Gfi-1) bind and recruit histone deacetylases (HDAC1) and methyltransferases (HMT) to the MIEP. The reduced content of acetylated H4 and the increase of dimethylated H3 histones (Me), promotes recruitment of the silencing protein (heterochromatin protein 1 -HP1β-), thus determining repression of the MIEP transcriptional activity.

In contrast to lytic replication, only a small subset of HCMV genes are expressed during latency (e.g., transcripts for the latency-associated viral IL-10/UL111A, LUNA [Latency Unique Natural Antigen], US28, UL138, and UL144) without any detectable production of infectious virus (Avdic et al., 2011; Reeves, 2011; Poole et al., 2014). HCMV latency is restricted to primary myeloid progenitors, such as granulocyte macrophage progenitors (GMPs), $CD34^+$ hematopoietic progenitor cells, and $CD14^+$ monocytes (Hahn et al., 1998; Scrivano et al., 2011; Rossetto et al., 2013). In these infected cell-types the carriage of viral DNA occurs in the absence of the expression of lytic genes (Mocarski et al., 2014). Only upon the terminal differentiation of these cells into macrophages or dendritic cells (DCs), HCMV lytic genes are expressed: IE gene products activate the expression of other genes, leading to viral DNA replication and *de novo* virus production (Taylor-Wiedeman et al., 1994; Hahn et al., 1998; Soderberg-Nauclér et al., 2001; Reeves et al., 2005; Britt, 2008; Huang et al., 2012; Mocarski et al., 2014). Since the number of cells carrying the latent viral genome is extremely low *in vivo* (about 0.001–0.01% of monocytes), it has been necessary to develop complex experimental latency models in order to study the related mechanisms. Thus, experimental HCMV latency and reactivation analyses have been performed using *in vitro* models that exploit primary myeloid progenitors cells: namely GMP cells, $CD34^+$ hematopoietic cells, and $CD14^+$ monocytes (Reeves et al., 2005; Cheung et al., 2006; Reeves and Sinclair, 2010; Liu et al., 2013). All of these studies have highlighted that a crucial aspect of HCMV latency is the repression of MIEP following its association with repressive chromatin markers (Bain et al., 2003; Wright et al., 2005; Sinclair and Sissons, 2006), involving the recruitment of histones and transcriptional silencing factors to the MIEP (Reeves, 2011). In addition to several binding sites for cellular transcription factors (e.g., NF-κB, CREB/ATF, AP1, SRF, Elk-1) that stimulate its transcriptional activity (**Figure 1**; Caposio et al., 2007a, 2010; Lashmit et al., 2009; Isern et al., 2011; Mocarski et al., 2014), the MIEP contains a series of multiple binding sites for transcription factors that may lead to its repression (YY1, ERF and Gfi-1; **Figure 1**; Reeves, 2011). In fact, during HCMV latency, YY1 and ERF bind to MIEP, and recruit histone deacetylases and methyltransferases than then target histones associated with MIEP (Wright et al., 2005). The methylated histones then become targets for the recruitment of heterochromatin protein 1 (HP-1), which augments MIEP repression and contributes to the establishment of latency (Liu et al., 2010). Therefore, permissive or latent infection may be determined by the balance between activating and repressive transcription factors that control MIEP activity (**Figure 1**; Reeves and Sinclair, 2013). However, this balance is thought to be mainly under the control of host inflammatory responses, either in immunocompetent or immunosuppressed individuals (Liu et al., 2013). Various studies have indeed shown that HCMV reactivation, during the allogeneic response to a transplanted

organ, is mediated by the expression of inflammatory cytokines (i.e., TNF, LPS, and IL-6) that may, in turn, activate MIEP-interacting cellular transcription factors (e.g., NF-κB and AP-1), thus promoting MIEP activation and lytic HCMV gene expression (Hummel and Abecassis, 2002). However, in other experimental systems, the induction of IE gene expression has been associated with the state of cellular differentiation, as observed in non-permissive monocytes upon their differentiation into permissive macrophages in response to tumor necrosis factor (TNF) and IFN-γ (Soderberg-Nauclér et al., 1997).

MODULATION OF HOST IMMUNE RESPONSES BY HCMV

Among the HCMV genes not essential for growth in cultured fibroblasts, about 40 have been shown to be involved in the modulation of host immune responses to viral infection by interfering with innate immune mechanisms and counteracting adaptive antibody and T-cell mediated immunity (Mocarski et al., 2014). The ability of HCMV to establish persistent infection, in spite of a robust T-cell and neutralizing antiviral antibody response, is mainly ensured by the activity of many virus-encoded immunomodulatory proteins. HCMV immunomodulation is therefore of pathogenetic importance in the establishment of virus persistence in the host. In this regard, HCMV is considered a master of immune evasion strategies, as highlighted by the results of several functional genomic, transcriptomic, and proteomic analyses that have defined many immunomodulatory functions of several HCMV proteins. In particular, these viral proteins have been observed to: (1) interfere with antigen presentation by major histocompatibility complex-I (MHC-I) molecules (e.g., the viral gene products US2, US3, US6, US10, US11, and UL82); (2) permit immune evasion through interference with and T and NK cells functions (e.g., the proteins encoded by genes UL16, UL18, UL40, UL83, UL141, UL142, US18, and US20); and (3) mimic the activities of cellular cytokines/chemokines (e.g., proteins UL111A, UL128, UL146, and UL147) or act as fake host cytokine/chemokine receptors (as observed for proteins UL144, UL21.5, US27, US28, UL33, and UL78; McSharry et al., 2012; Van Damme and Van Loock, 2014).

The best-characterized mechanisms of HCMV immunoevasion can be summarized as follows:

(1) Interference with antigen presentation by MHC-I molecules. A remarkable example is provided by glycoproteins US2, US3, US6, and US11, characterized by a single transmembrane (TM)-spanning and an immunoglobulin (Ig) domain, which most likely arose by duplication of a single viral ancestor gene (Gewurz et al., 2001). These HCMV glycoproteins, when expressed in fibroblasts, endothelial/epithelial cells, or DCs, induce, through different mechanisms, a decrease in the cell-surface expression of MHC-proteins required for the activation of $CD8^+$ T lymphocytes (Mocarski, 2002). In particular, both US2 and US11 proteins are able to redirect nascent MHC class I proteins from the ER into the cytosol until ubiquitin-dependent proteasome-mediated degradation occurs. The US2 glycoprotein has also been observed to inhibit MHC class II protein translocation, thus inducing a substantial block of $CD4^+$ T cell expansion. On the other hand, the US3 gene product prevents the egress of MHC class I proteins from the endoplasmic reticulum (ER) to the Golgi apparatus, whereas the US6 protein is able to bind the transporter of antigen processing (TAP) in the lumen of the ER, preventing ATP-dependent peptide loading on the cell membrane (Mocarski, 2002). In conclusion, this type of immunoevasion prevents the immune recognition of HCMV-infected cells.

(2) Immune evasion through interference with T and NK cells functions. Lytically HCMV-infected cells exhibit a noteworthy resistance to NK cell-mediated cytolysis *in vitro* (Wilkinson et al., 2008). HCMV is, in fact, able to alter and modify NK cell activity through different 'evasion' strategies, such as those exerted by: (i) UL16, a viral glycoprotein able to directly bind and sequester in the ER MICB, ULBP1, and ULBP2 -ligands for the activating NKG2D receptor present on NK cells- thus preventing their expression on the surface of HCMV-infected cells (Wilkinson et al., 2008); (ii) UL18, a viral-MHC-I homolog, which was found to bind the NK cell inhibitory receptor LIR1/ILT2 with a 1000-fold higher affinity than HLA-I molecules (Wilkinson et al., 2008); (iii) UL40, a viral protein able to promote the HLA-E upregulation on the surface of HCMV-infected cells. Since HLA-E acts as a NK cell-mediated cytotoxicity suppressor through the NK cell inhibitory receptor complex CD94/NKG2A (Prod'homme et al., 2012), its upregulation contributes to the NK cell immunoevasion of HCMV-infected cells; (iv) UL83, that encodes for the major HCMV tegument protein pp65 that suppresses the induction of several interferons (IFNs) and proinflammatory chemokine transcripts; pp65 may also directly bind to the activator receptor NKp30, present on the surface of NK cells, thus suppressing NK cell activation (Wilkinson et al., 2008); (v) UL141, a viral glycoprotein able to sequester CD155 molecule in the ER. CD155 expression leads to NK cell activation by binding to NK receptors CD226 and CD96 (Nemčovičová et al., 2013). Therefore, sequestration of CD155 prevents NK cell activation and survival of infected cells; (vi) US18 and US20 proteins recently observed to promote lysosomal MICA degradation (Fielding et al., 2014). MICA, the MHC class I polypeptide-related sequence A, is a natural ligand of the NKG2D receptor. NKG2D-MICA binding induces NK cell activation, leading to a cytolytic response against HCMV-infected cells. Therefore, MICA degradation reduces the capability of NK cells to recognize infected cells (Fielding et al., 2014).

(3) Mimic physiological activities of cellular cytokines/chemokines or host cytokine/chemokine receptors. HCMV encodes several homologs of chemokine/cytokine and/or their receptors that may interfere with the corresponding

host counterparts. In this regard, an important example of a HCMV-encoded chemokine receptor homolog is represented by the multifunctional US28 protein. US28 is considered a putative immunoevasion molecule due to its capability to bind and internalize a range of cellular chemokines, including a broad spectrum of CC and CX3C chemokines, thus limiting the ability of host chemotactic molecules to elicit their effects in immune cells stimulation (Mocarski et al., 2014). However, US28 makes use of both the extracellular chemokine milieu and different intracellular G-protein pathways to produce a wide variety of pathophysiological cell responses (Beisser et al., 2008). In fact, upon stimulation with some chemokines, US28 specifically promotes smooth muscle cells and macrophage migration, thus contributing to both virus dissemination and pathogenesis of HCMV-associated vascular diseases (Vomaske et al., 2009). On the other hand, constitutive expression of US28 induces, in a ligand-independent manner, cyclooxygenase-2 (COX-2) expression via NF-κB activation, leading to the production of VEGF, one of the most abundant angiogenic factors (Maussang et al., 2009).

Moreover, the release of virus-encoded cytokines/chemokines homologs (see HCMV-Induced Secreted Cellular Proteins) permits the virus to modulate specific host immune defense mechanisms in infected tissues, since these bioactive soluble molecules act in a paracrine fashion.

BIOACTIVE MOLECULES RELEASED FROM HCMV-INFECTED CELLS

The pathogenesis of acute HCMV diseases is related to end-organ damage that results from both lytic virus replication and host immune responses, whereas diseases associated to persistent infections in both immunocompetent and immunocompromised patients (transplant recipients and AIDS patients) are related to chronic inflammation (Britt, 2008). However, besides the direct cytopathic effect of virus replication on host tissues, the pathogenesis of HCMV diseases may be influenced by the activity of virus-induced molecules secreted from virus-infected cells. These secreted factors, of both viral and cellular origin, by acting in a paracrine fashion alter and modify the local microenvironment, thus contributing to the development of HCMV-related diseases (Streblow et al., 2008).

HCMV-Induced Secreted Cellular Proteins

Early *in vitro* studies on the ability of HCMV to stimulate the secretion of bioactive cellular factors identified several cytokines and growth factors released from different types of infected cells. First, Almeida et al. (1994) observed that HCMV infection of HUVECs (Human Umbilical Vein Endothelial Cells) greatly increased the expression of IL-6 mRNA and the secretion of this cytokine into the supernatant of infected cells. Dengler et al. (2000) went on to demonstrate the paracrine activity of IL-1β released from HCMV-infected cells, which resulted in an upregulation of pro-inflammatory adhesion molecules on non-infected neighboring cells. In fact, by analyzing the expression of plasma membrane proteins on the surface of both HCMV-infected HUVECs and human vascular smooth muscle cells (hvSMC), they observed a 200-fold overexpression of the Inter Cellular Adhesion Molecule-1 (ICAM-1) and *de novo* induction of both Vascular Cell Adhesion Molecule 1 (VCAM-1) and E-selectin (Dengler et al., 2000). In regard to the EC model, we observed an upregulation of ICAM-1, IL-8, CCL5/RANTES, CXCL10/IP-10, CXCL11/I-TAC, and COX-2 gene expression in HUVECs cells infected with a low-passage HCMV strain, thus indicating a direct involvement of HCMV in the modification of the extracellular vascular environment through the release of inflammatory mediators (Caposio et al., 2007b).

Using astrocyte and microglia cell models, Maxim et al. (2001) observed that HCMV stimulated the release of CCL2/MCP-1 and IL-8 in the supernatants of infected astrocytes, whereas infection of microglia cells led to an increased secretion of TNF-α, IL-6, CCL2/MCP-1, IL-8, CCL5/RANTES, and CCL3/MIP-1α. These findings thus suggest the ability of infected astrocytes to recruit microglia cells, through the release of the chemoattractant CCL2/MCP-1 (Maxim et al., 2001).

However, a different picture emerged from the investigation of the HCMV-mediated modulation of chemokine gene expression in the context of HCMV retinitis – a virus-induced inflammation of the retina characterized by vasculitis and retina degeneration that leads to retinal detachment in immunocompromised hosts. In the *in vitro* cell model provided by human retinal pigment epithelial cells, Momma et al. (2003) observed, by means of RT-PCR and ELISA assays, that HCMV infection caused an upregulation of IL-8, whereas CCL2/MCP-1 and CCL7/MCP-3 levels were downregulated. The authors thus suggested that the altered secretion of IL-8, CCL2/MCP-1 and CCL7/MCP-3 by retinal cells may be involved in the initiation and development of the inflammatory process responsible for the pathogenesis of HCMV retinitis. In this scenario, the increased secretion of IL-8 may stimulate the recruitment and trafficking of leukocytes to the site of infection within the retina; on the other hand, the decrease in CCL2/MCP-1 and CCL7/MCP-3 levels in the extracellular environment may limit the migration of leukocytes, thus contributing to the virus escape of the host innate immune response (Momma et al., 2003).

Together, these earlier studies demonstrated how HCMV is able to alter in a cell-type specific manner the extracellular microenvironment surrounding infected cells by stimulating the secretion of cellular bioactive factors.

However, only after the introduction of high-throughput large-scale proteomic approaches including antibody-based arrays and liquid chromatography-mass spectrometry (MS/LC), was it possible to drawn up an in-depth depiction of the complexity of the bioactive molecules released upon HCMV infection (Dumortier et al., 2008; Botto et al., 2011; Caposio et al., 2011, 2013; Fiorentini et al., 2011; Mason et al., 2012; MacManiman et al., 2014; Noriega et al., 2014; Gustafsson et al., 2015). These global biochemical characterizations confirmed the cell-type specific patterns of secreted cellular factors from HCMV-infected cells and most of the alterations of types and

quantities of bioactive molecules already observed in earlier studies.

Thanks to proteomics studies, the entire array of bioactive proteins of present in supernatants from different types of HCMV-infected cells, has now been defined, qualitatively and quantitatively, and designated the HCMV secretome (Streblow et al., 2008).

In the first of these studies, Dumortier et al. (2008) analyzed virus-free supernatants from lytically infected fibroblasts (NHDF) to investigate the complexity and heterogeneity of the HCMV-induced secretome. Using gel-free liquid chromatography (LCQ)-MS-MS, they identified more than 1,200 proteins in the secretome of HCMV-infected cells. Among them, several factors involved in angiogenesis (AG) and wound healing (WH) were further confirmed using a wide range human cytokine antibody array (RayBio G Series 2000 arrays). A conspicuous number of cytokines (IL-5, IL-6, IL-1α, IL-1β, GM-CSF, osteoprotegerin, TNF-α, TNF-RI and -RII), growth factors (angiopoietin, angiotensinogen, FGF, GDNF, HGF, IGF-BP, osteopontin, PDGF, PIGF, SPARC, and VEGF), extracellular matrix proteins, chemokines (IL-8/CXCL8 and CXCL1/GRO-α), enzymes, and adhesion molecules were identified (**Table 1**). The biological effects of HCMV secretome-derived proteins on AG and WH were then assessed using a modified matrigel *in vitro* tubule formation assay and an electric WH assay, respectively (Dumortier et al., 2008). However, many of the identified factors in the NHDF-derived secretome are also involved in the inflammatory response and EC activation, thus leading to cell proliferation, adhesion, and inflammatory response, all of which are indeed linked to TVS pathogenesis (**Figure 2**; Dumortier et al., 2008; Streblow et al., 2008). Thus, it was suggested that HCMV infection alters the local microenvironment through the secretion of cellular factors from infected cells that, in turn, by acting in a paracrine fashion, may stimulate AG and WH, both associated to the development of vascular diseases, such as TVS (Streblow et al., 2008).

Subsequent work by Botto et al. (2011) analyzed the secretome of infected HUVECs and identified by means of a cytokine/growth factors antibody array, 29 soluble factors, including IL-6, GM-CSF, and IL-8/CXCL8. A comparison of the HCMV secretomes derived from NHDF and HUVEC, highlighted that the presence in both secretomes of abundant cytokines/chemokines involved in AG, such as IL-6, GM-CSF, IL-8/CXCL8, CCL4/MIP-1β, CCL7/MCP-3, CCL20/MIP-3α, and CXCL11/I-TAC (Dumortier et al., 2008; Botto et al., 2011; Caposio et al., 2011, 2013). Among the EC-derived factors, IL-6 was identified as the major bioactive molecule involved in neovessel formation since its selective depletion severely decreased tubule formation (Botto et al., 2011). IL-6 is an inflammatory cytokine involved in different pathophysiological contexts, such as inflammation, lymphocytes differentiation, cell proliferation, and the inhibition of apoptotic signals (Scheller et al., 2011). IL-6 binding to its receptor (gp130) activates three different intracellular transduction pathways: (i) the JAK/STAT cascade, involved in growth regulation, survival, differentiation, and long-term inflammation-promoting effects (Horvath, 2004); (ii) the ERK1/2-MAPK pathway, and (iii) the phosphoinositol 3-kinase (PI3K)/Akt pathway, both of which are implicated in the regulation of cell growth and differentiation (Wegiel et al., 2008; Rose-John, 2012). In particular, IL-6 binding to its receptor leads to the phosphorylation of STAT3, which subsequently prevents apoptosis by blocking the activation of caspase 3 and 7, and most importantly, by increasing the expression of an apoptosis inhibitor, such as survivin (Hodge et al., 2005). Addition of the secretome derived from HCMV-infected ECs to uninfected ECs was found to stimulate expression of survivin, while antibody neutralization of IL-6 in the same secretome, abolished its capability to induce survivin or activate caspase 3 or 7 (Botto et al., 2011). Therefore, it was concluded that EC-derived HCMV secretome promotes EC survival through the expression of survivin via activation of the IL-6 pathway (Botto et al., 2011).

At the same time, in a study aimed at investigating the susceptibility of lymphatic endothelial cells (LECs) to HCMV infection, we characterized in detail the secretome of this particular type of EC (Fiorentini et al., 2011). Although, early reports have shown the presence of HCMV antigens and DNA in lymphoid tissues, the ability of HCMV to infect LECs remained unaddressed due to the lack of a suitable *in vitro* system. In this study, we observed that a clinical isolate of HCMV productively infected purified lymph node-derived LECs and dysregulated the expression of several LEC genes involved in the inflammatory response to viral infection (Fiorentini et al., 2011). Qualitative and quantitative cytokine antibody array analysis of virus-free supernatants from HCMV-infected LECs revealed the virus-induced secretion of several cytokines, chemokines, and growth factors that may be involved in the regulation of EC physiological properties. Among the released bioactive cellular proteins, the 20 most abundant included several cytokines (IL-1α, IL-3, IL-5, IL-6, IL-13, IL-15, GM-CSF, and TNF-β), several chemokines (CCL1/I-309, CCL7/MCP-3, CCL20/MIP-3α, CXCL1/GROα, CXCL5/ENA-78, CXCL9/MIG, and CXCL11/I-TAC), receptors (ICAM-1 and TNF-R1), and growth factors (TGF-β1 and bFGF). Functional assays then allowed us to establish that the secretome produced by HCMV-infected LECs indeed stimulated AG in both LECs and blood vessel ECs. Furthermore, neutralization of either IL-6 or GM-CSF in the secretome brought about the loss of its angiogenic properties. Involvement of IL-6 and GM-CSF in the HCMV-mediated lymphoangiogenesis was further supported by the finding that recombinant IL-6 and GM-CSF reproduce the same angiogenic effects as seen for the whole LEC-derived secretome. Thus, these results suggest that HCMV can stimulate both hemangiogenesis and lymphangiogenesis through an indirect mechanism that relies on the stimulation of IL-6 and GM-CSF secretion from virus-infected cells (Fiorentini et al., 2011).

A comparison of the HCMV-secretomes from different types of ECs (LECs and HUVEC) and fibroblasts (NHDF) highlights the presence of several common factors involved in both AG and WH processes, such as IL-6, GM-CSF, CCL7/MCP-3, CCL20/MIP-3α, CCL4/MIP-1β, and CXCL11/I-TAC (**Table 1**; Caposio et al., 2013).

TABLE 1 | The most abundant bioactive factors determined by Ray Biotech protein assay analysis in the secretome of lytic HCMV-infected cells.

				Cell types		
Classification	**Symbol**	**Gene annotation**	**Biological function**	**NHDF[1]**	**HUVEC[2]**	**LEC[3]**
Cytochine/chemochine	CXCL1; GROα	Chemokine (C-X-C motif) ligand 1	Chemoattractant for neutrophils	•	•	•
	CXCL5; ENA-78	Chemokine (C-X-C motif) ligand 5	Recruitment and activation of leukocytes	•		•
	CXCL6; GCP-2	Chemokine (C-X-C motif) ligand 6	Chemoattractant for neutrophilic granulocytes		•	
	CXCL9; MIG	Chemokine (C-X-C motif) ligand 9	Leukocyte trafficking			•
	CXCL10; IP-10	Chemokine (C-X-C motif) ligand 10	Stimulation of monocytes, natural killer, and T-cell migration	•	•	
	CXCL11; I-TAC	Chemokine (C-X-C motif) ligand 11	Chemoattractant	•	•	•
	CXCL16	Chemokine (C-X-C motif) ligand 16	Recruitment of leukocytes	•	•	
	CCL1; I-309	Chemokine (C-C motif) ligand 1	Leukocyte trafficking			•
	CCL3; MIP-1α	Chemokine (C-C motif) ligand 3	Inflammatory responses	•	•	
	CCL4; MIP-1β	Chemokine (C-C motif) ligand 4	Chemokinetic and inflammatory functions	•	•	•
	CCL5; RANTES	Chemokine (C-C motif) ligand 5	Chemoattractant for blood monocytes, memory T helper cells and eosinophils	•	•	
	CCL7; MCP-3	Chemokine (C-C motif) ligand 7	Chemoattractant for macrophages	•	•	•
	CCL8; MCP-2	Chemokine (C-C motif) ligand 8	Leukocyte trafficking		•	•
	CCL15; MIP-1δ	Chemokine (C-C motif) ligand 15	T-cells and monocyte chemoattractant			•
	CCL20; MIP-3α	Chemokine (C-C motif) ligand 20	Leukocyte trafficking	•	•	•
	CCL23; MPIF-1	Chemokine (C-C motif) ligand 23	Chemotactic activity on resting T lymphocytes and monocytes		•	
	IL-1α	Interleukin-1α	Role in immune responses and inflammatory processes			•
	IL-3	Interleukin-3	Role in cell growth, differentiation, and apoptosis			•
	IL-5	Interleukin-5	Growth and differentiation factor for B cells and eosinophils	•		•
	IL-6	Interleukin-6	Anti-inflammatory cytokine	•	•	•
	IL-8	Interleukin-8	Leukocyte trafficking	•	•	
	IL-10	Interleukin-10	Anti-inflammatory cytokine			•
	IL-13	Interleukin-13	Anti-inflammatory cytokine			•
	IL-15	Interleukin-15	T and natural killer cells activation and proliferation			•
	LTA; TNF-β	Lymphotoxin A	Proinflammatory cytokine			•
	TNF-α	Tumor necrosis factor alpha	Proinflammatory cytokine		•	•
	Osteoprotegerin	PG/TNFRSF11B	Lymph-node organogenesis and vascular calcification	•	•	
Growth factor	GM-CSF	Granulocyte-Macrophage Colony-Stimulating-Factor	Bone and cartilagine development	•	•	•
	bFGF	Fibroblast Growth Factor 2 (basic)	Mitogenic and angiogenic activities			•
	TGF-β1	Transforming growth factor beta 1	Cell growth, proliferation, differentiation, adhesion, and migration	•		•
	HGF	Hepatocyte growth factor	Angiogenesis	•		
Receptor	ICAM-1	Intercellular adhesion molecule 1	Cell adhesion for endothelial and immune system cells	•	•	•
	TNF-RI	Tumor necrosis factor receptor 1	Regulator of inflammation	•		•
	TNF-RII	Tumor necrosis factor receptor 2	Regulator of inflammation	•		
ECM	MMP-1	Matrix metallopeptidase 1	Disruption of extracellular matrix	•		•
	TIMP-1	TIMP metallopeptidase inhibitor 1	Inhibitor of the matrix metalloproteinases	•		
	TIMP-2	TIMP metallopeptidase inhibitor 2	Inhibitor of the matrix metalloproteinases	•		
	TIMP-4	TIMP metallopeptidase inhibitor 4	Inhibitor of the matrix metalloproteinases	•		
Enzyme	ANG	Angiogenin, ribonuclease, RNase A family, 5	Angiogenesis	•		

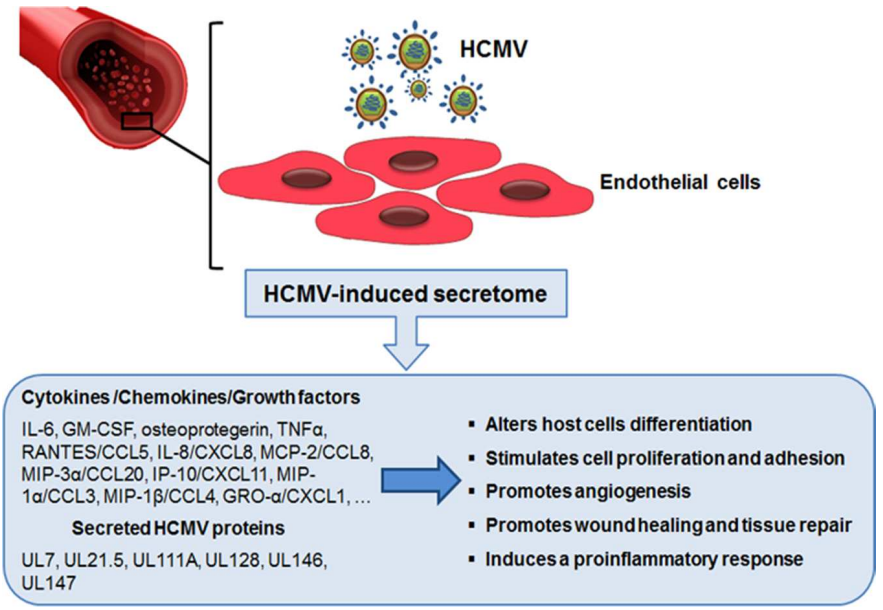

FIGURE 2 | The role of endothelial cell-derived HCMV secretome in accelerating vascular diseases. Productive HCMV infection of endothelial cells leads to the release of many bioactive proteins that can modify the microenvironment around host cells. The ability of these secreted factors to promote neo-vessel formation, wound healing, and the inflammatory response may help explain the role of HCMV infection in the development of HCMV-associated vascular diseases.

Taking into consideration the results of the aforementioned studies, the overall effect of HCMV-derived secretomes on AG and WH in ECs is very likely related to the combination of experimental conditions and different bioactive factors, which may cooperate in a synergetic manner. In this regard, Gustafsson et al. (2015) recently observed the influence of different experimental settings, such as the cell type from which the HCMV-secretome is derived, the culture conditions, the time points of supernatant collection, and the concentration of relevant bioactive molecules, in promoting AG and WH in cultured ECs.

Whilst a number of studies have provided evidence for functional roles of HCMV-derived secretomes produced by lytically infected fibroblasts and ECs (Dumortier et al., 2008; Botto et al., 2011; Fiorentini et al., 2011; MacManiman et al., 2014), little is known about the activities of secretomes released from latently HCMV-infected cells. However, exploitation of a new experimental *in vitro* model of HCMV latency allowed Mason et al. (2012) to obtain some first insights into how latent infection is able to stimulate the release of bioactive molecules. This experimental model involves the infection of $CD34^+$ hematopoietic progenitor cells cultivated in the absence of stimuli that may promote their differentiation into mature DCs or macrophages, that are fully permissive to lytic HCMV infection. These experimentally latently infected $CD34^+$ cells showed long-term carriage of viral genomes in which the MIEP remained associated with transcriptionally repressive chromatin, thus hampering further HCMV lytic gene expression. These observations correlate with those made *ex vivo* of natural HCMV latent infections (Reeves et al., 2005), and provide evidence in support of the use of experimentally latently infected $CD34^+$ cells as an effective model for the study of HCMV latent infection and reactivation (Reeves et al., 2005; Reeves and Sinclair, 2010). Indeed, analysis of supernatants from HCMV-infected $CD34^+$ cells by large-scale cytokine antibody array has revealed the presence of many bioactive cellular proteins known to be involved in both regulation of the immune response and chemoattraction of T cells (**Table 2**). The most highly abundant cellular factors identified in this latent HCMV-secretome included: several cytokines/chemokines (XCL1/LYMPHOTACTIN, CXCL9/MIG, CXCL12/SDF-1, CXCL13/BLC, CCL1/I-309, CCL2/MCP-1, CCL8/MCP-2, CCL13/MCP-4, CCL15/MIP-1δ, CCL17/TARC, CCL20/MIP-3α, CCL27/CTACK, IL-8, IL-10/CSIF, IL-13, IL-15, LEPTIN, LTA/TNF-β, and TNF-α), growth factors (BMP-4, BMP-6, CSF-1/M-CSF, IGF-1/MGF, IGFBP3/IBP3; NT-3/NTF3, TGF-β1, and TGF-β3), a receptor (ICAM-1), and the angiogenic RNase angiogenin. However, an increase in secreted CCL2 was also observed from latently infected GMPs, and related to the stimulation of $CD14^+$ monocytes migration toward the site of latency (Stern and Slobedman, 2008); whereas secretion of both CCL2 and CCL8 was measured during a short-term experimental latent infection of $CD14^+$ monocytes, and linked to the recruitment of $CD14^+$ monocytes and $CD4^+$ Th1 cells to latently infected cells (Noriega et al., 2014). Functional analysis of the HCMV-secretome derived from latently infected $CD34^+$ cells, however, highlighted that chemoattraction of $CD4^+$ T cells exclusively depended on the binding of CCL8 to CC chemokine receptors CCR3 and CCR5 expressed on $CD4^+$ T cells. In fact, the depletion of $CD4^+$ T cells bearing CCR3 or CCR5 receptors, as well as treatment of the secretome with a neutralizing anti-CCL8 antibody, resulted in a substantially blockade of $CD4^+$

TABLE 2 | Main bioactive proteins assessed in the HCMV secretome of latently infected CD34+ cells.

Classification	Symbol	Gene annotation	Biological function
Cytochine/chemochine	XCL1; LTN	Chemokine (C motif) ligand 1	Leukocyte trafficking
	CXCL9; MIG	Chemokine (C-X-C motif) ligand 9	Leukocyte trafficking
	CXCL12; SDF1	Chemokine (C-X-C motif) ligand 12	Leukocyte trafficking
	CXCL13; BLC	Chemokine (C-X-C motif) ligand 13	B lymphocyte chemoattractant
	CCL1; I-309	Chemokine (C-C motif) ligand 1	Leukocyte trafficking
	CCL2; MCP-1	Chemokine (C-C motif) ligand 2	Leukocyte trafficking
	CCL8; MCP-2	Chemokine (C-C motif) ligand 8	Leukocyte trafficking
	CCL13; MCP-4	Chemokine (C-C motif) ligand 13	Leukocyte trafficking
	CCL15; MIP-1δ	Chemokine (C-C motif) ligand 15	T-cells and monocyte chemoattractant
	CCL17; TARC	Chemokine (C-C motif) ligand 17	T cells chemotaxis and trafficking
	CCL20; MIP-3α	Chemokine (C-C motif) ligand 20	Leukocyte trafficking
	CCL27; CTACK	Chemokine (C-C motif) ligand 27	T-cell-mediated inflammation, T lymphocyte chemoattractant
	IL-8	Interleukin-8	Leukocyte trafficking
	IL-10; CSIF	Interleukin-10	Anti-inflammatory cytokine
	IL-13	Interleukin-13	Anty-inflammatory cytokine
	IL-15	Interleukin-15	T and natural killer cells activation and proliferation
	LEP	Leptin	Angiogenesis and wound healing
	LTA; TNF-β	Lymphotoxin A	Proinflammatory cytokine
	TNF-α	Tumor necrosis factor alpha	Proinflammatory cytokine
Growth factor	BMP-4	Bone morphogenetic protein 4	Bone and cartilagine development
	BMP-6	Bone morphogenetic protein 6	Bone and cartilagine growth
	CSF-1; M-CSF	Colony stimulating factor 1	Monocytes proliferation, differentiation, and survival
	IGF-1; MGF	Insulin-like growth factor 1	Cell growth and development
	IGFBP3; IBP3	Insulin-like growth factor binding protein 3	Cell growth and development
	NT-3; NTF3	Neurotrophin-3	Neurogenesis
	TGF-β1	Transforming growth factor beta 1	Cell growth, proliferation, differentiation, adhesion and migration
	TGF-β3	Transforming growth factor beta 3	Cell adhesion, proliferation, differentiation
Receptor	ICAM-1	Intercellular adhesion molecule 1	Cell adhesion for endothelial and immune system cells
Enzyme	ANG	Angiogenin, ribonuclease, RNase A family	Angiogenesis

T-cell migration toward latently infected cells (Mason et al., 2012). Moreover, in the same HCMV-secretome, high levels of TGF-β and cIL-10, both involved in the induction of immune tolerance by suppressing proliferation and T-cells functions were detected (Bettini and Vignali, 2009). This observation, led to the hypothesis that the secretome of latently infected $CD34^+$ cells may create an immunosuppressive microenvironment, thus interfering with the immune recognition of latently infected $CD34^+$ and promoting viral persistence. This hypothesis was indeed supported by the finding that TGF-β and cIL-10 in the latent secretome inhibited both the $CD4^+$ Th1-mediated cytotoxicity and the production of antiviral Th1-type cytokines, such as IFN-γ, TNF-α, and TNF-β. Furthermore, treatment of latent secretome with neutralizing antibodies specific for cIL-10 and TGF-β significantly restored IFN-γ production and the cytotoxic functions of $CD4^+$ cells, thus confirming that the observed inhibition of $CD4^+$ T cells functions strictly depended on the secretion of TGF-β and cIL-10 by latently infected $CD34^+$ cells (Mason et al., 2012). In addition, cIL-10 may prevent cell death and promote survival of latently infected cells (Weber-Nordt et al., 1996; Poole et al., 2011), since it was observed that cIL-10 is required for upregulation of the anti-apoptotic protein PEA-15 – which abolishes FAS-induced apoptosis (Poole et al., 2015). As described in Section "HCMV-Induced Secreted Cellular Proteins" above, the viral homolog LAvIL10 also stimulates the release of cIL-10 in latently infected $CD34^+$ cells. Subsequently, cIL-10 may downregulate the expression of cellular microRNA hsa-miR-92a, thus inducing the secretion of the cellular CCL8 (Poole et al., 2014). Together these findings suggest that TGF-β and cIL-10 in the latent HCMV secretome by acting in a paracrine manner on uninfected bystander $CD34^+$ cells, create and further expand a microenvironment conducive to the latent carriage of HCMV genomes. In fact, it was observed that uninfected $CD34^+$ cells synthetized TGF-β and cIL-10, when cultured in the presence of the latent secretome (Mason et al., 2012). Overall, these findings support the existence of a complex virus strategy during latency aimed at the generation of a heavily immunosuppressive microenvironment around latently infected $CD34^+$ that allows them to evade host immune recognition (**Figure 3**). In fact, even if HCMV infections leads to the recruitment of $CD4^+$ T cells to site of latency through the release of CCL8, it then inhibits their antiviral functions by generating the strong immunosuppressive microenvironment, thereby avoiding the elimination of latently infected cells (Sinclair and Reeves, 2013). Furthermore, the upregulation of cIL-10 protects latently infected $CD34^+$ from apoptosis, thus

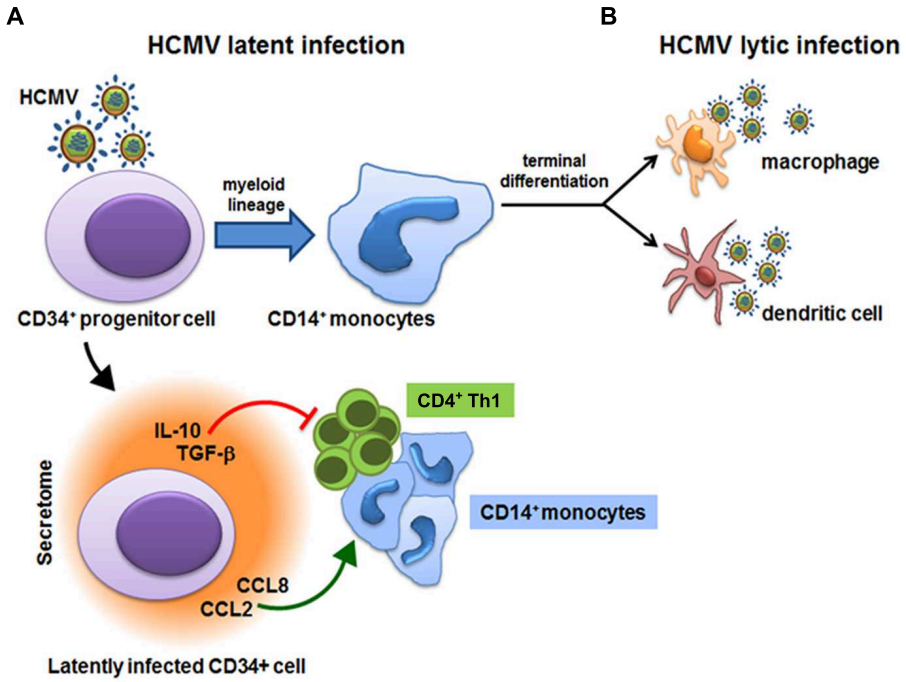

FIGURE 3 | Human cytomegalovirus (HCMV)-derived secretome and virus latency. HCMV latency is established in bone marrow cells and is carried out in $CD34^+$ hematopoietic progenitor cells and in $CD14^+$ monocytes. **(A)** Latently infected $CD34^+$ cells secrete CCL2/MCP-1 and CCL8/MCP-2 cytokines that recruit $CD14^+$ monocytes and $CD4^+$ Th1 cells to latently infected cells. However, the secretion of highly immunosuppressive cellular IL-10 and TGF-β inhibit proliferation and the antiviral functions of $CD4^+$ Th1 cells. **(B)** Upon differentiation of $CD14^+$ monocytes into mature macrophages or dendritic cells, reactivation of HCMV IE gene expression is promoted and the viral lytic gene program is initiated.

promoting their survival in order to maintain latent viral genome carriage (Poole et al., 2015).

Although, HCMV lytic and latent host-cell interactions involve different target cell types and viral genetic programs, and lead to different outcomes, many bioactive factors are common between the HCMV-induced secretome derived from lytically and latently infected cells. In particular, various cytokines/chemokines, such as CXCL9/MIG, CCL1/I-309, CCL8/MCP-2, CCL15/MIP-1δ, CCL20/MIP-3α, IL-8, IL-10, IL-13, IL-15, LTA/TNF-β, and TNF-α, the growth factor TGF-β, the receptor ICAM-1, and the enzyme ANG are present in both lytically- and latently-infected cells secretomes. However, the presence of a set of common bioactive factors between the two functionally different secretomes is of great interest considering the opposite outcomes of both forms of infections; indeed, we might hypothesize that some of the pathophysiological alterations of the microenvironment surrounding HCMV-infected cells that are induced by the bioactive molecules that the two secretomes have in common, may occur during both lytic and latent infection.

Secreted HCMV Proteins

Many viral-encoded secreted bioactive proteins have been identified in the supernatants of herpesvirus-infected cells. Among them, cytokine-like proteins, such as the viral counterparts of cellular IL-10, IL-17, and IL-6 were found in the supernatants from cells infected with Epstein–Barr virus (EBV; Ryon et al., 1993; Swaminathan et al., 1993), herpesvirus saimiri (Yao et al., 1995), or human herpesvirus-8 (HHV-8; Neipel et al., 1997), respectively. In this section, we review the main biochemical and functional features of the HCMV-encoded proteins that have identified to date within the secretome of infected cells.

UL21.5

This was the first protein of viral origin identified as a secreted product in the supernatant of HCMV-infected cells (Müllberg et al., 1999). UL21.5 mRNA is abundantly expressed at the late stage of HCMV infection (Rawlinson and Barrell, 1993) and is transcribed by two exons; the first encodes a signal peptide of 20 amino acids followed by an additional sequence of eight amino acids, while the second exon codes the remaining 75 residues. Intriguingly, UL21.5 mRNA is also packaged into virions, thus suggesting a role of the encoded protein even before the infecting viral genome reaches the nucleus and becomes transcriptionally active (Bresnahan and Shenk, 2000).

Later, it was demonstrated that pUL21.5 is a soluble CC chemokine receptor able to selectively bind CCL5/RANTES with high affinity and thus preventing its interaction with cellular receptors (Wang et al., 2004). pUL21.5 by acting as a secreted receptor decoy even at a distance from infected cells, may therefore counteract chemokine-mediated host antiviral response (Wang et al., 2004).

UL146 and UL147

UL146 and UL147 proteins are considered as potential viral encoded CXC-chemokines due to the presence of putative signal sequences, cysteine spacing, and (in pUL146 only) an ELR-CXC sequence (glutamic acid -E-, leucine -L-, arginine -R-, nominated the ELR sequence, upstream of the CXC motif). Such features are common to cellular CXC cytokines, such as IL-8 and Gro-α (Penfold et al., 1999; McSharry et al., 2012). Sequence analysis of UL146 and UL147 proteins from the Toledo strain of HCMV reveals 24 and 16% amino acid identity to IL-8 (McSharry et al., 2012), respectively; accordingly, UL146 protein is also known as vCXCL-1, and UL147 protein as vCXCL-2.

While the functions of UL147 have not yet been characterized, light has been shed on those of UL146 (Miller-Kittrell et al., 2007; Heo et al., 2015). vCXCL-1 (UL146) is a 117 amino acids glycoprotein secreted into the culture medium with late kinetics. The UL146 gene product is highly polymorphic among low-passage strains (Heo et al., 2008) and absent in laboratory strains of the virus, such as AD169 and Towne (Cha et al., 1996). Recently, it was observed that vCXCL-1 is able to: (i) induce calcium flux via CXCR2 (Heo et al., 2015); (ii) selectively stimulate the expression of beta2 integrin (CD11b and CD11c), responsible for the adherence of leukocytes to the vascular endothelium through an interaction with ICAM-1 and for modulating the life span of neutrophils (Mayadas and Cullere, 2005; Heo et al., 2015); (iii) induce the migration of neutrophils *in vitro* (Heo et al., 2015); and (iv) upregulate CCL22, a chemokine involved in chemotaxis in monocytes, dendritic and NK cells, and chronically activated T lymphocytes (Heo et al., 2015).

Together, the capability of vCXCL-1 to alter the trafficking of HCMV-infected neutrophils, as potential HCMV carriers, may facilitate viral dissemination and promote the spread of HCMV through the host (McSharry et al., 2012).

UL128

The UL128 protein is part of the pentameric complex composed of gH (pUL115), gL (pUL75), pUL130, and pUL131A that is required for HCMV entry into epithelial, endothelial and DCs (Revello and Gerna, 2010). However, pUL128 contains four conserved cysteine amino acids at its N-terminus, similar to those found in the CC-chemokines (Akter et al., 2003). To this regard, it was observed that UL128 is able to induce the migration of human PBMCs (peripheral blood mononuclear cells) to levels comparable to those induced by the human CCL3/MIP-1α chemokine (Zheng et al., 2012). In addition, treatment with soluble pUL128 increases the expression of both IL-6 and TNF-α in PBMCs, and stimulates PBMCs proliferation through activation of the MAPK pathway (Zheng et al., 2012). Alteration in PBMCs trafficking and cytokines secretion by soluble pUL128 may thus facilitate HCMV dissemination through the recruitment of infected carrier cells.

IL-10

Among the HCMV-encoded cytokine-like molecules, the latency-associated viral cellular IL-10 homologu (LAcmvIL-10), encoded by HCMV UL111A gene is worthy of note (Ouyang et al., 2014). This HCMV gene encodes two IL-10 homologs generated by alternative splicing (Kotenko et al., 2000; Jenkins et al., 2004): cmvIL-10 of 175 amino acids, expressed during productive infection with late kinetics (Spencer et al., 2002; Chang et al., 2004), and LAcmvIL-10, of 139 amino acids, the result of a C-terminal truncation, reported to be expressed with early kinetics during both productive and latent infection (Jenkins et al., 2004).

As the cellular IL-10 (cIL10), cmvIL-10, and LAcmvIL-10 downregulate the expression of MHC-II molecules in latently infected GMPs (Spencer et al., 2008). However, cmvIL-10 shares more functions in common with cIL-10 than LAcmvIL-10 (Jenkins et al., 2008b). In fact, cmvIL-10, but not LAcmvIL-10, increases the expression of the IgG (FCγ) receptors CD32 and CD64, and increases FCγ receptor-mediated phagocytosis (Jaworowski et al., 2009). Similar to cIL-10, cmvIL-10 then inhibits the expression of proinflammatory cytokines in LPS-stimulated MDDCs (Monocyte-Derived Dendritic Cells; Jenkins et al., 2008a,b). Concerning the role of LAcmvIL-10, it was recently observed that its expression in latently infected $CD34^+$ cells resulted in the downregulation of cellular microRNA hsa-miR-92a, which upregulates the myeloid transcription factor GATA2 (Poole et al., 2013, 2014). GATA2, in turn, increases the transcription of the latency-associated HCMV genes LUNA and UL144 (Reeves, 2011; Poole et al., 2013), as well as the transcription of the cellular IL-10 (Poole et al., 2011). Since cIL-10 is one of the most abundant cytokines in the secretome produced by cells latently infected with HCMV, and believed to create an immune suppressive environment and to suppress apoptosis (Mason et al., 2012; Poole and Sinclair, 2015), LAcmvIL-10 is thought to reduce the ability of $CD4^+$ cells to recognize HCMV-infected cells during latent infection (Mason et al., 2012; Poole and Sinclair, 2015).

UL7

More recently, a novel HCMV-encoded secreted molecule, the protein encoded by UL7 gene, has been identified as a critical component of the HCMV-secretome responsible for vascular dysregulation associated with persistent HCMV infection (MacManiman et al., 2014). The UL7 gene belongs to the RL11 family, located in the left end of the viral genome, and characterized by early-late-phase kinetics during lytic HCMV infection (Engel et al., 2011). Structure prediction of UL7 protein identified a 222-amino-acid type I glycoprotein characterized by a putative leader peptide (35 amino acids), an extracellular immunoglobulin superfamily domain (Ig-like domain; 102 amino acids), a mucine-like stalk region (55 amino acids), a hydrophobic transmembrane sequence (22 amino acids) and a short cytoplasmic tail (eight amino acids; Engel et al., 2011). Engel et al. (2011) observed that the UL7 protein is proteolytically cleaved in correspondence with the stalk region, resulting in a heavily glycosylated ectodomain that is released from infected cells. Interestingly, the UL7 Ig-like domain shares significant amino acid identity with both CD229 (member of SLAM family), which is involved in T-cell signaling (Engel et al., 2011), and the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) protein, that is highly expressed during

vasculogenesis (MacManiman et al., 2014). However, UL7 is not able to bind directly CD229 or any other member of the SLAM family, but it can contact a putative UL7 ligand on the surface of monocyte-derived DCs, thus interfering with pro-inflammatory responses (Engel et al., 2011). In particular, UL7 has been observed to downregulate the production of pro-inflammatory cytokines, such as TNF, IL-6, and IL-8 in primary human monocyte-derived DCs and in the PMA-induced myeloid cell lines, U937 and THP-1 (Engel et al., 2011). These findings have thus suggested UL7 as a novel HCMV-encoded product able to contribute to the inhibition of the host antiviral defense in favor of the establishment of persistent infection (Engel et al., 2011). Moreover, the activity of CEACAM-1 in promoting vasculogenesis led MacManiman et al. (2014) to hypothesize a potential role of UL7 in this process. In support of this, using *in vitro* AG assays, they found that the secretome generated by an UL7-deficient HCMV produced a 50% reduction in tubulogenesis compared with that for the secretome from wild-type virus. Furthermore, following adenovirus-mediated transduction of human aortic endothelial cells (HEACs) with either the full length UL7 or the UL7 ectodomain, they observed the appearance of a robust network of interconnecting tubules, thus sustaining the involvement of UL7, and in particular its ectodomain, in the promotion of ECs differentiation (MacManiman et al., 2014). Moreover, the observation of an increased secretion of IL-6 and the activation of STATs and MAPK pathways in ECs overexpressing UL7 further substantiates the role of UL7 in angiogenesis (MacManiman et al., 2014). Taken together, these findings support a role of secreted UL7 protein in regulating some physiological properties of ECs and in the stimulation of angiogenesis.

The Importance of HCMV-Induced Bioactive Molecules in Virus Pathogenesis

Human cytomegalovirus infections are associated with the acceleration of various long-term vascular diseases, especially in immunocompromised hosts (Griffiths et al., 2015). In these settings, HCMV has been correlated to the acceleration of a number of vascular diseases, such as atherosclerosis, restenosis, and TVS, which are all determinants of chronic rejection (CR), one of the most important long-term conditions leading to graft failure and re-transplantation (Grahame-Clarke, 2005; Streblow et al., 2007). TVS is a complex phenomenon in which the leading event is a diffuse and concentric intimal proliferation, which, in turn, determines vessel occlusion due to perivascular inflammation, ECs dysfunction, and hvSMC proliferation with extracellular matrix deposition. In addition, the progression of TVS is promoted by the same mechanisms that lead to AG and WH (Streblow et al., 2007, 2008). In early TVS lesions, macrophages, T cells, B cells, and NK cells are present, while progression to late lesions is associated with a thinning intima containing SMC and macrophages (Streblow et al., 2005). Strong correlations between HCMV reactivation and the acceleration of vascular diseases development have been made on the basis of: (i) the high efficiency rate of infection for those cell types involved in TVS, including EC, SMC, and monocyte-derived macrophages (Lemström et al., 1993); (ii) the delay in the TSV progression and a prolonged graft survival in transplant recipients after treatment with ganciclovir, an approved anti-HCMV drug (Lemström et al., 1997); and (iii) *in vivo* studies on CMV animal models (Lemström et al., 1997; Streblow et al., 2003). In particular, in a rat model of aortic allografts, it was observed that infection with rat cytomegalovirus (RCMV) accelerates atherosclerosis, leading to an overall rearrangement of both the structural and functional architecture of vessel cells (Lemström et al., 1993; Streblow et al., 2003), the reversal of these effects through the use of an anti-CMV agent, such as ganciclovir, further support a role of the virus in the pathogenesis of experimental atherosclerosis (Lemström et al., 1994, 1997). RCMV infection has also been associated with an altered profile of released cytokines, in which higher levels of IL-2 and IL-4 were evident compared with controls (Zhou et al., 1999).

Furthermore, the observation that the HCMV-secretomes from both fibroblasts and ECs stimulate AG and WH in *in vitro* models suggests that HCMV infection of the allograft in transplant recipients and the subsequent release of a virus-altered secretome may promote AG and WH in the host, thus resulting in the acceleration of TVS (Streblow et al., 2008; Caposio et al., 2011, 2013). On the other hand, the stimulation of "*de novo*" lymphatic and blood vessels sprouting may represent a strategy adopted by the virus to enhance its replication and dissemination from the initial infection site to other host tissues.

In contrast, the available data about the activities of the HCMV-derived secretome from latently infected cells, indicate that the generation of a secretome-induced immunosuppressive microenvironment around latently infected cells may help the virus escape recognition by the hosts's immune system, thus favoring cell survival, viral persistence and potential reactivations.

Clearly, in both lytic and latent infections, the activities of the wide range of bioactive molecules in HCMV-secretomes may have severe pathogenetic consequences since they facilitate both virus dissemination and persistence in the host in spite of a robust antiviral response.

CONCLUSION

One of the main intriguing features of HCMV biology is the exceptionally wide arsenal of virus-encoded proteins that have been observed to be capable of counteracting host innate and adaptive immune defenses. Persistence of the virus, even in the immunocompetent host, and its ability to avoid immune clearance may be due in part to the activities of the many viral immunomodulatory proteins (Mocarski et al., 2014). In this review, we have examined what is known about the bioactive molecules, of both viral and cellular origin, secreted from HCMV-infected cells. These proteins are able to modify the extracellular environment of host infected tissues, thus promoting both virus dissemination and persistence, as well as some pathophysiological processes, such as AG and WH, that may drive the development of associated vascular diseases.

However, more work is needed to fully appreciate the importance of HCMV-derived secretomes in the pathogenesis of HCMV diseases.

To date, ECs, fibroblasts, and hematopoietic precursor cells have been mainly used to investigate the composition and functions of HCMV-derived secretomes; but, in the future, a deeper understanding of the importance of the HCMV secretome may come from the analysis of secretomes derived from other cell types. For example, little is known about the impact of HCMV infection on the secretion of bioactive molecules from epithelial cells, which represent one of the main cell targets for productive infection in the natural host. Does the HCMV-induced secretome of epithelial cells able to alter the host's immune recognition of infected cells or to catalyze organ damage in relation to lytic infection as occurs in the retina (Momma et al., 2003)?

The findings that we have reviewed here have led to a better comprehension of both the viral and cellular bioactive molecules involved in the host-HCMV relationship. Little is known, however, about the molecular mechanisms underlying the modulation and secretion of such cellular bioactive proteins. For example, the HCMV mechanisms that interfere directly or indirectly with the cellular secretion pathways remain to be identified. Knowledge of these molecular dynamics would be useful for the development of strategies aimed at attenuating or blocking the ability of HCMV to interfere with the host immune responses that contributes to the pathological outcome. Finally, it will also be necessary to confirm the *in vitro* findings in *in vivo* models, such as a humanized mouse model. The confirmation of experimental data and hypotheses would support the rationale to deepen our understanding of HCMV immunomodulation in order to identify targets for novel therapeutic strategies to prevent or control HCMV diseases.

AUTHOR CONTRIBUTIONS

AL wrote the manuscript; MT wrote the manuscript; and GG conceived of the review and wrote the manuscript.

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REFERENCES

Ahlfors, K., Ivarsson, S. A., and Harris, S. (2001). Secondary maternal cytomegalovirus infection-A significant cause of congenital disease. *Pediatrics* 107, 1227–1228. doi: 10.1542/peds.107.5.1227

Akter, P., Cunningham, C., McSharry, B. P., Dolan, A., Addison, C., Dargan, D. J., et al. (2003). Two novel spliced genes in human cytomegalovirus. *J. Gen. Virol.* 84, 1117–1122. doi: 10.1099/vir.0.18952-0

Almeida, G. D., Porada, C. D., St Jeor, S., and Ascensao, J. L. (1994). Human cytomegalovirus alters interleukin-6 production by endothelial cells. *Blood* 83, 370–376.

Avdic, S., Cao, J. Z., Cheung, A. K., Abendroth, A., and Slobedman, B. (2011). Viral interleukin-10 expressed by human cytomegalovirus during the latent phase of infection modulates latently infected myeloid cell differentiation. *J. Virol.* 85, 7465–7471. doi: 10.1128/JVI.00088-11

Bain, M., Mendelson, M., and Sinclair, J. (2003). Ets-2 Repressor Factor (ERF) mediates repression of the human cytomegalovirus major immediate-early promoter in undifferentiated non-permissive cells. *J. Gen. Virol.* 84, 41–49. doi: 10.1099/vir.0.18633-0

Beisser, P. S., Lavreysen, H., Bruggeman, C. A., and Vink, C. (2008). Chemokines and chemokines receptors encoded by cytomegaloviruses. *Curr. Top. Microbiol. Immunol.* 325, 221–242. doi: 10.1007/978-3-540-77349-8_23

Bettini, M., and Vignali, D. A. (2009). Regulatory T cells and inhibitory cytokines in autoimmunity. *Curr. Opin. Immunol.* 21, 612–618. doi: 10.1016/j.coi.2009.09.011

Botto, S., Streblow, D. N., DeFilippis, V., White, L., Kreklywich, C. N., Smith, P. P., et al. (2011). IL-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. *Blood* 117, 352–361. doi: 10.1182/blood-2010-06-291245

Bresnahan, W. A., and Shenk, T. (2000). A subset of viral transcripts packaged within human cytomegalovirus particles. *Science* 288, 2373–2376. doi: 10.1126/science.288.5475.2373

Britt, W. (2008). Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. *Curr. Top. Microbiol. Immunol.* 325, 417–470. doi: 10.1007/978-3-540-77349-8_23

Caposio, P., Luganini, A., Bronzini, M., Landolfo, S., and Gribaudo, G. (2010). The Elk-1 and serum response factor binding sites in the major immediate-early promoter of human cytomegalovirus are required for efficient viral replication in quiescent cells and compensate for inactivation of the NF-κB sites in proliferating cells. *J. Virol.* 84, 4481–4493. doi: 10.1128/JVI.02141-09

Caposio, P., Luganini, A., Hahn, G., Landolfo, S., and Gribaudo, G. (2007a). Activation of the virus-induced IKK/NF-κB signalling axis is critical for the replication of human cytomegalovirus in quiescent cells. *Cell. Microbiol.* 9, 2040–2054. doi: 10.1111/j.1462-5822.2007.00936.x

Caposio, P., Musso, T., Luganini, A., Inoue, H., Gariglio, M., Landolfo, S., et al. (2007b). Targeting the NF-κB pathway through pharmacological inhibition of IKK2 prevents human cytomegalovirus replication and virus-induced inflammatory response in infected endothelial cells. *Antiviral Res.* 73, 175–184. doi: 10.1016/j.antiviral.2006.10.001

Caposio, P., Orloff, S. L., and Streblow, D. N. (2011). The role of cytomegalovirus in angiogenesis. *Virus Res.* 157, 204–211. doi: 10.1016/j.virusres.2010.09.011

Caposio, P., Streblow, D. N., and Nelson, J. A. (2013). "Cytomegalovirus Proteomics," in *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, Vol. I, ed. M. J. Reddehase (Norfolk: Caister Academic Press), 86–108.

Cha, T. A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996). Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J. Virol.* 70, 78–83.

Chang, W. L., Baumgarth, N., Yu, D., and Barry, P. A. (2004). Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J. Virol.* 78, 8720–8731. doi: 10.1128/JVI.78.16.8720-8731.2004

Cheung, A. K., Abendroth, A., Cunningham, A. L., and Slobedman, B. (2006). Viral gene expression during the establishment of human cytomegalovirus latent infection in myeloid progenitor cells. *Blood* 108, 3691–3699. doi: 10.1182/blood-2005-12-026682

Crough, T., and Khanna, R. (2009). Immunobiology of human cytomegalovirus: from bench to bedside. *Clin. Microbiol. Rev.* 22, 76–98. doi: 10.1128/CMR.00034-08

Dengler, T. J., Raftery, M. J., Werle, M., Zimmermann, R., and Schönrich, G. (2000). Cytomegalovirus infection of vascular cells induces expression of pro-inflammatory adhesion molecules by paracrine action of secreted interleukin-1beta. *Transplantation* 69, 1160–1168. doi: 10.1097/00007890-200003270-00022

Dumortier, J., Streblow, D. N., Moses, A. V., Jacobs, J. M., Kreklywich, C. N., Camp, D., et al. (2008). Human cytomegalovirus secretome contains factors

that induce angiogenesis and wound healing. *J. Virol.* 82, 6524–6535. doi: 10.1128/JVI.00502-08

Engel, P., Pérez-Carmona, N., Mar Albà, M., Robertson, K., Ghazal, P., and Angulo, A. (2011). Human cytomegalovirus UL7, a homologue of the SLAM-family receptor CD229, impairs cytokine production. *Immunol. Cell. Biol.* 89, 753–766. doi: 10.1038/icb.2011.55

Fielding, C. A., Aicheler, R., Stanton, R. J., Wang, E. C. Y., Han, S., Seirafian, S., et al. (2014). Two novel human cytomegalovirus NK cell evasion functions target mica for lysosomal degradation. *PLoS Pathog.* 10:e1004058. doi: 10.1371/journal.ppat.1004058

Fiorentini, S., Luganini, A., Dell'Oste, V., Lorusso, B., Cervi, E., Caccuri, F., et al. (2011). Human cytomegalovirus productively infects lymphatic endothelial cells and induces a secretome that promotes angiogenesis and lymphangiogenesis through interleukin-6 and granulocyte-macrophage colony-stimulating factor. *J. Gen. Virol.* 92, 650–660. doi: 10.1099/vir.0.025395-0

Gewurz, B. E., Gaudet, R., Tortorella, D., Wang, E. W., and Ploegh, H. L. (2001). Virus subversion of immunity: a structural perspective. *Curr. Opin. Immunol.* 13, 442–450. doi: 10.1016/S0952-7915(00)00239-9

Grahame-Clarke, C. (2005). Human cytomegalovirus, endothelial function and atherosclerosis. *Herpes* 12, 42–45.

Griffiths, P., Baraniak, I., and Reeves, M. (2015). The pathogenesis of human cytomegalovirus. *J. Pathol.* 235, 288–297. doi: 10.1002/path.4437

Gustafsson, R. K. L., Jeffery, H. C., Yaiw, K. C., Wilhelmi, V., Kostopoulou, O. N., Davoudi, B., et al. (2015). Direct infection of primary endothelial cells with human cytomegalovirus prevents angiogenesis and migration. *J. Gen. Virol.* 96, 3598–3612. doi: 10.1099/jgv.0.000301

Hahn, G., Jores, R., and Mocarski, E. S. (1998). Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 3937–3942. doi: 10.1073/pnas.95.7.3937

Heo, J., Dogra, P., Masi, T. J., Pitt, E. A., de Kruijf, P., Smit, M. J., et al. (2015). Novel human cytomegalovirus viral chemokines, vCXCL-1s, display functional selectivity for neutrophil signaling and function. *J. Immunol.* 195, 227–236. doi: 10.4049/jimmunol.1400291

Heo, J., Petheram, S., Demmler, G., Murph, J. R., Adler, S. P., Bale, J., et al. (2008). Polymorphisms within human cytomegalovirus chemokine (UL146/UL147) and cytokine receptor genes (UL144) are not predictive of sequelae in congenitally infected children. *Virology* 378, 86–96. doi: 10.1016/j.virol.2008.05.002

Hodge, D. R., Hurt, E. M., and Farrar, W. L. (2005). The role of IL-6 and STAT3 in inflammation and cancer. *Eur. J. Cancer* 41, 2502–2512. doi: 10.4049/jimmunol.1400291

Horvath, C. M. (2004). The Jak-STAT pathway stimulated by interleukin 6. *Sci. STKE* 260:tr9. doi: 10.1126/stke.2602004tr9

Huang, M. M., Kew, V. G., Jestice, K., Wills, M. R., and Reeves, M. B. (2012). Efficient human cytomegalovirus reactivation is maturation dependent in the Langerhans dendritic cell lineage and can be studied using a CD14+ experimental latency model. *J. Virol.* 86, 8507–8515. doi: 10.1128/JVI.00598-12

Hummel, M., and Abecassis, M. M. (2002). A model for reactivation of CMV from latency. *J. Clin. Virol.* 25, S123–S136. doi: 10.1016/S1386-6532(02)00088-4

Isern, E., Gustems, M., Messerle, M., Borst, E., Ghazal, P., and Angulo, A. (2011). The activator protein 1 binding motifs within the human cytomegalovirus major immediate-early enhancer are functionally redundant and act in a cooperative manner with the nf-κb sites during acute infection. *J. Virol.* 85, 1732–1746. doi: 10.1128/JVI.01713-10

Jaworowski, A., Cheng, W. J., Westhorpe, C. L., Abendroth, A., Crowe, S. M., and Slobedman, B. (2009). Enhanced monocyte Fc phagocytosis by a homologue of interleukin-10 encoded by human cytomegalovirus. *Virology* 391, 20–24. doi: 10.1016/j.virol.2009.06.003

Jenkins, C., Abendroth, A., and Slobedman, B. (2004). A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J. Virol.* 78, 1440–1447. doi: 10.1128/JVI.78.3.1440-1447.2004

Jenkins, C., Garcia, W., Abendroth, A., and Slobedman, B. (2008a). Expression of a human cytomegalovirus latency-associated homolog of interleukin-10 during the productive phase of infection. *Virology* 370, 285–294. doi: 10.1016/j.virol.2007.09.002

Jenkins, C., Garcia, W., Godwin, M. J., Spencer, J. V., Stern, J. L., Abendroth, et al. (2008b). Immunomodulatory properties of a viral homolog of human interleukin-10 expressed by human cytomegalovirus during the latent phase of infection. *J. Virol.* 82, 3736–3750. doi: 10.1128/JVI.02173-07

Kenneson, A., and Cannon, M. J. (2007). Review and meta- analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev. Med. Virol.* 17, 253–276. doi: 10.1002/rmv.535

Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V., and Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc. Natl. Acad. Sci. U.S.A.* 97, 1695–1700. doi: 10.1073/pnas.97.4.1695

Landolfo, S., Gariglio, M., Gribaudo, G., and Lembo, D. (2003). The human cytomegalovirus. *Pharmacol. Ther.* 98, 269–297. doi: 10.1016/S0163-7258(03)00034-2

Lashmit, P., Shuhui, W., Hongmei, L., Hiroki, I., and Stinski, M. F. (2009). The CREB site in the proximal enhancer is critical for cooperative interaction with the other transcription factor binding sites to enhance transcription of the major intermediate-early genes in Human Cytomegalovirus-infected cells. *J. Virol.* 83, 8893–8904. doi: 10.1128/JVI.02239-08

Lemström, K., Sihvola, R., Bruggeman, C., Häyry, P., and Koskinen, P. (1997). Cytomegalovirus infection-enhanced cardiac allograft vasculopathy is abolished by DHPG prophylaxis in the rat. *Circulation* 95, 2614–2616. doi: 10.1161/01.CIR.95.12.2614

Lemström, K. B., Bruning, J. H., Bruggeman, C. A., Lautenschlager, I. T., and Häyry, P. J. (1993). Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. *J. Clin. Investig.* 92, 549–558. doi: 10.1172/JCI116622

Lemström, K. B., Bruning, J. H., Bruggeman, C. A., Lautenschlager, I. T., and Häyry, P. J. (1994). Triple drug immunosuppression significantly reduces immune activation and allograft arteriosclerosis in cytomegalovirus-infected rat aortic allografts and induces early latency of viral infection. *Am. J. Pathol.* 144, 1334–1347.

Liu, X. F., Xueqiong, W., Shixian, Y., Zheng, Z., Michael, A., and Hummel, M. (2013). Epigenetic control of cytomegalovirus latency and reactivation. *Viruses* 5, 1325–1345. doi: 10.3390/v5051325

Liu, X. F., Yan, S., Abecassis, M., and Hummel, M. (2010). Biphasic recruitment of transcriptional repressors to the murine cytomegalovirus major immediate-early promoter during the course of infection in vivo. *J. Virol.* 84, 3631–3643. doi: 10.1128/JVI.02380-09

MacManiman, J. D., Meuser, A., Botto, S., Smith, P. P., Liu, F., Jarvis, M. A., et al. (2014). Human cytomegalovirus-encoded pUL7 is a Novel CEACAM1-like molecule responsible for promotion of angiogenesis. *MBio* 5:e02035. doi: 10.1128/mBio.02035-14

Mason, G. M., Poole, E., Sissons, J. G. P., Wills, M. R., and Sinclair, J. H. (2012). Human cytomegalovirus latency alters the cellular secretome, inducing cluster of differentiation (CD)4+ T-cell migration and suppression of effector function. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14538–14543. doi: 10.1073/pnas.1204836109

Maussang, D., Langemeijer, E., Fitzsimons, C. P., Stigter-van Walsum, M., Dijkman, R., Borg, M. K., et al. (2009). The human cytomegalovirus-encoded chemokine receptor US28 promotes angiogenesis and tumor formation via cyclooxygenase-2. *Cancer Res.* 69, 2861–2869. doi: 10.1158/0008-5472.CAN-08-2487

Maxim, C. J. C., Shuxian, H., Yager, S. L., Gekker, G., Peterson, P. K., and Lokensgard, J. R. (2001). Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: antiviral implications. *J. Neuro Virol.* 7, 135–147. doi: 10.1080/13550280152058799

Mayadas, T. N., and Cullere, X. (2005). Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol.* 26, 388–395. doi: 10.1016/j.it.2005.05.002

McSharry, B. P., Avdic, S., and Slobedman, B. (2012). Human cytomegalovirus encoded homologs of cytokines, chemokines and their receptors: roles in immunomodulation. *Viruses* 4, 2448–2470. doi: 10.3390/v4112448

Miller-Kittrell, M., Sai, J., Penfold, M., Richmond, A., and Sparer, T. E. (2007). Functional characterization of chimpanzee cytomegalovirus chemokine, vCXCL-1 (CCMV). *Virology* 364, 454–465. doi: 10.1016/j.virol.2007.03.002

Mocarski, E. S. (2002). Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. *Trends Microbiol.* 10, 332–339. doi: 10.1016/S0966-842X(02)02393-4

Mocarski, E. S., Shenk, T., Griffith, P. D., and Pass, R. F. (2014). "Cytomegaloviruses," in *Fields Virology*, 6th Edn, ed. D. M. Knipe (Philadelphia, PA: Lippincott Williams and Wilkins), 1960–2014.

Momma, Y., Nagineni, C. N., Chin, M. S., Srinivasan, K., Detrick, B., and Hooks, J. J. (2003). Differential expression of chemokines by human retinal pigment epithelial cells infected with cytomegalovirus. *Invest. Ophthalmol. Vis. Sci.* 44, 2026–2033. doi: 10.1167/iovs.02-0980

Müllberg, J., Hsu, M. L., Rauch, C. T., Gerhart, M. J., Kaykas, A., and Cosman, D. (1999). The R27080 glycoprotein is abundantly secreted from human cytomegalovirus-infected fibroblasts. *J. Gen. Virol.* 80, 437–440. doi: 10.1099/0022-1317-80-2-437

Murphy, E., and Shenk, T. (2008). Human cytomegalovirus genomes. *Curr. Top. Microbiol. Immunol.* 325, 1–19. doi: 10.1007/978-3-540-77349-8_1

Neipel, F., Albrecht, J. C., Ensser, A., Huang, Y. Q., Li, J. J., Friedman-Kien, A. E., et al. (1997). Human herpesvirus 8 encodes a homolog of interleukin-6. *J. Virol.* 71, 839–842.

Nemčovičová, I., Benedict, C. A., and Zajonc, D. M. (2013). Structure of human cytomegalovirus UL141 binding to TRAIL-R2 reveals novel, non-canonical death receptor interactions. *PLoS Pathog.* 9:e1003224. doi: 10.1371/journal.ppat.1003224

Nogalski, M. T., Collins-McMillen, D., and Yurochko, A. D. (2014). Overview of human cytomegalovirus pathogenesis. *Methods Mol. Biol.* 1119, 15–28. doi: 10.1007/978-1-62703-788-4_2

Noriega, V. M., Haye, K. K., Kraus, T. A., Kowalsky, S. R., Ge, Y., Moran, T. M., et al. (2014). Human cytomegalovirus modulates monocyte-mediated innate immune responses during short-term experimental latency in vitro. *J. Virol.* 88, 9391–9405. doi: 10.1128/JVI.00934-14

Ouyang, P., Rakus, K., van Beurden, S. J., Westphal, A. H., Davison, A. J., Gatherer, D., et al. (2014). IL-10 encoded by viruses: a remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. *J. Gen. Virol.* 95, 245–262. doi: 10.1099/vir.0.058966-0

Penfold, M. E., Dairaghi, D. J., Duke, G. M., Saederup, N., Mocarski, E. S., Kemble, G. W., et al. (1999). Cytomegalovirus encodes a potent alpha chemokine. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9839–9844. doi: 10.1073/pnas.96.17.9839

Poole, E., Avdic, S., Hodkinson, J., Jackson, S., Wills, M., Slobedman, B., et al. (2014). Latency-associated viral interleukin-10 (IL-10) encoded by human cytomegalovirus modulates cellular IL-10 and CCL8 Secretion during latent infection through changes in the cellular microRNA hsa-miR-92a. *J. Virol.* 88, 13947–13955. doi: 10.1128/JVI.02424-14

Poole, E., Lau, J. C. H., and Sinclair, J. (2015). Latent infection of myeloid progenitors by human cytomegalovirus protects cells from FAS-mediated apoptosis through the cellular IL-10/PEA-15 pathway. *J. Gen. Virol.* 96, 2355–2359. doi: 10.1099/vir.0.000180

Poole, E., McGregor Dallas, S. R., Colston, J., Joseph, R. S., and Sinclair, J. (2011). Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in $CD34^+$ progenitors. *J. Gen. Virol.* 92, 1539–1549. doi: 10.1099/vir.0.031377-0

Poole, E., and Sinclair, J. (2015). Sleepless latency of human cytomegalovirus. *Med. Microbiol. Immunol.* 204, 421–429. doi: 10.1007/s00430-015-0401-6

Poole, E., Walther, A., Raven, K., Benedict, C. A., Mason, G. M., and Sinclair, J. (2013). The myeloid transcription factor GATA-2 regulates the viral UL144 gene during human cytomegalovirus latency in an isolate-specific manner. *J. Virol.* 87, 4261–4271. doi: 10.1128/JVI.03497-12

Prod'homme, V., Tomasec, P., Cunningham, C., Lemberg, M. K., Stanton, R. J., McSharry, B. P., et al. (2012). Human cytomegalovirus UL40 signal peptide regulates cell surface expression of the NK cell ligands HLA-E and gpUL18. *J. Immunol.* 188, 2794–2804. doi: 10.4049/jimmunol.1102068

Rahav, G., Gabbay, R., Ornoy, A., Shechtman, S., Arnon, J., and Diav-Citrin, O. (2007). Primary versus non primary cytomegalovirus infection during pregnancy. *Israel. Emerg. Infect. Dis.* 13, 1791–1793. doi: 10.3201/eid1311.061289

Rawlinson, W. D., and Barrell, B. G. (1993). Spliced transcripts of human cytomegalovirus. *J. Virol.* 67, 5502–5513.

Reeves, M., and Sinclair, J. H. (2013). Regulation of human cytomegalovirus transcription in latency: beyond the major immediate-early promoter. *Viruses* 5, 1395–1413. doi: 10.3390/v5061395

Reeves, M. B. (2011). Chromatin-mediated regulation of cytomegalovirus gene expression. *Virus Res.* 157, 134–143. doi: 10.1016/j.virusres.2010.09.019

Reeves, M. B., MacAry, P. A., Lehner, P. J., Sissons, J. G., and Sinclair, J. H. (2005). Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4140–4145. doi: 10.1073/pnas.0408994102

Reeves, M. B., and Sinclair, J. H. (2010). Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter. *J. Gen. Virol.* 91, 599–604. doi: 10.1099/vir.0.015602-0

Revello, M. G., and Gerna, G. (2010). Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev. Med. Virol.* 20, 136–155. doi: 10.1002/rmv.645

Rose-John, S. (2012). IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *Int. J. Biol. Sci.* 8, 1237–1247. doi: 10.7150/ijbs.4989

Rossetto, C. C., Tarrant-Elorza, M., and Pari, G. S. (2013). Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells. *PLoS Pathog.* 9:e1003366. doi: 10.1371/journal.ppat.1003366

Ryon, J. J., Hayward, S. D., MacMahon, E. M., Mann, R. B., Ling, Y., Charache, P., et al. (1993). In situ detection of lytic Epstein-Barr virus infection: expression of the NotI early gene and viral interleukin-10 late gene in clinical specimens. *J. Infect. Dis.* 168, 345–351. doi: 10.1093/infdis/168.2.345

Scheller, J., Chalaris, A., Schmidt-Arras, D., and Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta* 1813, 878–888. doi: 10.1016/j.bbamcr.2011.01.034

Scrivano, L., Sinzger, C., Nitschko, H., Koszinowski, U. H., and Adler, B. (2011). HCMV spread and cell tropism are determined by distinct virus populations. *PLoS Pathog.* 7:e1001256. doi: 10.1371/journal.ppat.1001256

Sinclair, J., and Sissons, P. (2006). Latency and reactivation of human cytomegalovirus. *J. Gen. Virol.* 87, 1763–1779. doi: 10.1371/journal.ppat.1001256

Sinclair, J. H., and Reeves, M. B. (2013). Human cytomegalovirus manipulation of latently infected cells. *Viruses* 5, 2803–2824. doi: 10.3390/v5112803

Sinzger, C. (2008). Entry route of HCMV into endothelial cells. *J. Clin. Virol.* 41, 174–179. doi: 10.1016/j.jcv.2007.12.002

Sinzger, C., Digel, M., and Jahn, G. (2008). Cytomegalovirus cell tropism. *Curr. Top. Microbiol. Immunol.* 325, 63–83. doi: 10.1007/978-3-540-77349-8_4

Soderberg-Nauclér, C., Fish, K. N., and Nelson, J. A. (1997). Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J. Clin. Invest.* 100, 3154–3163. doi: 10.1172/JCI119871

Soderberg-Nauclér, C., Streblow, D. N., Fish, K. N., Allan-Yorke, J., Smith, P. P., and Nelson, J. A. (2001). Reactivation of latent human cytomegalovirus in CD141 monocytes is differentiation dependent. *J. Virol.* 75, 7543–7554. doi: 10.1128/JVI.75.16.7543-7554.2001

Spencer, J. V., Cadaoas, J., Castillo, P. R., Saini, V., and Slobedman, B. (2008). Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10. *Virology* 374, 164–169. doi: 10.1016/j.virol.2007.11.031

Spencer, J. V., Lockridge, K. M., Barry, P. A., Lin, G., Tsang, M., Penfold, M. E., et al. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J. Virol.* 76, 1285–1292. doi: 10.1128/JVI.76.3.1285-1292.2002

Stern, J. L., and Slobedman, B. (2008). Human cytomegalovirus latent infection of myeloid cells directs monocyte migration by up-regulating monocyte chemotactic protein-1. *J. Immunol.* 180, 6577–6585. doi: 10.4049/jimmunol.180.10.6577

Stern-Ginossar, N., Weisburd, B., Michalski, A., Le, V. T., Hein, M. Y., Huang, S. X., et al. (2012). Decoding human cytomegalovirus. *Science* 338, 1088–1093. doi: 10.1126/science.1227919

Stinski, M. F., and Petrik, D. T. (2008). Functional roles of the human cytomegalovirus essential IE86 protein. *Curr. Top. Microbiol. Immunol.* 325, 133–152. doi: 10.1007/978-3-540-77349-8_8

Streblow, D. N., Dumortier, J., Moses, A. V., Orloff, S. L., and Nelson, J. A. (2008). Mechanisms of cytomegalovirus-accelerated vascular disease: induction of paracrine factors that promote angiogenesis and wound healing. *Curr. Top. Microbiol. Immunol.* 325, 397–416. doi: 10.1007/978-3-540-77349-8_23

Streblow, D. N., Kreklywich, C., Yin, Q., De La Melena, V. T., Corless, C. L., Smith, P. A., et al. (2003). Cytomegalovirus-mediated upregulation of chemokine expression correlates with the acceleration of chronic rejection in rat heart transplants. *J. Virol.* 77, 2182–2194. doi: 10.1128/JVI.77.3.2182-2194.2003

Streblow, D. N., Kreklywich, C. N., Smith, P., Soule, J. L., Meyer, C., Yin, M., et al. (2005). Rat cytomegalovirus-accelerated transplant vascular sclerosis is reduced with mutation of the chemokine-receptor R33. *Am. J. Transplant.* 5, 436–442. doi: 10.1111/j.1600-6143.2004.00711.x

Streblow, D. N., Orloff, S. L., and Nelson, J. A. (2007). Acceleration of allograft failure by cytomegalovirus. *Curr. Opin. Immunol.* 19, 577–582. doi: 10.1016/j.coi.2007.07.012

Swaminathan, S., Hesselton, R., Sullivan, J., and Kieff, E. (1993). Epstein-Barr virus recombinants with specifically mutated BCRF1 genes. *J. Virol.* 67, 7406–7413.

Taylor-Wiedeman, J., Sissons, P., and Sinclair, J. (1994). Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J. Virol.* 68, 1597–1604.

Van Damme, E., and Van Loock, M. (2014). Functional annotation of human cytomegalovirus gene products: an update. *Front. Microbiol.* 19:218. doi: 10.3389/fmicb.2014.00218

Vomaske, J., Nelson, J. A., and Streblow, D. N. (2009). Human cytomegalovirus US28: a functionally selective chemokine binding receptor. *Infect. Disord. Drugs Targets* 9, 548–556. doi: 10.2174/187152609789105696

Wang, D., Bresnahan, W. A., and Shenk, T. (2004). Human cytomegalovirus encodes a highly specific RANTES decoy receptor. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16642–16647. doi: 10.1073/pnas.0407233101

Weber-Nordt, R. M., Henschler, R., Schott, E., Wehinger, J., Behringer, D., Mertelsmann, R., et al. (1996). Interleukin-10 increases Bcl-2 expression and survival in primary human $CD34^{+}$ hematopoietic progenitor cells. *Blood* 88, 2549–2558.

Wegiel, B., Bjartell, A., Culig, Z., and Persson, J. L. (2008). Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. *Int. J. Cancer* 122, 1521–1529. doi: 10.1002/ijc.23261

Wilkinson, G. W., Tomasec, P., Stanton, R. J., Armstrong, M., Prod'homme, V., Aicheler, R., et al. (2008). Modulation of natural killer cells by human cytomegalovirus. *J. Clin. Virol.* 41, 206–212. doi: 10.1016/j.jcv.2007.10.027

Wright, E., Bain, M., Teague, L., Murphy, J., and Sinclair, J. (2005). Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells. *J. Gen. Virol.* 86, 535–544. doi: 10.1099/vir.0.80352-0

Yao, Z., Fanslow, W. C., Seldin, M. F., Rousseau, A. M., Painter, S. L., Comeau, M. R., et al. (1995). Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3, 811–821. doi: 10.1016/1074-7613(95)90070-5

Zheng, Q., Tao, R., Gao, H., Xu, J., Shang, S., and Zhao, N. (2012). HCMV-encoded UL128 enhances TNF–alpha and IL–6 expression and promotes PBMC proliferation through the MAPK/ERK pathway in vitro. *Viral Immunol.* 25, 98–105. doi: 10.1089/vim.2011.0064

Zhou, Y. F., Shou, M., Guetta, E., Guzman, R., Unger, E. F., Yu, Z. X., et al. (1999). Cytomegalovirus infection of rats increases the neointimal response to vascular injury without consistent evidence of direct infection of the vascular wall. *Circulation* 100, 1569–1575. doi: 10.1161/01.CIR.100.14.1569

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The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling

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Gut microbiota provides the host with multiple functions (e.g., by contributing to food digestion, vitamin supplementation, and defense against pathogenic strains) and interacts with the host organism through both direct contact (e.g., through surface antigens) and soluble molecules, which are produced by the microbial metabolism. The existence of the so-called gut–brain axis of bi-directional communication between the gastrointestinal tract and the central nervous system (CNS) also supports a communication pathway between the gut microbiota and neural circuits of the host, including the CNS. An increasing body of evidence has shown that gut microbiota is able to modulate gut and brain functions, including the mood, cognitive functions, and behavior of humans. Nonetheless, given the extreme complexity of this communication network, its comprehension is still at its early stage. The present contribution will attempt to provide a state-of-the art description of the mechanisms by which gut microbiota can affect the gut–brain axis and the multiple cellular and molecular communication circuits (i.e., neural, immune, and humoral). In this context, special attention will be paid to the microbial strains that produce bioactive compounds and display ascertained or potential probiotic activity. Several neuroactive molecules (e.g., catecholamines, histamine, serotonin, and trace amines) will be considered, with special focus on Glu and GABA circuits, receptors, and signaling. From the basic science viewpoint, "microbial endocrinology" deals with those theories in which neurochemicals, produced by both multicellular organisms and prokaryotes (e.g., serotonin, GABA, glutamate), are considered as a common shared language that enables interkingdom communication. With regards to its application, research in this area opens the way toward the possibility of the future use of neuroactive molecule-producing probiotics as therapeutic agents for the treatment of neurogastroenteric and/or psychiatric disorders.

Keywords: inter-kingdom communication, gut–brain axis, gut microbiota, enteric nervous system, enteroendocrine cells, neuroactive molecules, mood, anxiety

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COMMUNICATION SIGNALS THROUGH DIFFERENT BIOLOGICAL KINGDOMS

Living organisms exchange information through systems that are based upon signal-receptor interactions. The specific sense organs of higher animals have evolved to perceive signals, but the whole living world, including protozoans, plants, fungi and bacteria, efficiently communicates by exchanging information at a molecular level. Some universally sensed signals and widespread receptors can be found in different kingdoms.

It has been suggested that ghrelin-like molecules, i.e., those structurally related to the appetite-stimulating lipopeptide hormone which was first isolated from the gastrointestinal tract of rats (Kojima et al., 1999), are ubiquitously present in living organisms (Aydin et al., 2006). These molecules have been found in microorganisms (i.e., viruses, archaea, and phototrophic bacteria), animals and plants, where they fulfill different functions (e.g., inhibition of apoptosis), especially those connected with food intake and cell proliferation (Aydin, 2007). For instance, a structural homolog (*N*-octanoyl homoserine lactone) of ghrelin is produced by Gram-negative bacteria as a quorum sensing (QS) autoinducer and cell-to-cell communication molecule, which is also involved in food-searching (Aydin et al., 2006).

Bacteria produce a huge range of compounds that are involved in inter-microbial and host–microbe relationships. It is well-known that QS peptides, once interpreted as specific bacteria–bacteria communication signals (and regulating phenotypes such as competence, sporulation, bioluminescence, biofilm formation, bacteriocin/toxin production) also have metal (e.g., iron) binding and antibiotic properties, that is, additional functions which can promote the improvement of human health (Schertzer et al., 2009). It has been established that antimicrobial pigments, such as *Serratia* spp. prodigionines (active against fungal and protozoan infections), can also control cancer and immunity (Williamson et al., 2006). So, a single signal can be detected by different cellular systems. "Dark side" examples also exist, as in the case of farnesol, a QS-like molecule synthesized by the yeast *Candida albicans*. Farnesol controls yeast/mycelium transition, but also triggers the disruption of human erythrocytes, and interferes with cytokine expression (Hornby et al., 2001; Navarathna et al., 2007). The extremely long coevolution of host and colonizing microbes can account for these inter-kingdom effects.

The neurochemicals and their related receptors that are found in mammals are far from being an exclusive to these organisms, and are widely dispersed throughout nature, even in microorganisms and plants that do not have nervous systems. For instance, the stress-related neuro-endocrine hormone family of catecholamines has also been demonstrated in fish, insects, plants and bacteria (Lyte, 2011). Microorganisms are able to produce a wide variety of neurochemicals. This feature has been studied extensively by scientists engaged in food safety, since the presence of neuroactive molecules in food has been found to be responsible for cases of poisoning, such as the "cheese reaction" and the "scombroid syndrome" (related to food containing large amounts of tyramine and histamine, respectively) (Pessione et al., 2005, 2009). It has been suggested that at least some molecular neurotransmitters and/or neuromodulators and/or neurohormones have been conserved or shared during co-evolution as the "words" of a common language, thus allowing communication between phylogenetically very distant organisms (Lyte, 2011). It has been hypothesized that such a common language contributes to the homeostatic regulation of gut microbiota and possibly to the functioning of the brain and behavior (Lyte, 2011).

MICROBIAL NEURO-IMMUNO-ENDOCRINOLOGY: HOW GUT MICROBIOTA AND THE HOST CROSS-TALK

Microbial neuro-endocrinology is a branch of microbiology which has recently arisen, and it refers to the bidirectional communication that exists between commensal or parasite microbiota and the host. Although, some reports refer to the ability of bacteria to control the level of neurotransmitters through Toll-like receptors (TLRs) and heat-shock proteins (Sivamaruthi et al., 2015), this form of communication may occur directly by means of neurochemicals.

The first evidence that bacteria could respond to mammalian neuro-endocrine hormones dates back to 1929. These studies reported the effect of neuro-hormones, and in particular those belonging to the catecholamine family, on enhancing the pathogenic phenotype of several bacterial strains, such as *Clostridium perfringens*, during *in vivo* infections (Lyte, 2011). At that time, these observations were interpreted to mean that neurochemicals suppressed local immunity and thus favored the rapid unimpeded growth of infectious microbes (Lyte, 2011). However, evidence that prokaryotes can directly sense and respond to neurochemicals (e.g., altering their growth and/or virulence potential) has started to accumulate since the early 1990s. As an example, it is known that some intestinal molecules, such as serotonin [5-hydroxy tryptamin (5-HT)], can modulate the pathogenic potential of *Pseudomonas fluorescens* by affecting its motility and pyoverdin production, but without affecting its growth (Biaggini et al., 2015). Currently, this feature is not considered rare, but is widespread over a high number of diverse microorganisms (Lyte, 2011).

These findings have gained a great deal of attention in light of the parallel emerging evidence of the so-called microbiota-gut-brain axis, which allows bidirectional communication between the gut microbiota and the central nervous system (CNS) of animals, including humans (Cryan and Dinan, 2012). It has been reported that gut microbiota can control the tryptophan metabolism of the host by enhancing the fraction of tryptophan available for the kynurenine route and decreasing the amount available for 5-HT synthesis (O'Mahony et al., 2015). These authors suggested that the deficiencies observed in the serotonergic system of the elderly could be due to quantitative and qualitative modifications of gut microbiota during aging (O'Mahony et al., 2015). The cognitive and behavioral functions of germ-free mice can be altered following colonization with microbiota from a different mouse strain, and this can lead to a similar increased exploratory behavior to that of the donor mouse strain (Bercik et al., 2011b).

The observation of psychiatric co-morbidities in various chronic inflammatory intestinal disorders has been interpreted by some authors as further evidence of the influence of gut microbiota on the CNS (Bercik et al., 2012; Cryan and Dinan, 2012). Altered gut microbiota has been observed in individuals affected by severe psychiatric disorders, such as autism (Adams et al., 2011; Williams et al., 2011). Studies on

animals infected by pathogenic bacteria, or treated with probiotic or antibiotic agents have suggested that gut microbiota may play a role in the genesis of multiple sclerosis, anxiety and depression (Berer et al., 2011; Cryan and Dinan, 2012). However, many psychiatric co-morbidities are thought to be the result of perinatal infections, and should be interpreted within the context of altered predisposition rather than microbiota-CNS communication (Buka et al., 2008; Eßlinger et al., 2016; Gilman et al., 2016). Since clear evidence of the role of gut microbiota in the development of these syndromes is still lacking, this subject is still the topic of debate.

However, all these reports have contributed to the concept of the microbiota-gut-brain axis emerging as a "circular" interactive network consisting of multiple (immune, neural, and endocrine) interaction mechanisms. On one hand, the role of the psychological status in the pathogenesis of/recovery from infectious disease is recognized within the medical community (Lyte, 2011). On the other hand, these studies have revolutionized the traditional conception of human–microbiota relationships, and hopefully opened fascinating perspectives as regards the balancing of microbiota through the application of probiotics as adjuvant agents in both neuro-enteric and psychiatric disorders (Mazzoli, 2014). However, it should be pointed out that, due to their extreme complexity and several confounding factors, the understanding of the molecular mechanisms by which intestinal microbiota can influence gut–brain communication or respond to human-derived signals is still at its very beginning. And it is also for this reason that the efficacy of probiotics as therapeutic agents in psychiatric disorders is still a subject of massive debate within the circle of experts (Bron et al., 2011; Kelly et al., 2015; Santocchi et al., 2016).

The Gut–Brain Axis

The gut–brain axis defines a bidirectional communication system that connects the GI tract with the CNS through multiple pathways involving neural, endocrine, and immune cells. The gut brain axis allows the CNS to regulate GI functions, including motility and secretion, and the GI tract to signal sensations such as hunger, pain, or discomfort to the CNS (Julio-Pieper et al., 2013). However, new complex emotional (affective mood), cognitive (memory formation) and behavioral functions (food intake) are beginning to be discovered (Berntson et al., 2003), and bacteria could play a role in this scenario (Cani and Knauf, 2016). Direct evidence that gut stimuli can affect emotional states includes the fact that the intragastric infusion of fatty acids in humans has been shown to reduce the brain response to experimentally induced sad emotions (Van Oudenhove et al., 2011).

The reciprocal influence between the GI tract and the CNS is sustained by the enteric nervous system (ENS) (Furness, 2006; Cryan and Dinan, 2012), which is considered as the third branch of the autonomic nervous system and consists of about 200–600 million neurons. Owing to its high number of neurons, complexity, and similarity in signaling molecules with the brain, ENS has also been referred to as the "second brain" (Gershon, 1998). ENS interfaces with the gut-associated lymphoid tissue (GALT, which contains more than two-thirds of the body's immune cells) and with 1000s of entero-endocrine cells (EECs, which contain more than 20 identified hormones). Furthermore, the GI tract is directly innervated by the CNS through spinal and vagal afferents (Mayer, 2011). For this reason, gut-to-brain communications can occur through three main routes: (i) direct perception of stimuli by primary afferent neurons (belonging to the ENS or the CNS); (ii) immune system mediated connections; (iii) EEC mediated connections.

Direct Neuronal Perception of Gut Chemical Stimuli

It is generally believed that the afferent terminals that innervate the gut cannot sense luminal chemosignals directly, but can through intermediate cells (e.g., EECs, immune cells) (Mayer, 2011). Under physiological conditions, protein-sized molecules cannot pass the intestinal epithelium through the paracellular pathway. However, lipophilic and small hydrophilic compounds of up to 600 Da can cross the intestinal barrier through multiple routes, e.g., through transcellular (namely, passive diffusion into the lipid bilayer and/or small aqueous pores) and paracellular routes (Keita and Söderholm, 2010). It can therefore be hypothesized that small neuroactive compounds may also use the latter pathways to diffuse in the lamina propria, that is, in contact with intrinsic and/or extrinsic neural afferents, or into the portal circulation, and therefore possibly exert extra-intestinal effects. Some experimental evidence can in fact support this hypothesis (Psichas et al., 2015). For instance, FFAR3 receptors for short chain fatty acids (SCFAs) have been detected in submucosal and myenteric ganglia (Nøhr et al., 2013), and the responsiveness of enteric neurons to glucose, amino acids and fatty acids has been demonstrated (Liu et al., 1999; Furness et al., 2013; Neunlist and Schemann, 2014). Furthermore, pattern recognition receptors that are able to bind a variety of microbial antigens have been identified in neural cells, including enteric neurons, which could possibly enable a rapid activation without the intermediation of immune cells (Lim et al., 2016) (for more details, see Bacteria-Immune-Endocrine-Vagal Connections).

Neuroimmune Connections

About 70% of the body's immune cells are present within the GALT (Mayer, 2011). The existence of close connections between afferent nerve terminals and immune cells (e.g., plasma cells, eosinophils, and mast cells) within the gut mucosa has been well-established (Keita and Söderholm, 2010; Mayer, 2011). For example, the terminals of some vagal afferents can respond to a variety of neuroactive compounds secreted by lymphocytes and mast cells, such as histamine, 5-HT, prostaglandins and various cytokines (Keita and Söderholm, 2010). Furthermore, neuropeptide receptors have been identified in mast cells, thus suggesting bi-directional communication between the nervous and immune systems (Keita and Söderholm, 2010).

Neuro-endocrine Connections

Entero-endocrine cells account for less than 1% of epithelial cells in the gut; nonetheless, they constitute the largest endocrine organ of the body (Mayer, 2011). More than 20 different types of EECs have been identified, and these differ according to the type(s) of regulatory peptides (e.g., glucagon-like peptides,

GLPs, pancreatic peptide YY, PYY, cholecystokinin, CCK, secretin) or bioactive molecules that they secrete. EECs regulate digestive functions through ENS circuits, and communicate with the CNS (e.g., with the hypothalamus), either directly, i.e., through endocrine pathways, or through paracrine signaling to vagal afferents (Raybould, 2010). Although, EECs and afferent neurons have long been thought to communicate indirectly, i.e., through neuropeptides released by EECs, a recent study has also demonstrated direct synaptic contacts between EECs and neurons (Bohórquez et al., 2015). Experimental evidence has reported that suggests EECs could act as both pre-synaptic (to communicate gut feelings to the nervous system) and post-synaptic (e.g., for the possible modulation of the responsiveness of EECs by efferent neurons) elements.

Immune-Endocrine Connections

Immune and enteroendocrine pathways are not distinct routes of the gut–brain axis, but can influence each other to some extent. Indeed, the release of interleukin-4 and 13 from CD4+ T cells, in a mouse gut inflammation model has been reported to increase cholecystokinin secretion in EECs (McDermott et al., 2006). Enterochromaffin cells (ECs) have been described as being able to modulate gut inflammation through 5-HT signaling (Mawe et al., 2009).

Bacteria-Immune-Endocrine-Vagal Connections

Studies in germ-free animals have underlined the importance of gut microbiota in the early-life development of the gut–brain axis, with particular reference to the hypothalamic-pituitary-adrenal axis (Sudo et al., 2004). Pattern recognition receptors such as TLRs, which are transmembrane proteins that are able to recognize bacterial envelope components, such as lipopolysaccharide (LPS) and lipoteichoic acids (Medzhitov, 2007), have been detected in a number of neuronal cells which could potentially mediate the direct neuronal sensing of microbial antigens (Lim et al., 2016). TLR4, which can be activated by bacterial LPS, has been detected in the nodose ganglion of the vagus nerve of rats (Hosoi et al., 2005). TLRs 3, 7, and 4 are expressed in the myenteric and submucosal plexus of the murine intestine, the human ileum and the lower dorsal root ganglia (Barajon et al., 2009). Similar results were also obtained by Qi et al. (2011), who detected the expression of TLRs 3, 7, and 9 in both human and murine dorsal root ganglion neurons. High mRNA levels of TLRs 1, 4, 5, and 6 have been found in the colonic dorsal root ganglion neurons of mice (Ochoa-Cortes et al., 2010). Furthermore, a number of studies have reported the direct excitation of neurons by LPS (Lim et al., 2016). However, in yet other studies the stimulation of pattern recognition receptors of neurons did not cause any direct excitation, but instead sensitized them and potentiated their excitability (Lim et al., 2016). Nonetheless, these researches have highlighted the existence of other possible pathways for bacteria sensing and communication pathways between the nervous and the immune systems.

Microbial exocellular polysaccharides (EPSs) are essential for protecting bacteria from the host immune response, but also for interacting with intestinal mucosal cells, including epithelial cells and EECs, thus inducing the release of molecular messengers that are able to modulate neural signaling or directly act on primary afferent axons (Forsythe and Kunze, 2013).

Mucosal immune cells are able to distinguish commensal from pathogenic bacteria (Artis, 2008) through TLRs, which are able to detect microbial antigens. Similarly, dendritic immune cells (extending their dendrites between epithelial cell tight junctions so as to directly sense the luminal environment) and some EECs can respond to the presence and activity of intraluminal microbial organisms through TLRs (Mayer, 2011). The intestinal infusion of *Escherichia coli* proteins has been reported to increase the secretion of anorexigenic GLP-1 and PYY, thus providing direct evidence of the ability of the gut microbiota to control appetite (Tennoune et al., 2014; Breton et al., 2016). In addition, EECs possess G-protein-coupled receptors that are able to sense microbial metabolites, such as amino acids (for example glutamate) (Bezençon et al., 2007), protein hydrolysates (Choi et al., 2007) and both long and short fatty acids (Tanaka et al., 2008; Nøhr et al., 2013). The expression of GPR41 receptor of SCFAs has been detected in several types of EECs (e.g., those secreting CCK, ghrelin, gastrin, GLP-1, PYY, neurotensin, secretin) throughout the GI tract of mice, from the stomach to the colon (Nøhr et al., 2013). Both the GPR41 and 43 receptors of SCFAs are expressed by enteroendocrine L-cells (Nøhr et al., 2013). Their stimulation promotes the secretion of GLP-1 and PYY (Samuel et al., 2008; Tolhurst et al., 2012; Nøhr et al., 2013). Furthermore, gut microbiota indirectly regulates the release of GLP-1, GLP-2 and PYY by modulating the differentiation of stem cells into EECs, and thus modifying the number of GLPs and PYY secreting L-cells (Everard et al., 2011). Another group of gut microbiota metabolites, namely indole and tryptamine derived from tryptophan, are known to modulate gut hormone release. Indole triggers GLP-1 secretion by enteroendocrine L-cells (Chimerel et al., 2014). Tryptamine induces ECs to secrete 5-HT (Takaki et al., 1985). Gut microbiota also contributes to regulating sensitivity to leptin, the so-called "satiety hormone" (Schéle et al., 2013).

Several microbial metabolites that display neuroactive properties have been described: (i) gaseous molecules, such as carbon monoxide, hydrogen sulfide and nitric oxide (Bienenstock and Collins, 2010); (ii) SCFAs such as *n*-butyrate, propionate, and acetate (Engelstoft et al., 2008; Nicholson et al., 2012); (iii) amines, such as putrescine, spermidine, spermine and cadaverine, which have been shown to be involved in CNS responses to stress (Bienenstock and Collins, 2010).

Curiously, bacteria can produce a wide range of molecules which mimic human hormones. For instance, muramyl dipeptide (similar to serotonin) (Masek and Kadlec, 1983) and indole (similar to melatonin) (Norris et al., 2013) can both cause sleep and drowsiness, while LPS from Gram-negative bacteria can directly act on thyroid cells, *via* type 4 TLRs, and up-regulate thyroglobulin gene expression (Nicola et al., 2009).

On the other hand, the direct response of bacteria to some of the regulatory peptides/neuroactive molecules that are secreted by EECs and/or the human nervous system has been demonstrated, which indicates the presence of

neuromodulator/neurotrasmitter receptors in the bacterial envelope (Lyte, 2011). For example, the stress hormones epinephrine and norepinephrine increase the *in vitro* growth of *E. coli* by more than four orders of magnitude (Freestone et al., 2002) and the *Clostridium*/*Bacteroides* ratio in the human gut (Bailey et al., 2011). A recent report has demonstrated that *Vibrio cholerae* can respond to epinephrine and norepinephrine (enhancing the growth rate, swimming motility, and production of virulence factors such as iron sequestrating phenotype) by means of specific sensor proteins (Halang et al., 2015).

Actually, the most forefront hypotheses consider that brain and gut commensal bacteria communicate with each other through shared chemical mediators, and this is part of the homeostasis mechanisms that help to maintain gut microbiota stability and possibly brain functions and behavior (Bienenstock and Collins, 2010; Lyte, 2011). This neurochemical-mediated "two-way street" (**Figure 1**) is one of the principles that supports the microbial endocrinology construct (Lyte, 2011).

BACTERIAL PRODUCTION OF NEUROACTIVE MOLECULES

The molecules that have assessed neuroactive properties produced by bacteria are reported in **Table 1**. This list is far from being exhaustive, and will likely include a progressively higher number of strains and molecules as the results of a greater number of studies become available. The bacteria that are able to produce catecholamines include *Bacillus* spp. (producing dopamine), *Escherichia* spp., and *Bacillus* spp. (producing noradrenalin) (Lyte, 2011). The presence of the complete biosynthetic pathway for catecholamines in these organisms has raised the hypothesis that cell-to-cell signaling in vertebrates may be the result of a late horizontal gene transfer from bacteria (Lyte, 2011).

Strains belonging to *Streptococcus* spp., *Escherichia* spp. and lactic acid bacteria (LAB) are known to synthesize 5-HT (Lyte, 2011; Matur and Eraslan, 2012). It should be underlined that, unlike what is generally believed, the gut (and not the brain) is the main producer of 5-HT in mammals. The ECs of the gut mucosa are the predominant site for the synthesis and storage of 5-HT in humans (Gershon and Tack, 2007). The release of 5-HT occurs in response to chemical stimuli, e.g., the presence of nutrients (e.g., glutamate, glucose), or food-related/bacterial toxins in the intestinal lumen (Mayer, 2011; Kitamura et al., 2012). However, 5-HT produced by gut microbiota could possibly contribute to the overall 5-HT pool in the gut. It is known that 5-HT is involved in the regulation of gut peristalsis, in vagal circuits associated with nausea and vomiting and in the perception of visceral discomfort and pain through spinal afferents (Costedio et al., 2007). For these reasons, 5-HT has been the target of several treatments of gastrointestinal and gut–brain associated disorders such as inflammatory bowel disease, irritable bowel syndrome, post-infectious irritable bowel syndrome, and idiopathic constipation (Costedio et al., 2007).

Several LAB strains are able to synthesize β-phenylethylamine and/or tyramine and/or tryptamine that belong to a group of structurally related neuroactive compounds which are commonly known as trace amines (Mazzoli, 2014). Trace amines (which also include octopamine) are physiologically present in the human nervous system, although in low amounts compared to 5-HT and catecholamines, with which they display close metabolic and neurophysiological relationships (Burchett and Hicks, 2006). Tryptamine production has been detected in *Lactobacillus bulgaricus* (Pessione et al., 2009), while *Leuconostoc* and *Enterococcus* species have been reported to produce both tyramine and β-phenylethylamine (Pessione et al., 2009). It is worth noting that tryptophan decarboxylases have been found in at least 10% of the samples tested from the NIH Human Microbiome Project (Williams et al., 2014). Despite their low amounts, trace amines, such as β-phenylethylamine, could play a significant role as the co-transmitters and neuromodulators (Burchett and Hicks, 2006) that are involved in mood control, appetite/satiety circuits, and attention deficit/hyperactive disorders (Shimazu and Miklya, 2004). Alterations in the trace amine network also seem to be involved in psychiatric syndromes such as bipolar disorder (BD), parkinsonism, and hepatic encephalopathy (Burchett and Hicks, 2006).

Lactic acid bacteria and several other bacteria, such as those that can contaminate fish or shellfish products, produce large amounts of histamine, which is widely known as a mediator of allergy and anaphylaxis but also plays a role as a neurotransmitter in both the CNS and the ENS (Lyte, 2011).

Molecules that control QS communication between bacteria (mainly peptides or acyl-homoserine lactones) have also been shown to be involved in neuronal functioning (Hughes and Sperandio, 2008).

Bacterial Production of Glutamate and γ-Aminobutyric Acid (GABA)

L-Glutamate (Glu) and γ-aminobutyric acid (GABA) are mainly known for their role as the main neuro-transmitters in the mammalian CNS, with excitatory and inhibitory roles, respectively (Hyland and Cryan, 2010; Julio-Pieper et al., 2013). However, these molecules are widespread in nature and have multiple functions, including plant signaling and communication between bacteria (Dagorn et al., 2013).

Several bacterial strains are able to produce Glu. Coryneform bacteria, such as *Corynebacterium glutamicum*, *Brevibacterium lactofermentum* and *Brevibacterium flavum*, have been used extensively for the industrial fermentative production of Glu (Sano, 2009). LAB strains belonging to *Lactobacillus plantarum, Lactobacillus paracasei,* and *Lactococcus lactis* are also able to synthesize Glu (Tanous et al., 2005). A recent study has revealed that about 15% of the LAB strains isolated from Asian fermented foods are Glu producers (Zareian et al., 2012).

Both prokaryotes and eukarya synthesize GABA through the decarboxylation of Glu by glutamate decarboxylase (GAD). GAD has been found in both Gram-positive and Gram-negative bacteria, where it is associated with systems that are involved in pH homeostasis and the generation of metabolic energy (i.e., proton motive force) (Pessione, 2012; Tsai et al., 2013). Marine microorganisms (Morse et al., 1980), *E. coli* (Richard and Foster, 2003) and *Pseudomonas* (Chou et al., 2008) can synthesize GABA.

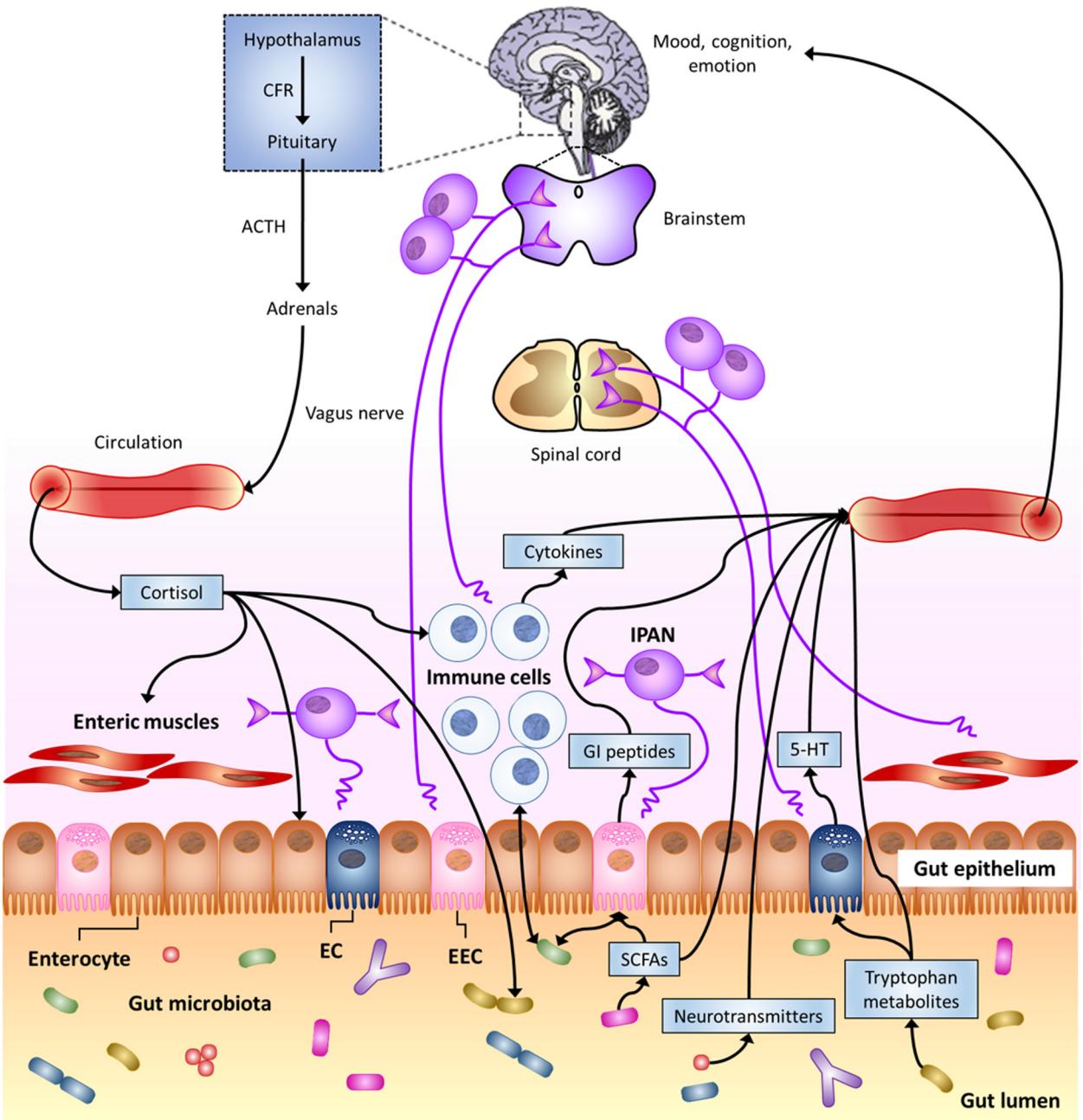

FIGURE 1 | The Microbiota-Gut–Brain Axis. Bi-directional communication between gut microbiota and the central nervous system (CNS) can occur through either direct or indirect multiple pathways. These include endocrine, immune, and neural mechanisms (see text). Here, special focus is given to gut-to-brain communication. 5-HT, serotonin; ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; EC enterochromaffin cell; EEC, enteroendocrine cell; GI, gastro-intestinal; IPAN; intrinsic primary afferent neuron; SCFAs, short-chain fatty acids (modified from Cryan and Dinan, 2012).

Among those microorganisms that are generally recognized as safe or health-promoting, several LAB (e.g., strains belonging to *Lactobacillus*, *Lactococcus,* and *Streptococcus* genera) and *Bifidobacterium* strains have been reported to biosynthesize GABA (Siragusa et al., 2007; Li and Cao, 2010; Mazzoli et al., 2010; Lyte, 2011). Recently, one *Lactobacillus* strain and four strains of *Bifidobacterium* isolated from the human intestine have been reported to being able to produce GABA (Barrett et al., 2012). Furthermore, a very recent analysis on metagenomic data from the Human Microbiome Project suggests that genes encoding GAD could be present in a significant proportion of human gut microbiota (Pokusaeva et al., 2016). Lactobacilli

TABLE 1 | Main neuroactive amines and amino acids released by bacteria.

Genus/species	Bioactive molecule and (precursor)	Physiological effects	Reference
Lactobacillus spp., *Enterococcus*	Histamine (His)	Hypotension, allergies	Pessione et al., 2005
Enterococcus faecalis	Tyramine (Tyr)	Hypertension, headaches	Pessione et al., 2009
Enterococcus faecalis	β-phenylethylamine (Phe)	Appetite/satiety control, mood control	Shimazu and Miklya, 2004
Bacillus	Dopamine (Tyr)	Multiple	Lyte, 2011
Bacillus, Escherichia coli	Nor-adrenaline (Tyr)	Multiple	Lyte, 2011
Bifidobacteria, LAB	Melatonin (Trp)	Smooth muscle relaxation, Sleep/wake cycle regulator	Wong et al., 2015
Lactobacillus bulgaricus, Streptococcus, Escherichia coli	Serotonin/triptamin (Trp)	Vagal circuit regulation, peristalsis	Matur and Eraslan, 2012
Corynebacterium glutamycum, Lactobacillus plantarum, Lactobacillus paracasei, Lactococcus lactis	Glu	Multiple	Tanous et al., 2005; Sano, 2009; Zareian et al., 2012
LAB, *Escherichia coli, Pseudomonas*	GABA (Glu)	Anxiolytic, miorelaxant	Richard and Foster, 2003; Chou et al., 2008; Mazzoli et al., 2010

include the strains with the highest GABA production, although this metabolic ability is more likely strain- rather than genus-related (Li and Cao, 2010).

Glu/GABA: RECEPTORS, SIGNALING, AND TRANSPORT

Bacterial Targets: Glu and GABA Receptors in Prokaryotes

The role of GABA in the communication between bacteria is in line with the identification of GABA-binding proteins in different prokaryotes. These proteins consist not only of transporters, such as GabP in *E. coli* or *Bacillus subtilis*) (Brechtel and King, 1998; Hu and King, 1998) and Bra in *Agrobacterium tumefaciens* (Chevrot et al., 2006), but also of possible specific receptors (Guthrie and Nicholson-Guthrie, 1989). It has been reported that metabotropic Glu and $GABA_B$ receptors, as well as bacterial periplasmic amino acid binding proteins, may have evolved from a common ancestor (Cao et al., 2009). In addition, as reported in the previous section, many bacteria (including those colonizing the human gut) can synthesize GABA, thus suggesting that, as in the case of eukaryotes, GABA might be a conserved and ubiquitous communication molecule.

The fact that GABA has an effect on the physiology of *Pseudomonas* can be supported by several observations. A periplasmic protein showing high affinity for GABA and displaying similar biochemical features to a subunit of the mammalian $GABA_A$ ionotropic receptor was identified in an environmental strain of *P. fluorescens* (Guthrie and Nicholson-Guthrie, 1989; Guthrie et al., 2000). Specific receptors for sensing GABA have been found in other Pseudomonads including: the PctC of *P. aeruginosa* and the McpG of *P. putida,* which are involved in GABA chemoreception (Reyes-Darias et al., 2015). Another study has demonstrated that GABA increases the cytotoxicity and virulence of *P. aeruginosa* through a sophisticated modulation of the protein expression (Dagorn et al., 2013). A functional proteomic analysis revealed that six proteins were differentially expressed in *P. aeruginosa* when exposed to GABA (Lyte, 2011). Hence, some bacteria are able to respond to GABA, of both prokaryote and mammalian origin.

A structurally similar ion channel to eukaryotic ionotropic $GABA_A$ receptors was identified in the plant pathogen *Erwinia chrysanthemi* (Zimmermann and Dutzler, 2011). The prokaryotic protein is activated by different amines, including GABA, and is modulated by benzodiazepines in a similar manner to its eukaryotic homolog (Zimmermann and Dutzler, 2011; Spurny et al., 2012).

Less information is available on prokaryotic Glu receptors. As far as we know, only a single case of a Glu-activated potassium channel in a bacterium (i.e., GluR0 from *Synechocystis* PCC 6803) has been reported to date (Chen et al., 1999). However, 100 prokaryotic channel proteins with putative Glu binding domains have recently been identified through a bioinformatic study (Ger et al., 2010). Among them, 22 proteins have been found to be homologs of vertebrate ionotropic Glu receptors (Ger et al., 2010).

Based on these findings, it is possible to hypothesize that members of the human gut microbiota can also sense and respond to GABA/Glu, although this has yet to be confirmed through dedicated studies. Furthermore, human-derived or dietary Glu can affect the balance of gut microbial populations by stimulating the growth of certain bacterial species at the expense of others. It is known, for instance, that LAB decarboxylate Glu to GABA in order to create a protonic gradient and enhance metabolic energy. Hence, Glu can constitute a privileged energy source that can be used to expand LAB populations.

Host-Targets: the Glutamatergic/GABAergic System in the CNS

Glu and GABA are the major excitatory and inhibitory neurotransmitters found in the human CNS, respectively (Julio-Pieper et al., 2013). These compounds are the molecular effectors of a homeostatic neuronal circuit that is characterized by extreme complexity and sophistication at both a physiological and a

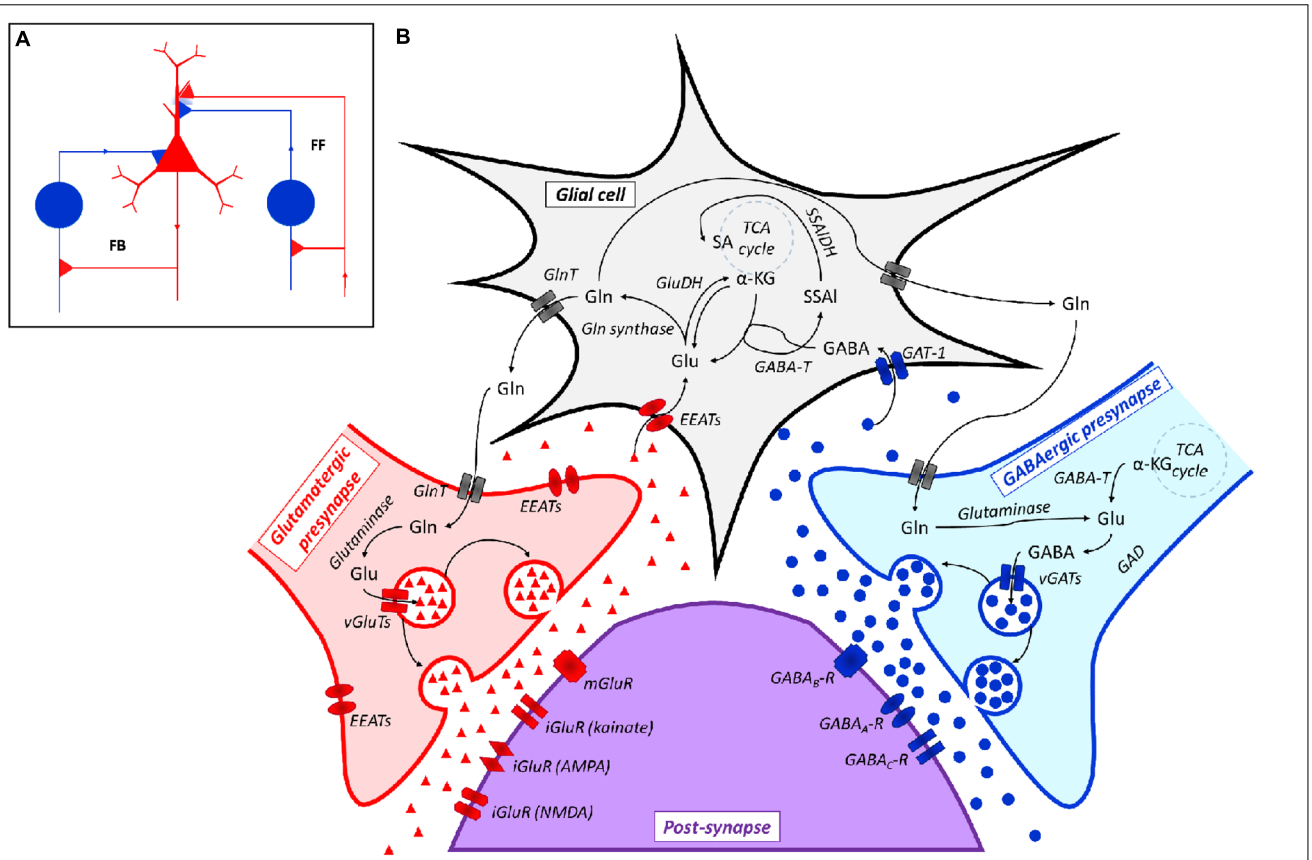

FIGURE 2 | (A) Representation of a Glutamatergic/GABAergic neural network, including an excitatory (Glutamatergic) pyramidal neuron (red), inhibitory (GABAergic) neurons (blue) and the related connections. The inhibitory neuron on the left is integrated into a feedback (FB) circuit, while the inhibitory neuron on the right is integrated into a feed-forward (FF) loop. Inhibitory synapses targeted to the soma or dendrites of the pyramidal cell are surrounded by light blue staining, which indicates a background GABA concentration that contributes to the overall inhibition (adapted from Roth and Draguhn, 2012). **(B)** Scheme of the Glutamatergic/GABAergic neurotransmission in the CNS. α-KG, α-keto-glutarate; EEATs, excitatory amino acid transporters; GABA, γ-aminobutyric acid; $GABA_A$-R, $GABA_A$ receptor; $GABA_B$-R, $GABA_B$ receptor; $GABA_C$-R, $GABA_C$ receptor; GABA-T, GABA transaminase; GAD, Glu decarboxylase; GAT-1, GABA transporter 1; GlnT, glutamine transporter; Glu, glutamate; iGluR, ionotropic Glu receptor; mGluR, metabotropic Glu receptor; SSAl, succinic semialdehyde; SSAIDH, succinic semialdehyde dehydrogenase; TCA cycle, the Krebs cycle; vGAT, vesicular GABA transporter; vGluT, vesicular Glu transporter. Symbols: ●, GABA; ▲, Glu.

biochemical level. This system also includes glutamine (Gln), which plays a key role as a non-neuroactive intermediate in the recycling of neurotrasmitters in the brain, mainly Glu and GABA (Soeiro-de-Souza et al., 2015).

At a physiological level, this network consists of feed forward (FF) and feedback (FB) neuronal connections in which the main role of inhibitory (i.e., GABAergic) neurons is currently considered as that of balancing the activity of excitatory (i.e., Glutamatergic) neurons (Roth and Draguhn, 2012; **Figure 2A**). Both Glutamatergic and GABAergic neurotransmissions are complex systems in which glial cells (mainly astrocytes), pre- and post-synaptic neurons and a large spectrum of different receptors, transporters and enzymes are involved (Cherlyn et al., 2010; Femenía et al., 2012; **Figure 2B**).

The biochemical relationship between Glu and GABA goes far beyond this reciprocally modulating activity, since Glu and GABA are easily interconverted. Actually, the biochemical reactions involved in GABA-to-Glu-to-Gln conversion are integral features of such a regulatory network, which also involves the central energy metabolism of neurons (Soeiro-de-Souza et al., 2015; **Figure 2B**).

Glu is synthesized *in situ* in the CNS since it cannot pass the blood–brain barrier (BBB) (Hawkins, 2009). One of the main Glu biosynthetic pathways consists of the conversion of glucose (through the Embden-Meyerhof-Parnas pathway and the Krebs cycle) to α-ketoglutarate (α-KG), which is subsequently transaminated (Cherlyn et al., 2010). Glu is accumulated in vesicles and, after suitable stimulation, released to the synaptic space, where it can bind to a panel of Glu receptors (GluRs) that may be found in post-synaptic neurons. GluRs differ on the basis of their mechanism of action (e.g., ionotropic, iGluR, or metabotropic, mGluR, Glu receptors) and pharmacological responsiveness [e.g., *N*-methyl D-aspartate, NMDA, or α-amino-3-hydroxy-5-methylisoxazole-4-proprinoic acid (AMPA) or kainate sensitive receptors]. Ligand-gated ionotropic receptors mediate fast synaptic responses by directly regulating the ion influx. Metabotropic receptors are coupled to G-proteins and modulate slower signal transduction cascades

(Hyland and Cryan, 2010; Julio-Pieper et al., 2013). Any excess Glu present in the synaptic cleft is taken up by glial cells or neurons to prevent the prolonged excitation of post-synaptic neurons, which can lead to cell death (Cherlyn et al., 2010). Glu in the glia is either oxidatively deaminated to α-KG [by Glu dehydrogenase (GluDH)], or converted to Gln by Gln synthetase. Gln is released from glial cells, uptaken by pre-synaptic neurons [*via* Gln transporters (GlnT)], and is then converted back to Glu by glutaminase. Apart from its role in neurotransmission, Glu is also involved in other neural processes, such as neuronal development and synaptic plasticity (Cherlyn et al., 2010).

Inhibitory pre-synaptic neurons convert Glu to GABA through GAD and pack it into vesicles *via* vesicular GABA transporters (vGATs). Again in this case, Gln is an alternative Glu source to α-KG. The multiple GABA receptors that are present in post-synaptic neurons include ionotropic ($GABA_A$, $GABA_C$) and metabotropic ($GABA_B$) receptors. Apart from GABA, these receptors are possibly modulated by a number of other compounds, e.g., barbiturates, baclofen, benzodiazepines and steroids (Simeone et al., 2003; Goetz et al., 2007). Any excess unbound GABA is cleared by glial cells through GABA transporters (GAT-1). GABA is then converted by GABA transaminase (GABA-T) into succinic semialdehyde, with concomitant Glu biosynthesis (Sivilotti and Nistri, 1991; Michels and Moss, 2007).

Glu/GABA: From the Gut to the Brain

As described above, several bacteria, including probiotic bacteria and bacteria that colonize the human GI tract, are able to produce Glu or GABA (Barrett et al., 2012). As far as we know, only one study concerning the modulation of host Glutamate/GABAergic systems by GABA/Glu producing gut-colonizing bacteria is available in the literature (Bravo et al., 2011). A recent study has demonstrated that a GABA-producing *Bifidobacterium dentium* is able to attenuate sensitivity of dorsal root ganglia neurons in a rat model of visceral pain (Pokusaeva et al., 2016). It is worth noting that these few examples refer to the limited number of studies on interaction between gut microbiota and the host nervous system which is currently available. Experimental evidence is likely to grow as a higher number of reports become available. To date, most studies on the Glu/GABA effects on humans concern dietary Glu/GABA.

Glu and GABA can be found in food as natural components or as food supplements. Glu is among the most abundant amino acids (8–10%) found in dietary proteins. Furthermore, monosodium glutamate is employed extensively as a flavor additive in food (Brosnan et al., 2014). In Germany, Glu average intake (comprinsing both free Glu and Glu as protein constituent in foods) of about 10 g/day has been estimated (Beyreuther et al., 2007). In asian consumers this value is likely higher since free Glu intake can reach up to 4 g/day in these countries (Beyreuther et al., 2007). GABA is relatively abundant in plant food, such as brown rice germs and sprouts, spinach, barley and bean sprouts, where GABA concentrations of between 300 and 720 nmol/g dry weight have been detected (Oh et al., 2003), while higher levels are found in fermented foods (Abdou et al., 2006). In recent years, the utilization of GABA as a food supplement has progressively increased in both Asian and Western (USA and Europe) countries (Boonstra et al., 2015). GABA concentration of few micrograms per gram of cecal content has been detected in mice (Pokusaeva et al., 2016).

Hundreds of people have reported the benefits of using GABA-supplemented food, for instance in alleviating anxiety and/or improving sleep quality (Boonstra et al., 2015). Moreover, at least five different studies have reported that the oral administration of GABA or GABA-supplemented food/beverages (corresponding GABA amounts of about 50–100 mg) has had positive effects on human health. These effects include: (i) the reduction of psychological stress in people who performed arithmetic tasks (Nakamura et al., 2009; Kanehira et al., 2011; Yoto et al., 2012); (ii) the reduction of stress in acrophobic subjects exposed to heights (Abdou et al., 2006); (iii) an increased ability to perform prioritized planned actions (Steenbergen et al., 2015).

Multiple pathways by which gut luminal Glu/GABA may affect the CNS can be hypothesized, including their transport across intestinal and BBB and their sensing through afferent terminals that innervate the GI tract (**Figure 3**). In the next sections, evidence possibly supporting these mechanisms will be illustrated.

Direct Influence of the Luminal Glu/GABA in the Gut on the CNS

Multiple transporters that mediate Glu absorption have been found in the apical membrane of GI epithelial cells, mainly in the small intestine, but also in the stomach (Burrin and Stoll, 2009), while little or no transport of amino acids from the lumen to portal blood occurs in the colon (Julio-Pieper et al., 2013). Studies on pigs and rodents have indicated the excitatory amino acid carrier 1 is the most abundant Glu transporter in the mucosa of the small intestine (Burrin and Stoll, 2009). A study performed in infant pigs has indicated that rate of Glu absorption and transport to the portal circulation linearly increases with increasing intraduodenal Glu intake (Janeczko et al., 2007). Portal absorption rate increased from 3.80 μmol kg^{-1} $h^{-1}$for normal Glu intake (i.e., 510 μmol kg^{-1} h^{-1}) to 20.46 3.80 μmol kg^{-1} h^{-1} for piglets infused with 3.5-fold increased Glu intake (Janeczko et al., 2007). Glu is one of the main nutrients for enterocytes. Several studies on different animal models, including pre-term infants and human adults, have agreed on that most Glu present in the GI lumen is oxidized to CO_2 or, secondarily, converted to other amino acids by the gut mucosa (Burrin and Stoll, 2009). Only a small percentage (between 5 and 17%, depending on the studies) of the ingested Glu is transported into the portal circulation but this does not generally affect the Glu concentration in the plasma to any great extent (Hays et al., 2007; Brosnan et al., 2014). Furthermore, an increased plasma Glu concentration (following Glu intake) does not necessarily affect the Glu concentration in brain tissues, since it is generally recognized that Glu cannot pass the BBB (Janeczko et al., 2007). A 20-fold (or more) increase in plasma Glu concentration was found necessary to access the brain tissues in rodents (Brosnan et al., 2014). The reaching of such a high Glu concentration in plasma after dietary intake (or Glu biosynthesis by gut microbiota) seems rather unlikely.

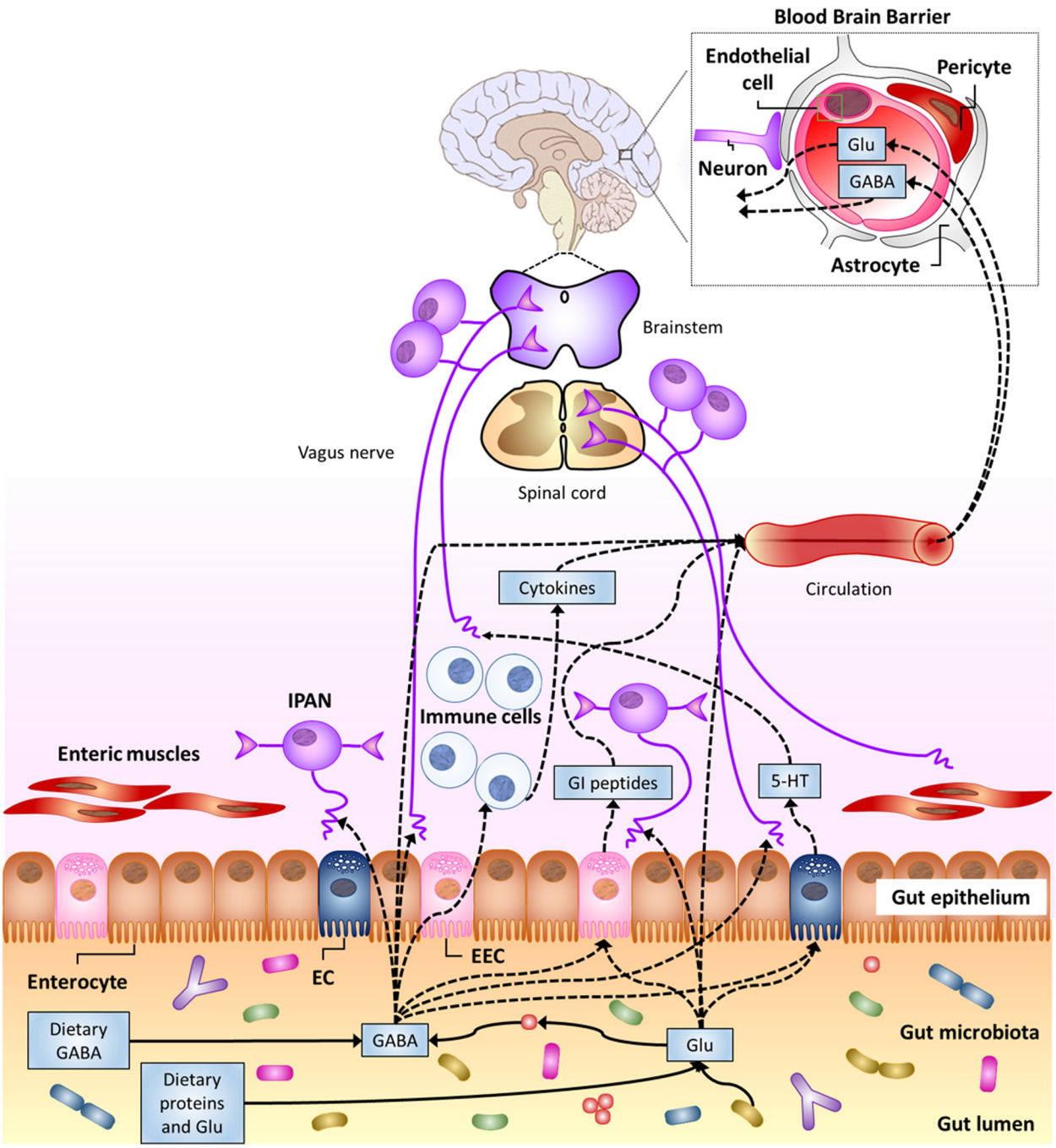

FIGURE 3 | Potential pathways for luminal Glu/GABA in the gut (of either dietary or gut–microbiota origin) to influence the nervous system (see text). 5-HT, serotonin; EC enterochromaffin cell; EEC, enteroendocrine cell; GI, gastro-intestinal; IPAN; intrinsic primary afferent neuron (from Cryan and Dinan, 2012).

As far as GABA transporters are concerned, they are mostly found in the CNS (Gadea and López-Colomé, 2001). However, H^+/GABA symport across the apical membrane of human intestinal epithelial (Caco-2) cells has been demonstrated (Thwaites et al., 2000). In fact, the hPAT1 H^+/amino acid symporter, which is thought to mediate the uptake of luminal GABA, is present throughout the human GI tract, from the stomach to the discending colon, with maximal expression in the small intestine (Chen et al., 2003). Furthermore, GABA carriers have been detected in the basolateral membrane of

Caco-2 cells (Nielsen et al., 2012). These findings suggest that luminal GABA should be able to cross the intestinal barrier and possibly reach extra-intestinal targets. In fact, orally administered GABA in rats was reported to increased GABA concentration in their blood, with a peak after 30 min (Abdou et al., 2006). Nonetheless, GABA ability to cross the BBB is still controversial (Boonstra et al., 2015). Most early studies, performed between 1950 and 1980, agreed on the impermeability of BBB to GABA, but more recent investigations have reported that GABA may actually cross this barrier, although in small amounts (Boonstra et al., 2015). These discrepancies have been ascribed to the different method of administration (e.g., oral versus injection), or animal model employed. Some studies have also suggested that BBB permeability to GABA could diminish with increasing age (Boonstra et al., 2015). Interestingly, GABA-transporters have been found in the BBB of mice (Kakee et al., 2001). Direct evidence of GABA facilitated transport across the BBB of mice has been reported, although the efflux rate from the brain was 17 times higher than the influx rate (Kakee et al., 2001). Unfortunately, no information about the possible presence of similar transporters in the BBB of humans is currently available.

All together, these findings suggest that a direct action of luminal gut Glu and/or GABA on the CNS cannot be excluded, although further *in vivo* studies are required to confirm this hypothesis. These investigations should also take into account that gut and/or BBB permeability are affected by several factors, including stress, diet and gut microbiota (Braniste et al., 2014; Kelly et al., 2015).

Sensing of Luminal Glu/GABA in the Gut

Multiple Glu receptor types (including ionotropic, types 1 and 4 metabotropic receptors and heterodimeric, TAS1R1 + TAS1R3, L-Glu taste receptors) have been detected in GI epithelial cells and/or enteric neurons in the stomach, small intestine and colon (Kondoh et al., 2009; San Gabriel et al., 2009; Kitamura et al., 2012). More in detail, $mGlu_4$ receptors have been detected in the mucosa of both the gastric antrum and duodenum (Akiba et al., 2009), while both $mGlu_4$ and $mGlu_7$ receptors have been identified in the colon epithelium (Chang et al., 2005; Julio-Pieper et al., 2010). TAS1R3, a subunit of umami taste receptors, has been found in enteroendocrine L-cells and X/A-like cells (Jang et al., 2007; Hass et al., 2010). This evidence suggests a possible role in luminal Glu sensing. In fact, small intestine luminal L-Glu seems to play a role in the defense mechanisms of duodenal mucosa in rats, by increasing the intracellular pH and mucus gel thickness (Akiba et al., 2009). A role of mGluRs in the human colon in the control of colon peristalsis and electrolyte transport has been described (Julio-Pieper et al., 2013). High levels of $mGlu_7$ and $mGlu_8$ have been detected in myenteric neurons, where they are possibly involved in the regulation of gut motility (Julio-Pieper et al., 2013). However, results on the ability of gut luminal Glu to directly excite enteric neurons still appear contradictory (Kirchgessner, 2001; Wang et al., 2014). On the other hand, an indirect activation of vagal afferents (i.e., *via* the production and release of nitric oxide and 5-HT) through the administration of Glu to the gastric lumen has been demonstrated (Uneyama et al., 2006). These data strongly suggest a role of ECs in the mediation of luminal Glu sensing. This luminal Glu sensing mechanism stimulates different areas in the CNS, including the cerebral cortex, basal ganglia, limbic system, and hypothalamus (for a comprehensive review, refer to Kondoh et al., 2009), and induces flavor preference in rats (Kitamura et al., 2012).

$GABA_B$ receptors are abundantly expressed in the GI tract (Hyland and Cryan, 2010). GABA and its ionotropic and metabotropic receptors are widely distributed throughout the ENS, in both submucosal and myenteric neurons, from the stomach to the ileum (Auteri et al., 2015). In addition, Nakajima et al. (1996) reported the expression of $GABA_B$ receptors in cells morphologically similar to EECs, where they co-localized with cells containing somatostatin (in the stomach) or serotonin (in the duodenum). However, a later study failed to detect any positive EEC for $GABA_B$ receptors (Casanova et al., 2009). Schwörer et al. (1989) showed that the release of 5-HT by ECs in the small intestine of guinea-pig is modulated by $GABA_A$ and $GABA_B$ receptors. Involvement of $GABA_B$ receptors in modulation of sensitivity of vagal and spinal afferents has been reported (Hyland and Cryan, 2010). Actually, the $GABA_B$ metabotropic receptors in the GI tract are thought to regulate several functions, including gut motility and gut-to-brain signaling (Hyland and Cryan, 2010). Experimental evidence of $GABA_B$/μ-type opiod receptor interaction, which mediates the synergistic potentiation of the anti-nociceptive action of GABA/morphine co-administration, has been reported (Hara et al., 1999). A similar $GABA_B$ receptor-mediated potentiation effect has also been described for drugs used to reduce visceral pain (Hara et al., 2004).

Since GABA receptors have been found in a wide range of immune cells, such as dendritic cells, mast cells and T-cells (Auteri et al., 2015), and are involved in regulating immunological processes, such as the down-regulation of pro-inflammatory cytokine release (Bjurström et al., 2008), their role in neuro-immune dialog in the gut has been proposed (Auteri et al., 2015).

It is worth reminding that GABA is produced through Glu decarboxylation in both eukarya and prokaryotes. Hence, GABA-producing microorganisms that are present in the gut may significantly affect luminal Glu/GABA ratio and, therefore, gut signaling.

PROBIOTICS FOR THE TREATMENT OF PSYCHIATRIC DISORDERS?

As far as its application to the field of probiotics is concerned, microbial neuro-endocrinology addresses the ability of probiotics to both synthesize and respond to neuroactive compounds, and thus to affect the host neurological processes and sometimes the overall physiology of the host. A microbial endocrinology-based approach may be relevant to help understand how probiotics can influence the health of a host and how host can select commensal microbial populations.

A number of studies have provided evidence on the ability of probiotic strains to modulate the mood and stress responses of humans, and to reduce anxiety and depression (Logan and Katzman, 2005; Messaoudi et al., 2011; Arseneault-Breard et al., 2012). As an example, the administration of Bifidobacteria and LAB as probiotic supplements to humans can increase the levels of morning salivary melatonin, thus reducing the symptoms of irritable bowel syndrome (Wong et al., 2015). Actually, most of the currently recognized probiotic strains belong to Bifidobacteria and LAB, with lactobacilli being among the most abundant members of such health-promoting strains (Vaughan et al., 2011). A direct modulation of myenteric neuron activity by unidentified *Bifidobacterium longum* fermentation product(s) displaying anxiolytic effects has also been reported (Bercik et al., 2011a).

Alterations in the Glu/ Gln/ GABA circuits in the CNS have been reported for generalized anxiety disorders, as well as in other psychiatric conditions, such as the major depressive disorder (MDD), BD (also known as manic depressive disorder) and schizophrenia (SZ) (Cherlyn et al., 2010; Femenía et al., 2012; Soeiro-de-Souza et al., 2015). It is worth noting that these complex neurobehavioral disorders affect a significant portion of the overall population throughout the world (e.g., individuals affected by BD or SZ account for about 2–3% of the global population) (Cherlyn et al., 2010). Different classes of drugs addressed to different steps of Glutamate/GABA-ergic neurotransmission (e.g., reducing Glu release, increasing Glu re-uptake, or increasing GABA levels by enhancing GAD or inhibiting GABA transaminase and succinic semialdehyde dehydrogenase) or serendipitously discovered (e.g., valproic acid) have been used in the treatment of such mood disorders (Terbach and Williams, 2009; Femenía et al., 2012). However, drugs frequently have negative secondary effects. For instance, most antidepressants (such as tricyclic antidepressants), while having a positive effect on the mood of humans, do not alleviate and can even aggravate cognitive symptoms, or impair vigilance (e.g., selective 5-HT reuptake inhibitors), and may cause dependence (e.g., benzodiazepines) (Femenía et al., 2012). Finding alternative or adjuvant therapies is therefore a research priority and, in this respect, the use of neuromodulating bacteria or so-called psychobiotics is a fascinating perspective.

Interestingly, the anxiolytic and anti-depressant-like effects of *Lactobacillus rhamnosus* JB-1 ingestion in mice involve a reduction in the plasma corticosterone levels and alterations of both $GABA_A$ and $GABA_B$ receptor expression patterns in specific brain areas (Bravo et al., 2011). Furthermore, increases in the Glx (i.e., Glu + Gln) and GABA levels in the CNS have been observed after 2 and 4 weeks of *L. rhamnosus* JB-1 treatment, respectively (Janik et al., 2016). These effects were mediated by gut-to-brain communication through the vagus nerve (Bravo et al., 2011). However, whether *L. rhamnosus* stimulates the vagus nerve with its own GABA or induces an endogenous GABA production still has to be clarified.

Modulation of the glutamatergic system in the central amygdala, cortex and hippocampus of mice by gut microbiota has been described (Sudo et al., 2004; Neufeld et al., 2011).

Although, GABA production has been speculated as the key factor in the ability of a *Lactobacillus helveticus–B. longum* mixture to reduce anxiety-like behavior(s) (Lyte, 2011), the observed probiotic effects on the psychological health of a host are generally likely the result of multiple interactions with the host (ENS, EEC, GALT). On the other hand, anorexigenic and anxiolytic soymorphin peptides (produced by LAB proteolytic action toward soy betaconglycinine) act *via* the $GABA_B$ receptor and, in this overall action, multiple circuits (including 5-HT and dopamine receptors) are involved (Kaneko et al., 2010). A recent investigation have suggested that a GABA-producing *B. dentium* is able to reduce nociception in a rat model of visceral pain (Pokusaeva et al., 2016).

Because of the wide range of potential neuro-active compounds (orexant, anorexant, opioid, opioid antagonist) produced by bacteria and the complex network that exists between them, a deep characterization of the proteome and metabolome of probiotic strains is one key aspect that would help to understand the different effects exerted by each strain on human health. Since not only Glu/GABA receptors but also other circuits can be potential targets for bacterial-derived molecules, it is tempting to hypothesize the use of probiotics that produce bioactive compounds (e.g., GABA, 5-HT, opioid peptides) as a novel frontline strategy for the adjuvant treatment of gastrointestinal, neuroenteric, neurological and psychiatric conditions (Hyland and Cryan, 2010; Lyte, 2011; Reid, 2011).

Together with this optimistic viewpoint and the enthusiasm that these findings have generated in the scientific domain, it is worth stating that a number of issues suggests caution in application of psychobiotics. The comprehension of the mechanisms by which gut microbiota and the nervous system communicate is still at its beginning. Multiple levels of complexity hinder the detailed understanding of this network and these include (but are not limited to) the fact that: the gut–brain axis comprises multiple (i.e., neural, immune, and endocrine) pathways with several reciprocal interactions; multiple molecular mediators, such as GABA and Glu, are involved, which play roles in a number of different biological processes; gut microbiota and even single commensal microorganisms interact with the host through multiple pathways and chemical mediators (e.g., cell envelope components, production/metabolism of neurotransmitters or neuroactive molecules). Therefore, it may be difficult to specifically address health problems with such microbial agents without the risk of unbalancing other pathways. As for other potential therapeutic agents, rigorous evaluation of the potential beneficial effects together with possible negative side effects should be performed for each probiotic/psychobiotic agent.

CONCLUSION

The increasing knowledge about gut bacteria–brain bi-directional communication has provided scientific evidence that can explain the popular statement that somebody has

made a decision based his/her on gut feeling, but also the saying "we are what we eat," as it is supposed that diet can affect the microbiota composition of the gut. It is now evident that gut microbiota is able to interfere, through its cellular components or by secreting bioactive compounds, with the gut–brain axis through immune, neural and humoral pathways. As the knowledge of this domain increases, new unexpected and exciting perspectives for both basic and applied science will likely emerge. At the same time, the observed effects of human-derived neuro-active compounds on gut microbiota would seem to suggest that some neurochemicals share a common language that enables interkingdom communication, as proposed by Lyte and Freestone (2010). The ability of a microbial strain to affect the overall patho-physiology of a host thus appears to be the result of a long and sophisticated synergic evolution between gut bacteria and the host.

AUTHOR CONTRIBUTIONS

Authors equally contributed to writing the manuscript. EP mainly contributed to describe bioactive molecules produced by bacteria and involved in interkingdom signaling, with particular attention to microbiota-gut-brain axis. RM focused his contribution mainly on Glutamate- and GABA-mediated communication, including Glu and GABA biosynthesis and receptors in both bacteria and humans, gut-to-brain transport and sensing and Glu-ergic/GABA-ergic neural transmission in the CNS.

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REFERENCES

Abdou, A. M., Higashiguchi, S., Horie, K., Kim, M., Hatta, H., and Yokogoshi, H. (2006). Relaxation and immunity enhancement effects of γ-aminobutyric acid (GABA) administration in humans. *Biofactors* 26, 201–208. doi: 10.1002/biof.5520260305

Adams, J. B., Johansen, L. J., Powell, L. D., Quig, D., and Rubin, R. A. (2011). Gastrointestinal flora and gastrointestinal status in children with autism—comparisons to typical children and correlation with autism severity. *BMC Gastroenterol.* 11:22. doi: 10.1186/1471-230X-11-22

Akiba, Y., Watanabe, C., Mizumori, M., and Kaunitz, J. D. (2009). Luminal L-glutamate enhances duodenal mucosal defense mechanisms via multiple glutamate receptors in rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G781–G791. doi: 10.1152/ajpgi.90605.2008

Arseneault-Breard, J., Rondeau, I., Gilbert, K., Girard, S. A., Tompkins, T. A., Godbout, R., et al. (2012). Combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. *Br. J. Nutr.* 107, 1793–1799. doi: 10.1017/S0007114511005137

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat. Rev. Immunol.* 8, 411–420. doi: 10.1038/nri2316

Auteri, M., Zizzo, M. G., and Serio, R. (2015). GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. *Pharmacol. Res.* 93, 11–21. doi: 10.1016/j.phrs.2014.12.001

Aydin, S. (2007). Ghrelin may be a universal peptide in all living organisms. *Turk. J. Med. Sci.* 37, 123–124.

Aydin, S., Geckil, H., Zengin, F., Ibrahim Ozercan, H., Karatas, F., Aydin, S., et al. (2006). Ghrelin in plants: what is the function of an appetite hormone in plants? *Peptides* 27, 1597–1602. doi: 10.1016/j.peptides.2006.01.005

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G., and Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain Behav. Immun.* 25, 397–407. doi: 10.1016/j.bbi.2010.10.023

Barajon, I., Serrao, G., Arnaboldi, F., Opizzi, E., Ripamonti, G., Balsari, A., et al. (2009). Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. *J. Histochem. Cytochem.* 57, 1013–1023. doi: 10.1369/jhc.2009.953539

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., and Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. *J. Appl. Microbiol.* 113, 411–417. doi: 10.1111/j.1365-2672.2012.05344.x

Bercik, P., Collins, S. M., and Verdu, E. F. (2012). Microbes and the gut-brain axis. *Neurogastroenterol. Motil.* 24, 405–413. doi: 10.1111/j.1365-2982.2012.01906.x

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., et al. (2011b). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology* 141, 599–609. doi: 10.1053/j.gastro.2011.04.052

Bercik, P., Park, A. J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., et al. (2011a). The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut– brain communication. *Neurogastroenterol. Motil.* 23, 1132–1139. doi: 10.1111/j.1365-2982.2011.01796.x

Berer, K., Mues, M., Koutrolos, M., Rasbi, Z. A., Boziki, M., Johner, C., et al. (2011). Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 479, 538–541. doi: 10.1038/nature10554

Berntson, G. G., Sarter, M., and Cacioppo, J. T. (2003). Ascending visceral regulation of cortical affective information processing. *Eur. J. Neurosci.* 18, 2103–2109. doi: 10.1046/j.1460-9568.2003.02967.x

Beyreuther, K., Biesalski, H. K., Fernstrom, J. D., Grimm, P., Hammes, W. P., Heinemann, U., et al. (2007). Consensus meeting: monosodium glutamate - an update. *Eur. J. Clin. Nutr.* 61, 304–313. doi: 10.1038/sj.ejcn.1602526

Bezençon, C., le Coutre, J., and Damak, S. (2007). Taste-signaling proteins are coexpressed in solitary intestinal epithelial cells. *Chem. Senses* 32, 41–49. doi: 10.1093/chemse/bjl034

Biaggini, K., Barbey, C., Borrel, V., Feuilloley, M., Déchelotte, P., and Connil, N. (2015). The pathogenic potential of *Pseudomonas fluorescens* MFN1032 on enterocytes can be modulated by serotonin, substance P and epinephrine. *Arch. Microbiol.* 197, 983–990. doi: 10.1007/s00203-015-1135-y

Bienenstock, J., and Collins, S. (2010). 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: psycho-neuroimmunology and the intestinal microbiota: clinical observations and basic mechanisms. *Clin. Exp. Immunol.* 160, 85–91. doi: 10.1111/j.1365-2249.2010.04124.x

Bjurström, H., Wang, J., Ericsson, I., Bengtssonm, M., Liu, Y., Kumar-Mendu, S., et al. (2008). GABA, a natural immunomodulator of T lymphocytes. *J. Neuroimmunol.* 205, 44–50. doi: 10.1016/j.jneuroim.2008.08.017

Bohórquez, D. V., Shahid, R. A., Erdmann, A., Kreger, A. M., Wang, Y., Calakos, N., et al. (2015). Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. *J. Clin. Invest.* 125, 782–786. doi: 10.1172/JCI78361

Boonstra, E., de Kleijn, R., Colzato, L. S., Alkemade, A., Forstmann, B. U., and Nieuwenhuis, S. (2015). Neurotransmitters as food supplements: the effects of GABA on brain and behavior. *Front. Psychol.* 6:1520. doi: 10.3389/fpsyg.2015.01520

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., et al. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Sci. Transl. Med.* 6:263ra158. doi: 10.1126/scitranslmed.3009759

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., et al. (2011). Ingestion of *Lactobacillus* strain regulates emotional behavior and

central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16050–16055. doi: 10.1073/pnas.1102999108

Brechtel, C. E., and King, S. C. (1998). 4-Aminobutyrate (GABA) transporters from the amine-polyamine-choline superfamily: substrate specificity and ligand recognition profile of the 4-aminobutyrate permease from *Bacillus subtilis*. *Biochem. J.* 333, 565–571. doi: 10.1042/bj3330565

Breton, J., Tennoune, N., Lucas, N., Francois, M., Legrand, R., Jacquemot, J., et al. (2016). Gut commensal *E. coli* Proteins Activate Host Satiety Pathways following nutrient-induced bacterial growth. *Cell Metab.* 23, 324–334. doi: 10.1016/j.cmet.2015.10.017

Bron, P. A., van Baarlen, P., and Kleerebezem, M. (2011). Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat. Rev. Microbiol.* 10, 66–78.

Brosnan, J. T., Drewnowski, A., and Friedman, M. I. (2014). Is there a relationship between dietary MSG and obesity in animals or humans ? *Amino Acids* 46, 2075–2087. doi: 10.1007/s00726-014-1799-7

Buka, S. L., Cannon, T. D., Torrey, E. F., Yolken, R. H., and Collaborative Study Group on the Perinatal Origins of Severe Psychiatric Disorders (2008). Maternal exposure to herpes simplex virus and risk of psychosis among adult offspring. *Biol. Psychiatry* 63, 809–815. doi: 10.1016/j.biopsych.2007.09.022

Burchett, S. A., and Hicks, T. P. (2006). The mysterious trace amines: protean neuromodulators of synaptic transmission in mammalian brain. *Prog. Neurobiol.* 79, 223–246. doi: 10.1016/j.pneurobio.2006.07.003

Burrin, D. G., and Stoll, B. (2009). Metabolic fate and function of dietary glutamate in the gut. *Am. J. Clin. Nutr.* 90, 850S–856S. doi: 10.3945/ajcn.2009.27462Y

Cani, P. D., and Knauf, C. (2016). How gut microbes talk to organs: the role of endocrine and nervous routes. *Mol. Metab.* 5, 743–752. doi: 10.1016/j.molmet.2016.05.011

Cao, J. H., Huang, S. L., Qian, J., Huang, J. L., Jin, L. X., Su, Z., et al. (2009). Evolution of the class C GPCR Venus flytrap modules involved positive selected functional divergence. *BMC Evol. Biol.* 9:67. doi: 10.1186/1471-2148-9-67

Casanova, E., Guetg, N., Vigot, R., Seddik, R., Julio-Pieper, M., Hyland, N. P., et al. (2009). A mouse model for visualization of GABA(B) receptors. *Genesis* 47, 595–602. doi: 10.1002/dvg.20535

Chang, H. J., Yoo, B. C., Lim, S. B., Jeong, S. Y., Kim, W. H., and Park, J. G. (2005). Metabotropic glutamate receptor 4 expression in colorectal carcinoma and its prognostic significance. *Clin. Cancer Res.* 11, 3288–3295. doi: 10.1158/1078-0432.CCR-04-1912

Chen, G. Q., Cui, C., Mayer, M. L., and Gouaux, E. (1999). Functional characterization of a potassium-selective prokaryotic glutamate receptor. *Nature* 402, 817–821. doi: 10.1038/45568

Chen, Z., Fei, Y. J., Anderson, C. M., Wake, K. A., Miyauchi, S., Huang, W., et al. (2003). Structure, function and immunolocalization of a proton-coupled amino acid transporter (hPAT1) in the human intestinal cell line Caco-2. *J. Physiol.* 546, 349–361. doi: 10.1113/jphysiol.2002.026500

Cherlyn, S. Y., Woon, P. S., Liu, J. J., Ong, W. Y., Tsai, G. C., and Sim, K. (2010). Genetic association studies of glutamate, GABA and related genes in schizophrenia and bipolar disorder: a decade of advance. *Neurosci. Biobehav. Rev.* 34, 958–977. doi: 10.1016/j.neubiorev.2010.01.002

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., et al. (2006). GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7460–7464. doi: 10.1073/pnas.0600313103

Chimerel, C., Emery, E., Summers, D. K., Keyser, U., Gribble, F. M., and Reimann, F. (2014). Bacterial metabolite indole modulates incretin secretion from intestinal enteroendocrine L cells. *Cell Rep.* 9, 1202–1208. doi: 10.1016/j.celrep.2014.10.032

Choi, S., Lee, M., Shiu, A. L., Yo, S. J., and Aponte, G. W. (2007). Identification of a protein hydrolysate responsive G protein-coupled receptor in enterocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292, G98–G112. doi: 10.1152/ajpgi.00295.2006

Chou, H. T., Kwon, D. H., Hegazy, M., and Lu, C. D. (2008). Transcriptome analysis of agmatine and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 190, 1966–1975. doi: 10.1128/JB.01804-07

Costedio, M. M., Hyman, N., and Mawe, G. M. (2007). Serotonin and its role in colonic function and in gastrointestinal disorders. *Dis. Colon Rectum* 50, 376–388. doi: 10.1007/s10350-006-0763-3

Cryan, J. F., and Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* 13, 701–712. doi: 10.1038/nrn3346

Dagorn, A., Hillion, M., Chapalain, A., Lesouhaitier, O., Duclairoir Poc, C., Vieillard, J., et al. (2013). Gamma-aminobutyric acid acts as a specific virulence regulator in *Pseudomonas aeruginosa*. *Microbiology* 159, 339–351. doi: 10.1099/mic.0.061267-0

Engelstoft, M. S., Egerod, K. L., Holst, B., and Schwartz, T. W. (2008). A gut feeling for obesity: 7TM sensors on enteroendocrine cells. *Cell Metab.* 8, 447–449. doi: 10.1016/j.cmet.2008.11.004

Everard, A., Lazarevic, V., Derrien, M., Girard, M., Muccioli, G. G., Neyrinck, A. M., et al. (2011). Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. *Diabetes Metab. Res. Rev.* 60, 2775–2786.

Eßlinger, M., Wachholz, S., Manitz, M. P., Plümper, J., Sommer, R., Juckel, G., et al. (2016). Schizophrenia associated sensory gating deficits develop after adolescent microglia activation. *Brain Behav. Immun.* 58, 99–106. doi: 10.1016/j.bbi.2016.05.018

Femenía, T., Gómez-Galán, M., Lindskog, M., and Magara, S. (2012). Dysfunctional hippocampal activity affects emotion and cognition in mood disorders. *Brain Res.* 1476, 58–70. doi: 10.1016/j.brainres.2012.03.053

Forsythe, P., and Kunze, W. A. (2013). Voices from within: gut microbes and the CNS. *Cell. Mol. Life Sci.* 70, 55–69. doi: 10.1007/s00018-012-1028-z

Freestone, P. P., Williams, P. H., Haigh, R. D., Maggs, A. F., Neal, C. P., and Lyte, M. (2002). Growth stimulation of intestinal commensal *Escherichia coli* by catecholamines: a possible contributory factor in trauma-induced sepsis. *Shock* 18, 465–470. doi: 10.1097/00024382-200211000-00014

Furness, J., Rivera, L., Cho, H., Bravo, D., and Callaghan, B. (2013). The gut as a sensory organ. *Nat. Rev. Gastroenterol. Hepatol.* 10, 729–740. doi: 10.1038/nrgastro.2013.180

Furness, J. B. (2006). *The Enteric Nervous System*. Oxford: Blackwell.

Gadea, A., and López-Colomé, A. M. (2001). Glial transporters for glutamate, glycine, and GABA: II, GABA transporters. *J. Neurosci. Res.* 63, 461–468. doi: 10.1002/jnr.1040

Ger, M. F., Rendon, G., Tilson, J. L., and Jakobsson, E. (2010). Domain-based identification and analysis of glutamate receptor ion channels and their relatives in prokaryotes. *PLoS ONE* 5:e12827. doi: 10.1371/journal.pone.0012827

Gershon, M. D. (1998). *The Second Brain*. New York, NY: Harper Collins.

Gershon, M. D., and Tack, J. (2007). The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 132, 397–414. doi: 10.1053/j.gastro.2006.11.002

Gilman, S. E., Cherkerzian, S., Buka, S. L., Hahn, J., Hornig, M., and Goldstein, J. M. (2016). Prenatal immune programming of the sex-dependent risk for major depression. *Transl. Psychiatry* 6:e822. doi: 10.1038/tp.2016.91

Goetz, T., Arslan, A., Wisden, W., and Wulff, P. (2007). GABA(A) receptors: structure and function in the basal ganglia. *Prog. Brain Res.* 160, 21–41. doi: 10.1016/S0079-6123(06)60003-4

Guthrie, G. D., and Nicholson-Guthrie, C. S. (1989). gamma- Aminobutyric acid uptake by a bacterial system with neurotransmitter binding characteristics. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7378–7381. doi: 10.1073/pnas.86.19.7378

Guthrie, G. D., Nicholson-Guthrie, C. S., and Leary, H. L. Jr. (2000). A bacterial high-affinity GABA binding protein: isolation and characterization. *Biochem. Biophys. Res. Commun.* 268, 65–68. doi: 10.1006/bbrc.1999.1960

Halang, P., Toulouse, C., Geißel, B., Michel, B., Flauger, B., Müller, M., et al. (2015). Response of *Vibrio cholerae* to the catecholamine hormones Epinephrine and Norepinephrine. *J. Bacteriol.* 197, 3769–3778. doi: 10.1128/JB.00345-15

Hara, K., Saito, Y., Kirihara, Y., and Sakura, S. (2004). The interaction between gamma-aminobutyric acid agonists and diltiazem in visceral antinociception in rats. *Anesth. Analg.* 98, 1380–1384. doi: 10.1213/01.ANE.0000107935.84035.48

Hara, K., Saito, Y., Kirihara, Y., Yamada, Y., Sakura, S., and Kosaka, Y. (1999). The interaction of antinociceptive effects of morphine and GABA receptor agonists within the rat spinal cord. *Anesth. Analg.* 89, 422–427. doi: 10.1213/00000539-199908000-00032

Hass, N., Schwarzenbacher, K., and Breer, H. (2010). T1R3 is expressed in brush cells and ghrelin-producing cells of murine stomach. *Cell Tissue Res.* 339, 493–504. doi: 10.1007/s00441-009-0907-6

Hawkins, R. A. (2009). The blood–brain barrier and glutamate. *Am. J. Clin. Nutr.* 90, 867S–874S. doi: 10.3945/ajcn.2009.27462BB

Hays, S. P., Ordonez, J. M., Burrin, D. G., and Sunehag, A. L. (2007). Dietary glutamate is almost entirely removed in its first pass through the splanchnic bed in premature infants. *Pediatr. Res.* 62, 353–356. doi: 10.1203/PDR.0b013e318123f719

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., et al. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* 67, 2982–2992. doi: 10.1128/AEM.67.7.2982-2992.2001

Hosoi, T., Okuma, Y., Matsuda, T., and Nomura, Y. (2005). Novel pathway for LPS-induced afferent vagus nerve activation: possible role of nodose ganglion. *Auton. Neurosci.* 120, 104–107. doi: 10.1016/j.autneu.2004.11.012

Hu, L. A., and King, S. C. (1998). Membrane topology of the *Escherichia coli* γ-aminobutyrate transporter: implications on the topography and mechanism of prokaryotic and eukaryotic transporters from the APC superfamily. *Biochem. J.* 336, 69–76. doi: 10.1042/bj3360069

Hughes, D. T., and Sperandio, V. (2008). Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* 6, 111–120. doi: 10.1038/nrmicro1836

Hyland, N. P., and Cryan, J. F. (2010). A gut feeling about GABA: focus on GABA(B) receptors. *Front. Pharmacol.* 1:124. doi: 10.3389/fphar.2010.00124

Janeczko, M. J., Stoll, B., Chang, X., Guan, X., and Burrin, D. G. (2007). Extensive gut metabolism limits the intestinal absorption of excessive supplemental dietary glutamate loads in infant pigs. *J. Nutr.* 137, 2384–2390.

Jang, H. J., Kokrashvili, Z., Theodorakis, M. J., Carlson, O. D., Kim, B. J., Zhou, J., et al. (2007). Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15069–15074. doi: 10.1073/pnas.0706890104

Janik, R., Thomason, L. A., Stanisz, A. M., Forsythe, P., Bienenstock, J., and Stanisz, G. J. (2016). Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *Neuroimage* 125, 988–995.

Julio-Pieper, M., Hyland, N. P., Bravo, J. A., Dinan, T. G., and Cryan, J. F. (2010). A novel role for the metabotropic glutamate receptor-7: modulation of faecal water content and colonic electrolyte transport in the mouse. *Br. J. Pharmacol.* 160, 367–375. doi: 10.1111/j.1476-5381.2010.00713.x

Julio-Pieper, M., O'Connor, R. M., Dinan, T. G., and Cryan, J. F. (2013). Regulation of the brain-gut axis by group III metabotropic glutamate receptors. *Eur. J. Pharmacol.* 698, 19–30. doi: 10.1016/j.ejphar.2012.10.027

Kakee, A., Takanaga, H., Terasaki, T., Naito, M., Tsuruo, T., and Sugiyama, Y. (2001). Efflux of a suppressive neurotransmitter, GABA, across the blood-brain barrier. *J. Neurochem.* 79, 110–118. doi: 10.1046/j.1471-4159.2001.00540.x

Kanehira, T., Yoshiko, N., Nakamura, K., Horie, K., Horie, N., Furugori, K., et al. (2011). Relieving occupational fatigue by consumption of a beverage containing γ-amino butyric acid. *J. Nutr. Sci. Vitaminol.* 57, 9–15. doi: 10.3177/jnsv.57.9

Kaneko, K., Iwasaki, M., Yoshikawa, M., and Ohinata, K. (2010). Orally administered soymorphins, soy-derived opioid peptides, suppress feeding and intestinal transit via gut mu(1)-receptor coupled to 5-HT(1A), D(2), and GABA(B) systems. *Am. J. Physiol. Gastrointest. Liver Physiol.* 299, G799–G805. doi: 10.1152/ajpgi.00081.2010

Keita, A. V., and Söderholm, J. D. (2010). The intestinal barrier and its regulation by neuroimmune factors. *Neurogastroenterol. Motil.* 22, 718–733. doi: 10.1111/j.1365-2982.2010.01498.x

Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G., and Hyland, N. P. (2015). Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front. Cell. Neurosci.* 9:392. doi: 10.3389/fncel.2015.00392

Kirchgessner, A. L. (2001). Glutamate in the enteric nervous system. *Curr. Opin. Pharmacol.* 1, 591–596. doi: 10.1016/S1471-4892(01)00101-1

Kitamura, A., Tsurugizawa, T., Uematsu, A., Torii, K., and Uneyama, H. (2012). New therapeutic strategy for amino acid medicine: effects of dietary glutamate on gut and brain function. *J. Pharmacol. Sci.* 118, 138–144. doi: 10.1254/jphs.11R06FM

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402, 656–660. doi: 10.1038/45230

Kondoh, T., Mallick, H. N., and Torii, K. (2009). Activation of the gut-brain axis by dietary glutamate and physiologic significance in energy homeostasis. *Am. J. Clin. Nutr.* 90, 832S–837S. doi: 10.3945/ajcn.2009.27462V

Li, H., and Cao, Y. (2010). Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids* 39, 1107–1116. doi: 10.1007/s00726-010-0582-7

Lim, J. Y., Choi, S. I., Choi, G., and Hwang, S. W. (2016). Atypical sensors for direct and rapid neuronal detection of bacterial pathogens. *Mol. Brain.* 9:26. doi: 10.1186/s13041-016-0202-x

Liu, M., Seino, S., and Kirchgessner, A. (1999). Identification and characterization of glucoresponsive neurons in the enteric nervous system. *J. Neurosci.* 19, 10305–10317.

Logan, A. C., and Katzman, M. (2005). Major depressive disorder: probiotics may be an adjuvant therapy. *Med. Hypotheses* 64, 533–538. doi: 10.1016/j.mehy.2004.08.019

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays* 33, 574–581. doi: 10.1002/bies.201100024

Lyte, M., and Freestone, P. P. E. (2010). *Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health*. New York, NY: Springer.

Masek, K., and Kadlec, O. (1983). Sleep factor, muramyl peptides, and the serotoninergic system. *Lancet* 1, 1277.

Matur, E., and Eraslan, E. (2012). "The impact of probiotics on the gastrointestinal physiology," in *New Advances in the Basic and Clinical Gastroenterology*, ed. T. Brzozowski (Rijeka: InTech), 51–74.

Mawe, G. M., Strong, D. S., and Sharkey, K. A. (2009). Plasticity of enteric nerve functions in the inflamed and postinflamed gut. *Neurogastroenterol. Motil.* 21, 481–491. doi: 10.1111/j.1365-2982.2009.01291.x

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut-brain communication. *Nat. Rev. Neurosci.* 12, 453–466. doi: 10.1038/nrn3071

Mazzoli, R. (2014). "Neuro-active compounds produced by probiotics. Towards a microbiota-(gut-) brain axis control?," in *Interactive Probiotics*, ed. E. Pessione (Boca Raton, FL: CRC Press), 148–176.

Mazzoli, R., Pessione, E., Dufour, M., Laroute, V., Giuffrida, M. G., Giunta, C., et al. (2010). Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. *Amino Acids* 39, 727–737. doi: 10.1007/s00726-010-0507-5

McDermott, J. R., Leslie, F. C., D'Amato, M., Thompson, D. G., Grencis, R. K., and McLaughlin, J. T. (2006). Immune control of food intake: enteroendocrine cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. *Gut* 55, 492–497. doi: 10.1136/gut.2005.081752

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449, 819–826. doi: 10.1038/nature06246

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., et al. (2011). Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Br. J. Nutr.* 105, 755–764. doi: 10.1017/S000711451000 4319

Michels, G., and Moss, S. J. (2007). GABAA receptors: properties and trafficking. *Crit. Rev. Biochem. Mol. Biol.* 42, 3–14. doi: 10.1080/10409230601146219

Morse, D. E., Duncan, H., Hooker, N., Baloun, A., and Young, G. (1980). GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae. *Fed. Proc.* 39, 3237–3241.

Nakajima, K., Tooyama, I., Kuriyama, K., and Kimura, H. (1996). Immunohistochemical demonstration of GABAB receptors in the rat gastrointestinal tract. *Neurochem. Res.* 21, 211–215. doi: 10.1007/BF02529137

Nakamura, H., Takishima, T., Kometani, T., and Yokogoshi, H. (2009). Psychological stress-reducing effect of chocolate enriched with gamma-aminobutyric acid (GABA) in humans: assessment of stress using heart rate variability and salivary chromogranin A. *Int. J. Food Sci. Nutr.* 60, 106–113. doi: 10.1080/09637480802558508

Navarathna, D. H., Nickerson, K. W., Duhamel, G. E., Jerrels, T. R., and Petro, T. M. (2007). Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect. Immun.* 75, 4006–4011. doi: 10.1128/IAI.00397-07

Neufeld, K. M., Kang, N., Bienenstock, J., and Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol. Motil.* 23:e119. doi: 10.1111/j.1365-2982.2010.01620.x

Neunlist, M., and Schemann, M. (2014). Nutrient-induced changes in the phenotype and function of the enteric nervous system. *J. Physiol.* 592, 2959–2965. doi: 10.1113/jphysiol.2014.272948

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Nicola, J. P., Velez, M. L., Lucero, A. M., Fozzatti, L., Pellizas, C. G., and Masini-Repiso, A. M. (2009). Functional toll-like receptor 4 conferring lipopolysaccharide responsiveness is expressed in thyroid cells. *Endocrinology* 150, 500–508. doi: 10.1210/en.2008-0345

Nielsen, C. U., Carstensen, M., and Brodin, B. (2012). Carrier-mediated γ-aminobutyric acid transport across the basolateral membrane of human intestinal Caco-2 cell monolayers. *Eur. J. Pharm. Biopharm.* 81, 458–462. doi: 10.1016/j.ejpb.2012.03.007

Nøhr, M. K., Pedersen, M. H., Gille, A., Egerod, K. L., Engelstoft, M. S., Husted, A. S., et al. (2013). GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology* 154, 3552–3564. doi: 10.1210/en.2013-1142

Norris, V., Molina, F., and Gewirtz, A. T. (2013). Hypothesis: bacteria control host appetites. *J. Bacteriol.* 195, 411–416. doi: 10.1128/JB.01384-12

Ochoa-Cortes, F., Ramos-Lomas, T., Miranda-Morales, M., Spreadbury, I., Ibeakanma, C., Barajas-Lopez, C., et al. (2010). Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *Am. J. Physiol. Gastrointest. Liver Physiol.* 299, G723–G732. doi: 10.1152/ajpgi.00494.2009

Oh, S. H., Moon, Y. J., and Oh, C. H. (2003). γ-Aminobutyric acid (GABA) content of selected uncooked foods. *Preven. Nutr. Food Sci.*8, 75–78. doi: 10.3746/jfn.2003.8.1.075

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., and Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav. Brain Res.* 277, 32–48. doi: 10.1016/j.bbr.2014.07.027

Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front. Cell. Infect. Microbiol.* 2:86. doi: 10.3389/fcimb.2012.00086

Pessione, E., Mazzoli, R., Giuffrida, M. G., Lamberti, C., Garcia-Moruno, E., Barello, C., et al. (2005). A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics* 5, 687–698. doi: 10.1002/pmic.200401116

Pessione, E., Pessione, A., Lamberti, C., Coïsson, D. J., Riedel, K., Mazzoli, R., et al. (2009). First evidence of a membrane-bound, tyramine and beta-phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a two-dimensional electrophoresis proteomic study. *Proteomics* 9, 2695–2710. doi: 10.1002/pmic.200800780

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., et al. (2016). GABA-producing *Bifidobacterium dentium* modulates visceral sensitivity in the intestine. *Neurogastroenterol. Motil.* doi: 10.1111/nmo.12904 [Epub ahead of print].

Psichas, A., Reimann, F., and Gribble, F. M. (2015). Gut chemosensing mechanisms. *J. Clin. Invest.* 125, 908–917. doi: 10.1172/JCI76309

Qi, J., Buzas, K., Fan, H., Cohen, J. I., Wang, K., Mont, E., et al. (2011). Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *J. Immunol.* 186, 6417–6426. doi: 10.4049/jimmunol.1001241

Raybould, H. E. (2010). Gut chemosensing: interactions between gut endocrine cells and visceral afferents. *Auton. Neurosci.* 153, 41–46. doi: 10.1016/j.autneu.2009.07.007

Reid, G. (2011). Neuroactive probiotics. *Bioessays* 33:562. doi: 10.1002/bies.201100074

Reyes-Darias, J. A., García, V., Rico-Jiménez, M., Corral-Lugo, A., Lesouhaitier, O., Juárez-Hernández, D., et al. (2015). Specific gamma-aminobutyrate chemotaxis in pseudomonads with different lifestyle. *Mol. Microbiol.* 97, 488–501. doi: 10.1111/mmi.13045

Richard, H. T., and Foster, J. W. (2003). Acid resistance in *Escherichia coli*. *Adv. Appl. Microbiol.* 52, 167–186. doi: 10.1016/S0065-2164(03)01007-4

Roth, F. C., and Draguhn, A. (2012). GABA metabolism and transport: effects on synaptic efficacy. *Neural Plast.* 2012:805830. doi: 10.1155/2012/805830

Samuel, B. S., Shaito, A., Motoike, T., Rey, F. E., Backhed, F., Manchester, J. K., et al. (2008). Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16767–16772. doi: 10.1073/pnas.0808567105

San Gabriel, A., Nakamura, E., Uneyama, H., and Torii, K. (2009). Taste, visceral information and exocrine reflexes with glutamate through umami receptors. *J. Med. Invest.* 56(Suppl.), 209–217. doi: 10.2152/jmi.56.209

Sano, C. (2009). History of glutamate production. *Am. J. Clin. Nutr.* 90, 728S–732S. doi: 10.3945/ajcn.2009.27462F

Santocchi, E., Guiducci, L., Fulceri, F., Billeci, L., Buzzigoli, E., Apicella, F., et al. (2016). Gut to brain interaction in autism spectrum disorders: a randomized controlled trial on the role of probiotics on clinical, biochemical and neurophysiological parameters. *BMC Psychiatry* 16:183. doi: 10.1186/s12888-016-0887-5

Schéle, E., Grahnemo, L., Anesten, F., Hallén, A., Bäckhed, F., and Jansson, J. O. (2013). The gut microbiota reduces leptin sensitivity and the expression of the obesity-suppressing neuropeptides proglucagon (Gcg) and brain-derived neurotrophic factor (Bdnf) in the central nervous system. *Endocrinology* 154, 3643–3651. doi: 10.1210/en.2012-2151

Schertzer, J. W., Boulette, M. L., and Whiteley, M. (2009). More than a signal: non-signaling properties of quorum sensing molecules. *Trends Microbiol.* 17, 189–195. doi: 10.1016/j.tim.2009.02.001

Schwörer, H., Racké, K., and Kilbinger, H. (1989). GABA receptors are involved in the modulation of the release of 5-hydroxytryptamine from the vascularly perfused small intestine of the guinea-pig. *Eur. J. Pharmacol.* 165, 29–37. doi: 10.1016/0014-2999(89)90767-X

Shimazu, S., and Miklya, I. (2004). Pharmacological studies with endogenous enhancer substances: beta phenylethylamine, tryptamine and their synthetic derivatives. *Prog. Neuropsychopharmacol. Biol. Psichiatry* 28, 421–427. doi: 10.1016/j.pnpbp.2003.11.016

Simeone, T. A., Donevan, S. D., and Rho, J. M. (2003). Molecular biology and ontogeny of gamma-aminobutyric acid (GABA) receptors in the mammalian central nervous system. *J. Child. Neurol.* 18, 39–48. doi: 10.1177/08830738030180012101

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R., and Gobbetti, M. (2007). Synthesis of gamma-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.* 73, 7283–7290. doi: 10.1128/AEM.01064-07

Sivamaruthi, B. S., Madhumita, R., Balamurugan, K., and Rajan, K. E. (2015). Cronobacter sakazakii infection alters serotonin transporter and improved fear memory retention in the rat. *Front. Pharmacol.* 6:188. doi: 10.3389/fphar.2015.00188

Sivilotti, L., and Nistri, A. (1991). GABA receptor mechanisms in the central nervous system. *Prog. Neurobiol.* 36, 35–92. doi: 10.1016/0301-0082(91)90036-Z

Soeiro-de-Souza, M. G., Henning, A., Machado-Vieira, R., Moreno, R. A., Pastorello, B. F., da Costa, et al. (2015). Anterior cingulate glutamate-glutamine cycle metabolites are altered in euthymic bipolar I disorder. *Eur. Neuropsychopharmacol.* 25, 2221–2229. doi: 10.1016/j.euroneuro.2015.09.020

Spurny, R., Ramerstorfer, J., Price, K., Brams, M., Ernst, M., Nury, H., et al. (2012). Pentameric ligand-gated ion channel ELIC is activated by GABA and modulated by benzodiazepines. *Proc. Natl. Acad. Sci. U.S.A.* 109, E3028–E3034. doi: 10.1073/pnas.1208208109

Steenbergen, L., Sellaro, R., Stock, A.-K., Beste, C., and Colzato, L. S. (2015). γ-Aminobutyric acid (GABA) administration improves action selection processes: a randomised controlled trial. *Sci. Rep.* 5:12770. doi: 10.1038/srep12770

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., et al. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. Physiol.* 558, 263–275. doi: 10.1113/jphysiol.2004.063388

Takaki, M., Mawe, G. M., Barasch, J. M., Gershon, M. D., and Gershon, M. D. (1985). Physiological responses of guinea-pig myenteric neurons secondary to the release of endogenous serotonin by tryptamine. *Neuroscience* 16, 223–240. doi: 10.1016/0306-4522(85)90059-4

Tanaka, T., Katsuma, S., Adachi, T., Koshimizu, T. A., Hirasawa, A., and Tsujimoto, G. (2008). Free fatty acids induce cholecystokinin secretion through GPR120. *Naunyn Schmiedebergs Arch. Pharmacol.* 377, 523–527. doi: 10.1007/s00210-007-0200-8

Tanous, C., Chambellon, E., Sepulchre, A. M., and Yvon, M. (2005). The gene encoding the glutamate dehydrogenase in *Lactococcus lactis* is part of a remnant

Tn3 transposon carried by a large plasmid. *J. Bacteriol.* 187, 5019–5022. doi: 10.1128/JB.187.14.5019-5022.2005

Tennoune, N., Chan, P., Breton, J., Legrand, R., Chabane, Y. N., Akkermann, K., et al. (2014). Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide α-MSH, at the origin of eating disorders. *Transl. Psychiatry* 4:e458. doi: 10.1038/tp.2014.98

Terbach, N., and Williams, R. S. (2009). Structure-function studies for the panacea, valproic acid. *Biochem. Soc. Trans.* 37, 1126–1132. doi: 10.1042/BST0371126

Thwaites, D. T., Basterfield, L., McCleave, P. M., Carter, S. M., and Simmons, N. L. (2000). Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers. *Br. J. Pharmacol.* 129, 457–464. doi: 10.1038/sj.bjp.0703069

Tolhurst, G., Heffron, H., Lam, Y. S., Parker, H. E., Habib, A. M., Diakogiannaki, E., et al. (2012). Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes Metab. Res. Rev.* 61, 364–371.

Tsai, M. F., McCarthy, P., and Miller, C. (2013). Substrate selectivity in glutamate-dependent acid resistance in enteric bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5898–5902. doi: 10.1073/pnas.1301442110

Uneyama, H., Niijima, A., San Gabriel, A., and Torii, K. (2006). Luminal amino acid sensing in the rat gastric mucosa. *Am. J. Physiol. Gastrointest. Liver Physiol.* 291, G1163–G1170. doi: 10.1152/ajpgi.00587.2005

Van Oudenhove, L., McKie, S., Lassman, D., Uddin, B., Paine, P., Coen, S., et al. (2011). Fatty acid-induced gut-brain signaling attenuates neural and behavioral effects of sad emotion in humans. *J. Clin. Invest.* 121, 3094–3099. doi: 10.1172/JCI46380

Vaughan, E. E., Tannock, G. W., Salminen, S., and Morelli, L. (2011). "Healthy heroes: LABs and gut microbes," in *Thirthy Years of Research on Lactic Acid Bacteria*, eds A. Ledeboer, J. Hugenholtz, J. Kok, W. Konings, and J. Wouters (Rotterdam: 24 Media Labs), 151–169.

Wang, G. D., Wang, X. Y., Xia, Y., and Wood, J. D. (2014). Dietary glutamate: interactions with the enteric nervous system. *J. Neurogastroenterol. Motil.* 20, 41–53. doi: 10.5056/jnm.2014.20.1.41

Williams, B. B., Van Benschoten, A. H., Cimermancic, P., Donia, M. S., Zimmermann, M., Taketani, M., et al. (2014). Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell Host Microbe* 16, 495–503. doi: 10.1016/j.chom.2014.09.001

Williams, B. L., Hornig, M., Buie, T., Bauman, M. L., Cho Paik, M., Wick, I., et al. (2011). Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PLoS ONE* 6:e24585. doi: 10.1371/journal.pone.0024585

Williamson, N. R., Fineran, P. C., Leeper, F. J., and Salmond, G. P. (2006). The biosynthesis and regulation of bacterial prodiginines. *Nat. Rev. Microbiol.* 4, 887–899. doi: 10.1038/nrmicro1531

Wong, R. K., Yang, C., Song, G. H., Wong, J., and Ho, K. Y. (2015). Melatonin regulation as a possible mechanism for probiotic (VSL#3) in irritable bowel syndrome: a randomized double-blinded placebo study. *Dig. Dis. Sci.* 60, 186–194. doi: 10.1007/s10620-014-3299-8

Yoto, A., Murao, S., Motoki, M., Yokoyama, Y., Horie, N., Takeshima, K., et al. (2012). Oral intake of γ-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks. *Amino Acids* 43, 1331–1337. doi: 10.1007/s00726-011-1206-6

Zareian, M., Ebrahimpour, A., Bakar, F. A., Mohamed, A. K., Forghan, B., Ab-Kadir, M. S., et al. (2012). A glutamic acid-producing lactic acid bacteria isolated from Malaysian fermented foods. *Int. J. Mol. Sci.* 13, 5482–5497. doi: 10.3390/ijms13055482

Zimmermann, I., and Dutzler, R. (2011). Ligand activation of the prokaryotic pentameric ligand-gated ion channel ELIC. *PLoS Biol.* 9:e1001101. doi: 10.1371/journal.pbio.1001101

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MINI REVIEW
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Melatonin and Other Tryptophan Metabolites Produced by Yeasts: Implications in Cardiovascular and Neurodegenerative Diseases

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Yeast metabolism produces compounds derived from tryptophan, which are found in fermented beverages, such as wine and beer. In particular, melatonin and serotonin, may be relevant due to their bioactivity in humans. Indeed, the former is a neurohormone related to circadian rhythms, which also has a putative protective effect against degenerative diseases. Moreover, serotonin is a neurotransmitter itself, in addition to being a precursor of melatonin synthesis. This paper summarizes data reported on fermented beverages, to evaluate dietary intake. Additionally, the article reviews observed effects of yeast amino acid metabolites on the prevention of neurodegenerative diseases (Alzheimer's and Parkinson's) and angiogenesis, focusing on evidence of the molecular mechanism involved and identification of molecular targets.

Keywords: wine, beer, VEGF, β-amyloid, α-synuclein, tryptophol, serotonin

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ORIGIN, OCCURRENCE, AND DIETARY INTAKE

The presence of bioactive compounds in fermented beverages has long been observed and they have been studied with great interest. A large body of research has focused on polyphenols, in particular, since these bioactive compounds are already present in plants and released into fermented products. Yeast also transforms certain other molecules into biologically active compounds. Among these, the case of amino acid tryptophan is of interest, since it is the precursor of at least three biologically active compounds: melatonin, serotonin, and tryptophol (Mas et al., 2014).

Tryptophol is an alcohol produced by the Ehrlich pathway and it has long been detected in appreciable concentrations in wines and beers in the mg/L range (Bartolomé et al., 2000; Monagas et al., 2007). Therefore, its occurrence in beverages is widely recognized. Moreover, tryptophol has also been indicated as a quorum sensing molecule for yeast (Sprague and Winans, 2006).

Just a few years ago, melatonin was detected in wines in much lower levels: within the ng/L range. Not only was it evidenced in wines, but also in other fermented foods, as summarized in **Table 1**. Furthermore, Rodriguez-Naranjo et al. (2011) highlighted that melatonin was produced after alcoholic fermentation, pinpointing the role *Saccharomyces* plays. Indeed, different strains synthetized melatonin at different levels (Rodriguez-Naranjo et al., 2012).

The synthetic pathway of melatonin in yeast is not completely elucidated, yet it seems the formation of serotonin might be an intermediate in the pathway (Mas et al., 2014). In addition, serotonin has been detected at mg/L levels in red wine following malolactic fermentation (Wang et al., 2014). Further research is required to explore the roles of yeast and bacteria in the occurrence of these bioactive compounds in fermented products.

One of the characteristics of bioactive compounds is the minimal concentration required for them to act. The reported concentrations in wine and beer would mean that someone consuming these beverages would obtain a low daily intake of these compounds. According to WHO, the daily intake of ethanol should not exceed 30 g and 20 g for men and women, respectively. That is to say that daily intake for a man of a wine can provide up to 0.00005–0.13 mg of melatonin. In a comprehensive review summarizing the results of human intervention studies, Harpsøe et al. (2015) concluded that the bioavailability of melatonin was 15%. In our example, its bioavailable concentration should result in 1.5–4000 pg/mL of melatonin in blood. Physiological values for day plasma melatonin are very low, accounting for several pg/mL (5–10 pg/mL in human plasma) (de Almeida et al., 2011). Thus, pg/mL in plasma might be expected after dietetic intake of wine or beer, considering the values displayed in **Table 1**. Indeed, Maldonado et al. (2009) determined an increase in the concentration of plasmatic melatonin after the ingestion of a moderate dose of beer (330 mL for women volunteers, 660 mL for men). To the best of our knowledge, there is no published data on the bioavailability of serotonin after food or beverage intake.

BIOLOGICAL EFFECTS AND PREVENTION OF CHRONIC DISEASES

Literature on the biological effects of these compounds is extensive and encompasses circadian rhythm, antioxidant properties, and reproductive function. Due to the length of this mini-review, we will focus on more recent findings on the prevention of the most prevalent degenerative diseases, such as cancer, and cardiovascular and neurodegenerative diseases.

IMPLICATIONS FOR CANCER AND CARDIOVASCULAR DISEASE: THE ROLE OF ANGIOGENESIS

Angiogenesis, which consists of the formation of new blood vessels from pre-existing ones, is crucial for organ growth during embryonic development and after birth. However, in adulthood, angiogenesis plays an essential role in the pathogenesis of diverse chronic diseases, such as cancer and cardiovascular disease, involving the progression and development of the tumor, and development and destabilization of atherosclerotic plaques (Celletti et al., 2001; Bergers and Benjamin, 2003).

Angiogenesis occurs when there is an imbalance between pro-angiogenic (e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor, alfa tumor necrotic factor, etc.) and anti-angiogenic (e.g., angiostatin and endostatin) factors. VEGF is the most active endogenous pro-angiogenic factor in humans (Giles, 2001; Dulak, 2005; Cebe-Suarez et al., 2006; Cook and Figg, 2010). It exerts its angiogenic effect by stimulating VEGF receptor 2 (VEGFR-2), which is critical for promoting the proliferation and differentiation of endothelial cells (Giles, 2001; Ferrara and Kerbel, 2005). It has been demonstrated that VEGF promotes atherosclerotic plaque progression (Celletti et al., 2001; Khurana et al., 2005) and tumor angiogenesis (Senger et al., 1993). Indeed, VEGF is a target for drug therapies that aim to

TABLE 1 | Concentration of melatonin and other tryptophan metabolites in fermented products.

Compound		Concentration		Reference
	Food			
Melatonin	Probiotic yogurt	126.7 ± 9.00	pg/g or pg/mL	Kocadağli et al. (2014)
	Kefir (fermented milk drink)	n.d	pg/g or pg/mL	
	Black olive (naturally fermented)	5.3 ± 0.10	pg/g or pg/mL	
	Bread (crumb)	341.7 ± 29.30	pg/g or pg/mL	
	Bread (crust)	138.1 ± 23.20	pg/g or pg/mL	
	Beer	94.5 ± 6.70	pg/g or pg/mL	
	Wine			
	Alaban, Sangiovese, Trebbiano (Italy)	0.6–0.4	ng/mL	Mercolini et al., 2012
	Chardonnay, Malbec, Cabernet Sauvignon (Argentina)	0.16–0.32		Stege et al., 2010
	Gropello, Merlot (Italy)	8.1–5.2		Vitalini et al., 2013
	Cabernet Saivignon, Merlot, Syrah, Tempranillo, Tintilla de Rota, Petit Verdot, Prieto Picudo, and Palomino fino (Spain)	5.1–420		Rodriguez-Naranjo et al., 2011
	Fermented orange beverage	20.0 ± 2.02	ng/mL	Fernández-Pachón et al., 2014
	Red wine	4.88–9.15	mg/L	Monagas et al., 2007
	Fermented lentils	2.70+0.25	mg/g dry material	Bartolomé et al., 2000
Tryptophol	Whole-wheat bread	–		Jiang and Peterson, 2013
	Beer	0.242 ± 0.200	mg/L	Bartolomé et al., 2000
Serotonin	Beer	3.5–24.2	mg/L	Kirschbaum et al., 1999
	Wine	2.94–5.93	mg/L	Wang et al., 2014
		1.93 ± 0.043	mg/L	Manfroi et al., 2009
		5.5	ng/mL	Mandrioli et al., 2011

inhibit VEGF signaling (Ferrara and Kerbel, 2005). Anti-VEGF antibodies, aptamers and small molecule VEGFR tyrosine kinase inhibitors have been developed and given regulatory approval for the treatment of colon, lung, breast, kidney, and liver cancer, in addition to neovascular age-related macular degeneration (Giles, 2001; Ferrara and Kerbel, 2005). However, serious side effects, such as hypertension, have been reported with prolonged use of anti-VEGF therapies (Zhu et al., 2007; Wu et al., 2008; Kappers et al., 2010). The use of natural products in reducing VEGF-induced angiogenesis may prove to be more beneficial than the current anti-VEGF drugs available (Moyle et al., 2015).

Melatonin has been associated with a decline in VEGF secretion levels in the serum of advanced cancer patients (Lissoni et al., 2001), in addition to markedly reducing the expression of VEGF in HUVEC and culture cancer cells at 1 μM and 1 mM (Dai et al., 2008; Cui et al., 2012; Álvarez-García et al., 2013; Gonçalves et al., 2014). Melatonin has also been proven to reduce endothelial cell proliferation, invasion, migration, and tube formation, through downregulation of VEGF at 1 mM (Álvarez-García et al., 2013). The possible cell signaling pathway when melatonin inhibits HUVEC proliferation has been related to the following pathway: melatonin receptors/ERK/PI3K/Akt/PKC/NF-kB (Cui et al., 2008). Additionally, Sohn et al. (2015) have recently demonstrated that melatonin (1 mM) upregulates miRNA3195 and miRNA374b, whose overexpression synergistically reduced VEGF production in hypoxic PC-3 prostate cancer cells, indicating the important role of miRNA3195 and miRNA374b in melatonin induced antiangiogenic activity. Melatonin (40 mg/kg) has also shown an antitumor effect on mammary tumor growth in mice after 21 days of treatment; the mice displayed significantly smaller tumor volume and tumor regression (Jardim-Perassi et al., 2014). Additionally, in the same study, a lower expression of VEGFR2 was observed in the melatonin-treated tumors compared to the vehicle-treated tumors. More research is consequently needed to focus on determining the molecular mechanism by which melatonin exerts its angiogenic effect and the molecular target involved.

NEURODEGENERATIVE DISEASES

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common human neurodegenerative diseases. In both cases, their incidence increases with age. The aggregation of proteins that results in different fibrillar structures is responsible for these disorders. Specifically, they are owing to the abnormal pathological assembly of amyloid- beta (Aβ), tau and α- synuclein (αS).

Indeed, several studies have demonstrated that protofibrils and oligomers of αS and Aβ are more neurotoxic than fibrils (Pike et al., 1993; Lashuel et al., 2002; Volles and Lansbury, 2003; Outeiro et al., 2008).

This review focus on the evidence of certain bioactives which can present in fermented products. However, to give a fair balance, alcohol effects on neurodegeneration have to be highlighted as it is a major component formed by yeast in alcoholic beverages. It is well-Known that alcohol intake crosses the BBB (Blood–Brain Barrier) easily producing the excessive release of neurotransmitters, oxidative stress and inflammatory response which turns out in neurotoxicity and finally cell death. (Persidsky and Potula, 2014).

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive and irreversible neurodegenerative disorder characterized by loss of memory and cognition, abstract thinking, and personality alteration. The etiology of AD is unknown in more than 90% of cases. In the pathogenesis of AD there are two principal hallmarks: neurofibrillary tangles (NFTs) and amyloid plaques. NFT are formed by the intracellular accumulation of phosphorylated tau protein and amyloid plaques, by extracellular accumulation of amyloid β peptides (Hardy and Selkoe, 2002). The amyloid beta peptide is formed via cleavage of the amyloid precursor protein (APP). In the non-amyloidogenic pathway (normal state), APP is cleaved by α-secretase, to generate sAPP (soluble N- terminal fragment), which is neuroprotective as it is involved in the enhancement of synaptogenesis, neurite outgrowth, and neuronal survival. Conversely, in the disease state, APP is cleaved by β and γ secretase, resulting in insoluble beta amyloid peptide, which has high potential for assembly and formation of toxic aggregates (Gandy, 2005).

Several mechanisms have been proposed to explain βA neurotoxicity, such as oxidative stress and loss of endogenous antioxidants (Behl et al., 1994; Abramov and Duchen, 2005; Hamel et al., 2008); mitochondrial damage, depolarization, and mitochondrial permeability transition pore opening (Moreira et al., 2001, 2010; Abramov et al., 2004, 2007); destabilization of intracellular calcium homeostasis in neurons (Bezprozvanny and Mattson, 2008); glial cells (Abramov et al., 2003, 2004), and neuroinflammation (McNaull et al., 2010).

Levels of melatonin and its precursors (serotonin and tryptophan) are significantly decreased in elderly AD individuals and are associated with the emergence of AD (Zhou et al., 2003; Greilberger et al., 2010). A growing body of evidence supports the protective role of melatonin in several molecular mechanisms implicated in the development of AD.

Among these mechanisms, the most significant one is that melatonin prevents amyloid aggregation and overproduction. This neurohormone has a great affinity for Aβ peptide, preventing amyloid fibril formation (Masilamoni et al., 2008), as determined by circular dichroism (CD) spectroscopy, electron microscopy, nuclear magnetic resonance (NMR) and electrospray ionization-mass spectrometry (ESI-MS). In particular, a hydrophobic interaction has been observed between melatonin and Aβ, specifically on the 29–40 residues of the Aβ segment (Skribanek et al., 2001). Additionally, melatonin has inhibitory effects on the formation of secondary β-sheet structures through the disruption of the histidine (His^+) and aspartate (Asp^-) salt bridges in Aβ peptide that promote fibril dissolution (Fraser et al., 1991; Huang et al., 1997; Pappolla et al., 1998).

Melatonin presents a great capacity to regulate the synthesis and maturation of APP at different levels by: decreasing its mRNA encoding β-APP (Song and Lahiri, 1997; Lahiri, 1999); blocking cAMP production, which is involved in activating the APP gene promoter, (Husson et al., 2002); and inactivating GSK-3, which promotes α-secretase mediated cleavage of APP, favoring the non-amyloidogenic pathway (McArthur et al., 1997; Zhu et al., 2001; Hoppe et al., 2010). *In vivo* studies with transgenic mice over-expressing APP (in 9–10 months they develop senile plaques) and fed with 0.5 mg/mL of melatonin in their drinking water (3 mL/day) found a reduction in important markers of the disease, including Aβ levels in the brain, and that some animals survived (Matsubara et al., 2003). The amount given to rodents are within the pharmacological dose and out of the range of the dose that can be achieved with moderate consumption of wine. Therefore it cannot be concluded that these effect will be observed in humans after wine intake. Further research is required to obtain the evidence at dietary doses.

Furthermore, melatonin exhibits a protective effect on the cholinergic system. In AD patients, a dramatic decrease of acetylcholine has been observed (Francis et al., 1999), which was related to a decrease in enzyme choline acetyltransferase (ChAT) activity and an increase in acetylcholinesterase (AChE) activity (Bieschke et al., 2005). Indeed, AChE inhibitors increase the synaptic levels of acetylcholine, which is why they are used as a treatment for mild to moderate AD. *In vivo* administration of melatonin in rats (50 mg/kg body weight) has led to significantly reduced AChE activity, with maintenance of calcium levels under conditions of oxidative stress (Masilamoni et al., 2008). Regarding ChAT, melatonin increased its activity, after 4 months of melatonin administration in rats (Feng et al., 2004).

Finally, melatonin reduces Aβ-induced oxidative stress related to reactive oxygen species (ROS) and proinflamatory cytokines, such as IL6 and IL1-β in *in vivo* studies (Masilamoni et al., 2008). As a result of these effects, melatonin protects brain neurons from damage and death by increasing viability in hippocampal neurons and glial cells following treatment with Aβ1–40, Aβ25–40, and Aβ1–28. Moreover, melatonin prevents the death of murine N2a neuroblastoma and PC12 cells by using Aβ25–35 (Pappolla et al., 1997; Ionov et al., 2011).

There is scarce literature available in relation to the activity of other tryptophan metabolites, with indole 3-acetic acid and tryptophol being the only bioactive molecules reported so far. Morshedi et al. (2007) proved the inhibitory effect of these indole derivatives on the amyloid fibrillation of hen egg-white lysozyme, which is another model for exploring the amyloidogenic mechanism.

PARKINSON'S DISEASE

Parkinson's disease is the second most common neurodegenerative disorder. Its diagnosis is based on motor abnormalities, such as resting tremor, bradykinesia, and rigidity (Duvoisin, 1992). Indeed, patients present other non-motor symptoms, such as depression, anxiety, and sleep disorders (Jenner et al., 2013). Only 10% of patients have a genetic basis, with 90% being considered sporadic cases. PD is characterized by the degeneration of the subcortical structure of the brain. Specifically, there are significant losses of dopaminergic neurons in the substantia nigra pars compacta (SNpc; Forno, 1996), although other cell populations are also susceptible to the neurodegeneration process.

α-Synuclein (αS) is a 140 amino acid and a highly abundant neuronal protein. It is found as a soluble cytoplasmatic protein associated with synaptic vesicles (Iwai et al., 1995). It is thought that it plays a role in neurotransmission and cognitive function. Although its physiological function is uncertain, the pathology is associated with the accumulation of αS aggregates, which are the main component of Lewy bodies (LBs; Spillantini et al., 1997). LBs are spherical inclusions formed by αS aggregate (99%) and other proteins.

Despite the main risk factor being aging, other possible risk factors include mutation in the SNCA (alpha-synuclein gene) and exposure to environmental toxins. The latter are also linked to metabolic abnormalities involving neurotransmitter systems (dopamine, serotonin, GABA, and glutamate), fatty acids, such as arachidonic acid-cascade, oxidative stress and mitochondrial function (Henchcliffe and Beal, 2008; Quinones and Kaddurah-Daouk, 2009; Kaidanovich-Beilin et al., 2012; Lei and Powers, 2013).

Furthermore, several studies suggest that αS oligomers and protofibrils are an important factor in neurotoxicity in PD. αS protofibrils cause membrane permeabilization, which alters cellular homeostasis and may activate an apoptotic process (Volles and Lansbury, 2003). Indeed, there is evidence to support the capacity of αS to inhibit proteosomal activity, which would prevent elimination of misfolded proteins (Giasson and Lee, 2003).

Substantial evidence also suggests that a significant factor in dopaminergic neuronal loss in the PD brain are ROS, which result from dopamine metabolism, low glutathione concentration and high levels of iron and calcium in the SNpc (Jenner and Olanow, 2006). Additionally, the brain contains high concentrations of polyunsaturated fatty acids, which, under oxidative stress, result in lipid peroxidation and generation of toxic products (Liu et al., 2008).

No treatment is currently available for the prevention or cure of PD. However, a combination of L-DOPA and antioxidants has been recommended to reduce the rate of progression of the disease, due to the decrease in dopamine levels and significant increase of oxidative stress commonly concomitant to this type of disorder (Zhu et al., 2004).

Concerning the role of melatonin in PD, several works have reported different mechanisms of action. Lin et al. (2007) demonstrated that melatonin attenuates arsenite-induced apoptosis by reducing aggregated αS levels in rat brains, by means of Western blot analysis. Additionally, Ishido (2007) showed that melatonin inhibits αS assembly, using immunostaining in rat pheochromocytoma cells.

It is also important to highlight that melatonin dose-dependently inhibits all steps of the αS assembly process. Ono et al. (2012) observed a reduction in the number of fibrils and the corresponding increase of the number of

short fibrils and amorphous aggregates (25–250 μM) using electron microscopy and thioflavin S experiments. Indeed, melatonin presents a significant destabilization effect (also dose-dependently), suggesting a decrease in beta-sheet levels. In the same study, the authors performed experiments with primary cultures of mesencephalon and neostriatum with MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) a colorimetric assay for assessing cell metabolic activity. The results showed that melatonin reduced the toxic effects of αS after pretreatment (2–6 days) with an increase in cell viability of between 56 and 97%.

In addition to this, it is well known that melatonin exhibits antioxidant properties (Reiter et al., 1997; Kotler et al., 1998). Cellular injury cause by αS-mediated perturbation of cellular redox reactions is an important mechanism proposed for PD (George et al., 2009). Melatonin has been suggested as a potential therapeutic agent in diseases where oxidative stress is thought to be a major pathogenic factor. Mayo et al. (1998) observed that this hormone was an effective free radical scavenger and that it prevented apoptosis in neuronal cells. Moreover, *in vitro* studies on MPTD-induced (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) PD in mice have shown that melatonin protects against excitotoxicity by reducing the autoxidation of dopamine. The administration of melatonin leads to normalization of complex I activity and oxidative status in mitochondria (Escames et al., 2010).

In conclusion, and based on the preceding evidence, we should consider that melatonin presents strong inhibitory effects on protofibril formation and peptide oligomerization.

AUTHOR CONTRIBUTIONS

RH-O and AC Literature search and first draft. AT, MG-P, and AM thorough revision and discussion and final document.

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REFERENCES

Abramov, A. Y., Canevar, L., and Duchen, M. R. (2003). Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. *J. Neurosci.* 23, 5088–5095.

Abramov, A. Y., Canevari, L., and Duchen, M. R. (2004). Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J. Neurosci.* 24, 565–575. doi: 10.1523/JNEUROSCI.4042-03.2004

Abramov, A. Y., and Duchen, M. R. (2005). The role of an astrocytic NADPH oxidase in the nurotoxicity of amyloid beta peptides. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 2309–2314. doi: 10.1098/rstb.2005.1766

Abramov, A. Y., Fraley, C., Diao, C. T., Winkfein, R., Colicos, M. A., Duchen, M. R., et al. (2007). Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18091–18096. doi: 10.1073/pnas.0708959104

Álvarez-García, V., González, A., Alonso-González, C., Martínez-Campa, C., and Cos, S. (2013). Regulation of vascular endothelial growth factor by melatonin in human breast cancer cells. *J. Pineal Res.* 54, 373–380. doi: 10.1111/jpi.12007

Bartolomé, B., Pena-Neira, A., and Gomez-Cordoves, C. (2000). Phenolics and related substances in alcohol-free beers. *Eur. Food Res. Technol.* 210, 419–423. doi: 10.1007/s002170050574

Behl, C., Davis, J. B., Lesley, R., and Schubert, D. (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* 77, 817–827. doi: 10.1016/0092-8674(94)90131-7

Bergers, G., and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer.* 3, 401–410. doi: 10.1038/nrc1093

Bezprozvanny, I., and Mattson, M. P. (2008). Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci.* 31, 454–463. doi: 10.1016/j.tins.2008.06.005

Bieschke, J., Zhang, Q., Powers, E. T., Lerner, R. A., and Kelly, J. W. (2005). Oxidative metabolites accelerate Alzheimer's amyloidogenesis by a two-step mechanism, eliminating the requirement for nucleation. *Biochemistry* 44, 4977–4983. doi: 10.1021/bi0501030

Cebe-Suarez, S., Zehnder-Fjallman, A., and Ballmer-Hofer, K. (2006). The role of VEGF receptors in angiogenesis; complex partnerships. *Cell. Mol. Life Sci.* 63, 601–615. doi: 10.1007/s00018-005-5426-3

Celletti, F. L., Waugh, J. M., Amabile, P. G., Brendolan, A., Hilfiker, P. R., and Dake, M. D. (2001). Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat. Med.* 7, 425–429. doi: 10.1038/86490

Cook, K. M., and Figg, W. D. (2010). Angiogenesis inhibitors: current strategies and future prospects. *CA-Cancer J. Clin.* 60, 222–243. doi: 10.3322/caac.20075

Cui, P., Yu, M., Luo, Z., Dai, M., Han, J., Xiu, R., et al. (2008). Intracellular signaling pathways involved in cell growth inhibition of human umbilical vein endothelial cells by melatonin. *J. Pineal Res.* 44, 107–114. doi: 10.1111/j.1600-079X.2007.00496.x

Cui, P., Yu, M., Peng, X., Dong, L., and Yang, Z. (2012). Melatonin prevents human pancreatic carcinoma cell PANC-1-induced human umbilical vein endothelial cell proliferation and migration by inhibiting vascular endothelial growth factor expression. *J. Pineal Res.* 52, 236–243. doi: 10.1111/j.1600-079X.2011.00933.x

Dai, M., Cui, P., Yu, M., Han, J., Li, H., and Xiu, R. (2008). Melatonin modulates the expression of VEGF and HIF-1α induced by CoCl 2 in cultured cancer cells. *J. Pineal Res.* 44, 121–126. doi: 10.1111/j.1600-079X.2007.00498.x

de Almeida, E. A., Di Mascio, P., Harumi, T., Spence, D. W., Moscovitch, A., Hardeland, R., et al. (2011). Measurement of melatonin in body fluids: standards, protocols and procedures. *Childs Nerv. Syst.* 27, 879–891. doi: 10.1007/s00381-010-1278-8

Dulak, J. (2005). Nutraceuticals as anti-angiogenic agents: hopes and reality. *J. Physiol. Pharmacol.* 1, 51–67.

Duvoisin, R. C. (1992). Overview of Parkinsonś disease Ann. *N. Y. Acad. Sci.* 648, 187–193. doi: 10.1111/j.1749-6632.1992.tb24537.x

Escames, G., López, A., García, J. A., García, L., Acuña-Castroviejo, D., García, J. J., et al. (2010). The role of mitocondria in brain aging and the effects of melatonin. *Curr. Neuropharmacol.* 8, 182–193. doi: 10.2174/157015910792246245

Feng, Z., Chang, Y., Cheng, Y., Zhang, B. L., Qu, Z. W., Qin, C., et al. (2004). Melatonin alleviates behavioral deficits associated with apoptosis and cholinergic system dysfunction in the APP 695 transgenic mouse model of Alzheimerś disease. *J. Pineal Res.* 37, 129–136. doi: 10.1111/j.1600-079X.2004.00144.x

Fernández-Pachón, M. S., Medina, S., Herrero-Martín, G., Cerrillo, I., Berná, G., Escudero-López, B., et al. (2014). Alcoholic fermentation induces melatonin synthesis in orange juice. *J. Pineal Res.* 56, 31–38. doi: 10.1111/jpi.12093

Ferrara, N., and Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. *Nature* 438, 967–974. doi: 10.1038/nature04483

Forno, L. S. (1996). Neuropathology of Parkinsonś disease. *J. Neurophatol. Exp. Neurol.* 55, 259–272. doi: 10.1097/00005072-199603000-00001

Francis, P. T., Palmer, A. M., Snape, M., and Wilcock, G. K. (1999). The cholinergic hypothesis of Alzheimers disease: a review of progress. *J. Neurol. Neurosurg. Psychiatry* 66, 137–147. doi: 10.1136/jnnp.66.2.137

Fraser, P. E., Nguyen, J. T., Surewicz, W. K., and Kirschner, D. A. (1991). pH-dependent structural transitions of Alzheimer's amyloid peptides. *Biophys. J.* 60, 1190–1201.

Gandy, S. (2005). The role of cerebral amyloid β accumulation in common forms of Alzheimer disesase. *J. Clin. Invest.* 115, 1121–1129. doi: 10.1172/JCI2005 25100

George, J. L., Mok, S., Moses, D., Wilkins, S., Bush, A. I., Cherny, R. A., et al. (2009). Targeting the progression of Parkinson's disease. *Curr. Neuropharmacol.* 7, 9–36. doi: 10.2174/157015909787602814

Giasson, B., and Lee, V. M. (2003). Are ubiquitination pathways central to Parkinson's disease? *Cell* 114, 1–8. doi: 10.1016/S0092-8674(03)00509-9

Giles, F. J. (2001). The vascular endothelial growth factor (VEGF) signaling pathway: a therapeutic target in patients with hematologic malignancies. *Oncologist* 6, 32–39. doi: 10.1634/theoncologist.6-suppl_5-32

Gonçalves, N. N., Rodrigues, R. V., Jardim-Perassi, B. V., Moschetta, M. G., Lopes, J. R., Colombo, J., et al. (2014). Molecular markers of angiogenesis and metastasis in lines of oral carcinoma after treatment with melatonin. *Anti Cancer Agents Med. Chem.* 14, 1302–1311. doi: 10.2174/187152061 4666140812110246

Greilberger, J., Fuchs, D., Leblhuber, F., Greilberger, M., Wintersteiger, R., and Tafeit, E. (2010). Carbonyl proteins as a clinical marker in Alzheimers disease and its relation to tryptophan degradation and immune activation. *Clin. Lab.* 56, 441–448.

Hamel, E., Nicolakakis, N., Aboulkassim, T., Ongali, B., and Tong, X. K. (2008). Oxidative stress and cerebrovascular dysfunction in mouse models of Alzheimerś disease. *Exp. Physiol.* 93, 116–120. doi: 10.1113/expphysiol. 2007.038729

Hardy, J., and Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimerśdisease: progress and problems on the road to therapeutics. *Science* 297, 353–356. doi: 10.1126/science.1072994

Harpsøe, N. G., Andersen, L. P., Gögenurm, I., and Rosenberg, J. (2015). Clinical pharmacokinetics of melatonin: a systematic review. *Eur. J. Clin. Pharmacol.* 71, 901–909. doi: 10.1007/s00228-015-1873-4

Henchcliffe, C., and Beal, M. F. (2008). Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. *Nat. Clin. Pract. Neurol.* 4, 600–609. doi: 10.1038/ncpneuro0924

Hoppe, J. B., Frozza, R. L., Horn, A. P., Comiran, R. A., Bernardi, A., Campos, M. M., et al. (2010). Amyloid-beta neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3beta, tau and neuroinflammation. *J. Pineal Res.* 48, 230–238. doi: 10.1111/j.1600-079X. 2010.00747.x

Huang, T. H., Fraser, P. E., and Chakrabartty, A. (1997). Fibrillogenesis of Alzheimer's Abeta peptides studied by fluorescence energy transfer. *J. Mol. Biol.* 269, 214–224. doi: 10.1006/jmbi.1997.1050

Husson, I., Mesples, B., Bac, P., Vamecq, J., Evrard, P., and Gressens, P. (2002). Melatoninergic neuroprotection of the murine periventricular white matter against neonatal excitotoxic challenge. *Ann. Neurol.* 51, 82–92. doi: 10.1002/ana.10072

Ionov, M., Burchell, V., Klajnert, B., Bryszewska, M., and Abramov, A. Y. (2011). Mechanism of neuroprotection of melatonin against beta-amyloid neurotoxicity. *Neuroscience* 180, 229–237. doi: 10.1016/j. neuroscience.2011.02.045

Ishido, M. (2007). Melatonin inhibits maneb-induced aggregation of alpha-synuclein in rat pheochromocytoma cells. *J. Pineal Res.* 42, 125–130. doi: 10.1111/j.1600-079X.2006.00390.x

Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H. A. R., et al. (1995). The precursor protein of non-Aβ component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14, 467–475. doi: 10.1016/0896-6273(95)90302-X

Jardim-Perassi, B. V., Arbab, A. S., Ferreira, L. C., Borin, T. F., Varma, N. R. S., Iskander, A. S. M., et al. (2014). Effect of melatonin on tumor growth and angiogenesis in xenograft model of breast Cancer. *PLoS ONE* 9:e85311. doi: 10.1371/journal.pone.0085311

Jenner, P., Morris, H. R., Robbins, T. W., Goedert, M., Hardy, J., Ben-Shlomo, Y., et al. (2013). Parkinson's disease–the debate on the clinical phenomenology, aetiology, pathology and pathogenesis. *J. Parkinson's Dis.* 3, 1–11. doi: 10.3233/JPD-130175

Jenner, P., and Olanow, W. (2006). The pathogenesis of cell death in Parkinson's disease. *Neurology* 66, S24–S36. doi: 10.1212/WNL.66.10_suppl_4.S24

Jiang, D., and Peterson, D. G. (2013). Identification of bitter compounds in whole wheat bread. *Food Chem.* 141, 1345–1353. doi: 10.1016/j.foodchem.2013. 03.021

Kaidanovich-Beilin, O., Cha, D. S., and McIntyre, R. S. (2012). Crosstalk between metabolic and neuropsychiatric disorders. *F1000 Biol. Repl.* 4:14. doi: 10.3410/B4-14

Kappers, M. H. W., Smedts, F. M. M., Horn, T., van Esch, J. H. M., Sleijfer, S., Leijten, F., et al. (2010). Hypertension induced by the tyrosine kinase inhibitor sunitinib is associated with increased circulating endothelin-1 levels. *Hypertension* 56, 675–681. doi: 10.1161/HYPERTENSIONAHA.109.149690

Khurana, R., Simons, M., Martin, J. F., and Zachary, I. C. (2005). Role of angiogenesis in cardiovascular disease - A critical appraisal. *Circulation* 112, 1813–1824. doi: 10.1161/CIRCULATIONAHA.105.535294

Kirschbaum, J., Meier, A., and Brtickner, H. (1999). Determination of biogenic amines in fermented beverages and vinegars by pre-column derivatization with para-Nitrobenzyloxycarbonyl Chloride (PNZ-CI) and Reversed-Phase LC. *Chromatographia* 49, 117–124. doi: 10.1007/BF02575272

Kocadağli, T., Yilmaz, C., and Gökmen, V. (2014). Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. *Food Chem.* 153, 151–156. doi: 10.1016/j.foodchem.2013.12.036

Kotler, M., Rodríguez, C., Sáinz, R. M., Antolín, I., and Menéndez-Peláez, A. (1998). Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. *J. Pineal Res.* 24, 83–89. doi: 10.1111/j.1600-079X.1998.tb00371.x

Lahiri, D. K. (1999). Melatonin affects the metabolism of the beta-amyloid precursor protein in different cell types. *J. Pineal. Res.* 26, 137–146. doi: 10.1111/j.1600-079X.1999.tb00575.x

Lashuel, H. A., Petre, B. M., Wall, J., Simon, M., Nowak, R. J., Walz, T., et al. (2002). α-synuclein, especially the Parkinson's disease- associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322, 1089–1102. doi: 10.1016/S0022-2836(02)00735

Lei, S., and Powers, R. (2013). NMR metabolomics analysis of Parkinson's Disease. *Curr. Metabolomics.* 1, 191–209. doi: 10.2174/2213235X113019990004

Lin, A. M., Fang, S. F., Chao, P. L., and Yang, C. H. (2007). Melatonin attenuates arsenite-induced apoptosis in rat brain: involvement of mitochondrial and endoplasmic reticulum pathways and aggregation of alpha-synuclein. *J. Pineal Res.* 43, 163–171. doi: 10.1111/j.1600-079X.2007.00456.x

Lissoni, P., Rovelli, F., Malugani, F., Bucovec, R., Conti, A., and Maestroni, G. J. M. (2001). Anti-angiogenic activity of melatonin in advanced cancer patients. *Neurol. Endocrinol. Lett.* 22, 45–47.

Liu, X., Yamada, N., Maruyama, W., and Osawa, T. (2008). Formation of dopamine adducts derived from brain polyunsaturated fatty acids: mechanism for Parkinson disease. *J. Biol. Chem.* 283, 34887–34895. doi: 10.1074/jbc.M805682200

Maldonado, M. D., Moreno, H., and Calvo, J. R. (2009). Melatonin present in beer contributes to increase the levels of melatonin and antioxidant capacity of the human serum. *Clin. Nutr.* 28, 188–191. doi: 10.1016/j.clnu.2009.02.001

Mandrioli, R., Morganti, E., Mercolini, L., Kenndler, E., and Raggi, M. A. (2011). Fast analysis of amino acids in wine by capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis* 32, 2809–2815. doi: 10.1002/elps.201100112

Manfroi, L., Silva, P. H. A., Rizzonc, L. A., Sabainid, P. S., and Glória, M. B. A. (2009). Influence of alcoholic and malolactic starter cultures on bioactive amines in Merlot wines. *Food Chem.* 116, 208–213. doi: 10.1016/j.foodchem.2009.02.034

Mas, A., Guillamon, J. M., Torija, M. J., Beltran, G., Cerezo, A. B. A. M., Troncoso, A. M., et al. (2014). Bioactive compounds derived from the yeast metabolism of aromatic amino acids during alcoholic fermentation. *Biomed. Res. Int.* 7. doi: 10.1155/2014/898045

Masilamoni, J. G., Jesudason, E. P., Dhandayuthapani, S., Ashok, B. S., Vignesh, S., Jebaraj, W. C., et al. (2008). The neuroprotective role of melatonin against

amyloid beta peptide injected mice. *Free Radic. Res.* 42, 661–673. doi: 10.1080/10715760802277388

Matsubara, E., Bryant-Thomas, T., Pacheco Quinto, J., Henry, T. L., Poeggeler, B., Herbert, D., et al. (2003). Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease. *J. Neurochem.* 85, 1101–1108. doi: 10.1046/j.1471-4159.2003.01654.x

Mayo, J. C., Sainz, R. M., Uria, H., Antolin, I., Esteban, M. M., and Rodriguez, C. (1998). Melatonin induced by 6-hydroxydopamine in neuronal cells: implications for Parkinson's disease. *J. Pineal Res.* 24, 179–192. doi: 10.1111/j.1600-079X.1998.tb00531.x

McArthur, A. J., Hunt, A. E., and Gillette, M. U. (1997). Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. *Endocrinology* 138, 627–634. doi: 10.1210/endo.138.2.4925

McNaull, B. B., Todd, S., McGuinness, B., and Passmore, A. P. (2010). Inflammation and anti-inflammatory strategies for Alzheimer's disease—a mini-review. *Gerontology* 56, 3–14. doi: 10.1159/000237873

Mercolini, L., Mandrioli, R., and Raggi, M. A. (2012). Content of melatonin and other antioxidants in grape-related foodstuffs: measurement using a MEPS-HPLC-F method. *J. Pineal Res.* 53, 21–28. doi: 10.1111/j.1600-079X.2011.00967.x

Monagas, M., Gomez-Cordoves, C., and Bartolome, B. (2007). Evaluation of different *Saccharomyces cerevisiae* strains for red winemaking. Influence on the anthocyanin, pyranoanthocyanin and non-anthocyanin phenolic content and colour characteristics of wines. *Food Chem.* 104, 814–823. doi: 10.1016/j.foodchem.2006.12.043

Moreira, P. I., Carvalho, C., Zhu, X., Smith, M. A., and Perry, G. (2010). Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. *Biochim. Biophys. Acta* 1802, 2–10. doi: 10.1016/j.bbadis.2009.10.006

Moreira, P. I., Santos, M., Moreno, A. J. M., and Oliveira, C. (2001). Amyloid beta-peptide promotes permeability transition pore in brain mitochondria. *Biosci. Rep.* 21, 789–800. doi: 10.1023/A:1015536808304

Morshedi, D., Rezaei-Ghaleh, N., Ebrahim-Habibi, A., Ahmadian, S., and Nemat-Gorgani, M. (2007). Inhibition of amyloid fibrillation of lysozyme by indole derivatives - possible mechanism of action. *FEBS J.* 274, 6415–6425. doi: 10.1111/j.1742-4658.2007.06158.x

Moyle, C. W. A., Cerezo, A. B., Winterbone, M. S., Hollands, W. J., Alexeev, Y., Needs, P. W., et al. (2015). Potent inhibition of VEGFR-2 activation by tight binding of green tea epigallocatechin gallate and apple procyanidins to VEGF: relevance to angiogenesis. *Mol. Nutr. Food Res.* 59, 401–412. doi: 10.1002/mnfr.201400478

Ono, K., Mochizuki, H., Ikeda, T., Nihira, T., Takasaki, J., Teplow, D. B., et al. (2012). Effect of melatonin on α-synuclein self-assembly and cytotoxicity. *Neurobiol. Aging* 33, 2172–2185. doi: 10.1016/j.neurobiolaging.2011.10.015

Outeiro, T. F., Putcha, P., Tetzlaff, J. E., Spoelgen, R., Koker, M., Carvalho, F., et al. (2008). Formation of toxic oligomeric α-synuclein species in living cells. *PLoS ONE* 3:e1867. doi: 10.1371/journal.pone.0001867

Pappolla, M., Bozner, P., Soto, C., Shao, H., Robakis, N. K., Zagorski, M., et al. (1998). Inhibition of Alzheimer beta-fibrillogenesis by melatonin. *J. Biol. Chem.* 273, 7185–7188. doi: 10.1074/jbc.273.13.7185

Pappolla, M. A., Sos, M., Omar, R. A., Bick, R. J., Hickson-Bick, D. L., Reiter, R. J., et al. (1997). Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide. *J. Neurosci.* 17, 1683–1690.

Persidsky, Y. G., and Potula, R. (2014). "Alcohol and neurodegeneration," in *Neuroinflammation and Neurodegeneration*, ed. P.K. Peterson and M. Toborek (New York, NY: Springer), 511–526.

Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993). Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* 13, 1676–1687.

Quinones, M. P., and Kaddurah-Daouk, R. (2009). Metabolomics tools for identifying biomarkers for neuropsychiatric diseases. *Neurobiol. Dis.* 35, 165–176. doi: 10.1016/j.nbd.2009.02.019

Reiter, R., Tang, L., Garcia, J. J., and Muñoz-Hoyos, A. (1997). Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci.* 60, 2255–2271. doi: 10.1016/S0024-3205(97)00030-1

Rodriguez-Naranjo, M. I., Gil-Izquierdo, A., Troncoso, A. M., Cantos-Villar, E., and Garcia-Parrilla, M. C. (2011). Melatonin is synthesised by yeast during alcoholic fermentation in wines. *Food Chem.* 126, 1608–1613. doi: 10.1016/j.foodchem.2010.12.038

Rodriguez-Naranjo, M. I., Torija, M. J., Mas, A., Cantos-Villar, E., and Garcia-Parrilla, M. C. (2012). Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions. *J. Pineal Res.* 53, 219–224. doi: 10.1111/j.1600-079X.2012.00990.x

Senger, D. R., Van De Water, L., Brown, L. F., Nagy, J. A., Yeo, K. T., Yeo, T. K., et al. (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metast. Rev.* 12, 303–324. doi: 10.1007/BF00665960

Skribanek, Z., Balaspiri, L., and Mak, M. (2001). Interaction between synthetic amyloid-beta-peptide (1-40) and its aggregation inhibitors studied by electrospray ionization mass spectrometry. *J. Mass Spectrom.* 36, 1226–1229. doi: 10.1002/jms.243

Sohn, E. J., Won, G., Lee, L., Lee, S., and Kim, S. H. (2015). Upregulation of miRNA3195 and miRNA374b mediates the anti-angiogenic properties of melatonin in hypoxic PC-3 prostate cancer cells. *J. Cancer.* 6, 19–28. doi: 10.7150/jca.9591

Song, W., and Lahiri, D. K. (1997). Melatonin alters the metabolism of the beta-amyloid precursor protein in the neuroendocrine cell line PC12. *J. Mol. Neurosci.* 9, 75–92. doi: 10.1007/BF02736852

Spillantini, M. G., Schmidt, M. L., Lee, V. M.-Y., Trojanowski, J. Q., Ross, J., and Michel, G. M. (1997). α-Synuclein in Lewy bodies. *Nature* 388, 839–840. doi: 10.1038/42166

Sprague, G. F. Jr., and Winans, S. C. (2006). Eukaryotes learn how to count: quorum sensing by yeast. *Genes Dev.* 20, 1045–1049. doi: 10.1101/gad.1432906

Stege, P. W., Sombra, L. L., Messina, G., Martinez, L. D., and Silva, M. F. (2010). Determination of melatonin in wine and plant extracts by capillary electrochromatography with immobilized carboxylic multi-walled carbon nanotubes as stationary phase. *Electrophoresis* 31, 2242–2248. doi: 10.1002/elps.200900782

Vitalini, S., Gardana, C., Simonetti, P., Fico, G., and Iriti, M. (2013). Melatonin, melatonin isomers and stilbenes in Italian traditional grape products and their antiradical capacity. *J. Pineal Res.* 54, 322–333. doi: 10.1111/jpi.12028

Volles, M. J., and Lansbury, P. T. Jr. (2003). Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's Disease. *Biochemistry* 42, 7871–7878. doi: 10.1021/bi030086j

Wang, Y. Q., Ye, D. O., Zhu, B. Q., Wu, G. F., and Duan, C. Q. (2014). Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. *Food Chem.* 163, 6–15. doi: 10.1016/j.foodchem.2014.04.064

Wu, S., Chen, J. J., Kudelka, A., Lu, J., and Zhu, X. (2008). Incidence and risk of hypertension with sorafenib in patients with cancer: a systematic review and meta-analysis. *Lancet Oncol.* 9, 117–123. doi: 10.1016/S1470-2045(08)70003-2

Zhou, J. N., Liu, R. Y., Kamphorst, W., Hofman, M. A., and Swaab, D. F. (2003). Early neuropathological Alzheimers changes in aged individuals are accompanied by decreased cerebrospinal fluid melatonin levels. *J. Pineal Res.* 35, 125–130. doi: 10.1034/j.1600-079X.2003.00065.x

Zhu, G., Wang, D., Lin, Y. H., McMahon, T., Koo, E. H., and Messing, R. O. (2001). Protein kinase C epsilon suppresses Abeta production and promotes activation of alphasecretase. *Biochem. Biophys. Res. Commun.* 285, 997–1006. doi: 10.1006/bbrc.2001.5273

Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., and Fink, A. L. (2004). The flavonoid baicalein inhibits fibrillation of alpha-synucleinand disaggregates existing fibrils. *J Biol. Chem.* 279, 26846–26857. doi: 10.1074/jbc.M403129200

Zhu, X., Wu, S., Dahut, W. L., and Parikh, C. R. (2007). Risks of Proteinuria and hypertension with Bevacizumab, an antibody against vascular endothelial growth factor: systematic Review and meta-analysis. *Am. J. Kidney Dis.* 49, 186–193. doi: 10.1053/j.ajkd.2006.11.039

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The broad-spectrum antibiotic, zeamine, kills the nematode worm *Caenorhabditis elegans*

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Soil bacteria can be prolific producers of secondary metabolites and other biologically active compounds of economic and clinical importance. These natural products are often synthesized by large multi-enzyme complexes such as polyketide synthases (PKSs) or non-ribosomal peptide synthases (NRPSs). The plant-associated Gram-negative bacterium, *Serratia plymuthica* A153, produces several secondary metabolites and is capable of killing the nematode worm *Caenorhabditis elegans*; a commonly used model for the study of bacterial virulence. In this study, we show that disruption of the hybrid PKS/NRPS zeamine (*zmn*) gene cluster results in the attenuation of "fast-killing" of *C. elegans*, indicating that zeamine has nematicidal activity. *C. elegans* also exhibits age-dependent susceptibility to zeamine, with younger worms being most sensitive to the bioactive molecule. The *zmn* gene cluster is widely distributed within *Serratia* and phytopathogenic *Dickeya* species and investigation of strains harboring the *zmn* gene cluster showed that several of them are highly virulent in *C. elegans*. Zeamine was described previously as a phytotoxin and broad-spectrum antibacterial compound. In addition to its nematicidal properties, we show here that zeamine can also kill *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The expression of the *zmn* gene cluster and regulation of zeamine production were also investigated. Transcription of the cluster was growth phase-dependent, and was modulated by the post-transcriptional RNA chaperone, Hfq. The results of this study show that zeamine is a highly toxic molecule with little, or no, apparent host specificity in very diverse biological systems. In its current form, zeamine(s) may be useful as a lead compound suitable for chemical modification and structure-activity assays. However, because of widespread non-selective toxicity in multiple bioassays, unmodified zeamine(s) is unlikely to be suitable as a therapeutic antibiotic.

Keywords: *Serratia plymuthica, Dickeya,* secondary metabolite, PKS, NRPS, antibiotic, *Caenorhabditis elegans,* zeamine

INTRODUCTION

Bacteria belonging to the Gram-negative genus *Serratia* are members of the γ-proteobacterial family *Enterobacteriaceae*. The best characterized member of the genus, *Serratia marcescens*, was first described in 1819 as a pigmented microbial isolate (Grimont and Grimont, 1978). *Serratia* species are ecologically diverse although strains of *Serratia plymuthica* are commonly isolated from soil as well as from the rhizosphere of wheat (Åström and Gerhardson, 1988), oilseed rape (Neupane et al., 2012a,b,c), melon (Kamensky et al., 2003), and pea (Gould et al., 2008), the anthosphere of oil pumpkin (Fürnkranz et al., 2012) and from rotting potato tissue (Czajkowski et al., 2012). Many strains of *S. plymuthica* are capable of producing compounds with antibiotic activity, such as the antifungal and anti-oomycete haterumalide, oocydin A (Levenfors et al., 2004), and the antifungal pyrrolnitrin (Liu et al., 2007). Several *S. plymuthica* strains were shown to be effective as biocontrol agents (De Vleesschauwer and Höfte, 2003), for example being capable of controlling the phytopathogenic gray mold *Botrytis cinerea* and white mold, *Sclerotina sclerotiorum,* on greenhouse-grown melon (Kamensky et al., 2003), as well as suppressing the growth of *Penicillium* blue and green mold on citrus fruit (Meziane et al., 2006). The strain used in this study, *S. plymuthica* A153, was isolated from the rhizosphere of wheat (Åström and Gerhardson, 1988), despite producing phytopathogen-antagonistic compounds (Thaning et al., 2001; Levenfors et al., 2004). Recently, the strain has also been shown to be virulent in the *Caenorhabditis elegans* animal infection model (Matilla et al., 2012).

Caenorhabditis elegans is a free-living nematode that reaches an adult length of 1–2 mm. Although first developed as a genetic model in the 1970s (Brenner, 1974), *C. elegans* has undergone a renaissance over the past decade as a model system for assaying and understanding bacterial pathogenesis (Sifri et al., 2005). The importance of this has been two-fold. Firstly, not only are some soil-dwelling nematodes agricultural pests that attack economically important crops (reviewed by Jones et al., 2013) but they are also hard to control with traditional pesticides, and that makes nematode-pathogenic bacteria attractive as potential biocontrol agents (Chitwood, 2002). Secondly, although nematodes and mammals are separated by over 900 million years of evolution (Hedges et al., 2006), there are a surprising number of

bacterial virulence factors affecting both taxa (Rahme et al., 2000). Altogether, *C. elegans* has become a useful model for the identification of novel virulence factors and the characterization of the relationships between pathogens and genetically amenable hosts (Sifri et al., 2005).

Bacteria are capable of antagonizing *C. elegans* by several mechanisms (Sifri et al., 2005). The most common of these is bacterial colonization of the nematode intestine, where bacteria accumulate in the intestinal lumen (causing it to expand) and interfere with its normal function (Sifri et al., 2005). This is the mechanism by which many human pathogens – such as *Salmonella typhimurium* (Aballay et al., 2000), *Pseudomonas aeruginosa* (Mahajan-Miklos et al., 1999), and *S. marcescens* (Kurz et al., 2003) – infect *C. elegans*. This type of 'worm killing,' also referred to as 'slow killing,' takes place over the span of several days and correlates with bacterial proliferation in the intestine (Sifri et al., 2005; Portal-Celhay et al., 2012). On the other hand, some bacteria are capable of killing worms over much shorter time-spans. This 'fast killing' is usually mediated by toxins that are produced by the bacteria (Sifri et al., 2005). Although toxic proteins have been described (Wei et al., 2003), the toxins are typically secondary metabolites. For example, *P. aeruginosa* PA14 is capable of killing *C. elegans* through oxidative stress by using the phenazine compound pyocyanin as a virulence factor (Mahajan-Miklos et al., 1999; Cezairliyan et al., 2013).

Secondary metabolites are traditionally considered to be non-essential organic molecules that are synthesized by cells during the later stages of growth, without playing any direct role in growth or development (Price-Whelan et al., 2006). Although the true roles of secondary metabolites remain elusive, some of them can confer fitness advantages to producing bacteria. Thus, secondary metabolites with antibiotic activity are thought to increase the fitness of bacteria in complex natural environments by antagonizing microbial competitors (Mazzola et al., 1992) or by deterring predation (Pradel et al., 2007). Additionally, since soil is a stressful environment where nutrients are limited (Challis and Hopwood, 2003), it has been speculated that soil bacteria are enriched for the production of secondary metabolites performing primary functions acting, for example, as synergistic siderophores (Price-Whelan et al., 2006). Many secondary metabolites are synthesized by large multidomain proteins such as non-ribosomal peptide synthases (NRPSs) or polyketide synthases (PKSs) (Sattely et al., 2008). Frequently, the genes encoding these enzymes are carried on the biosynthetic gene clusters together with the genes for tailoring enzymes responsible for the modification of the final structure of the molecule (Osbourn, 2010). The modular and mobile nature of biosynthetic gene clusters can enable the mixed assembly of biosynthetic genes from different sources to form hybrid gene clusters containing both NRPS- and PKS-encoding genes.

Preliminary work in this laboratory showed that *S. plymuthica* A153 is a nematode-pathogen capable of killing *C. elegans* rapidly (Matilla et al., 2012), suggesting that it produced a nematicide toxin. In this study we investigated the relationship between A153 and *C. elegans* to characterize the pathogen-worm interaction, with particular emphasis on identifying the A153 nematicide, genes involved in its biosynthesis and the regulation thereof.

MATERIALS AND METHODS

STRAINS, PLASMIDS, PHAGES, CULTURE MEDIA, AND GROWTH CONDITIONS

Bacterial strains, plasmids, and phages used in this study are listed in **Table 1**. *Serratia*, *Dickeya*, and derived strains were grown at 30°C, unless otherwise indicated, in L broth (LB, per liter: 5 g yeast extract, 10 g tryptone, 5 g NaCl), potato dextrose (16 g of potato dextrose broth l^{-1}), minimal medium [MM: 0.1% (w/v) $(NH_4)_2SO_4$, 0.41 mM $MgSO_4$, 15 mM carbon source, 40 mM K_2HPO_4, 14.7 mM KH_2PO_4, pH 6.9–7.1], optimized minimal medium [OMM: 0.2% (w/v) $(NH_4)_2SO_4$, 1.66 mM $MgSO_4$, 0.2% (w/v) mannitol, 0.2% (w/v) glycerol, 60.3 mM K_2HPO_4, 33.1 mM KH_2PO_4, 15.9 μM $MnCl_2$, 90.1 μM $CaCl_2$, 32.9 μM $FeSO_4$], 1-carbon OMM [1C-OMM: 0.2% (w/v) $(NH_4)_2SO_4$, 1.66 mM $MgSO_4$, 15 mM carbon source, 60.3 mM K_2HPO_4, 33.1 mM KH_2PO_4, 15.9 μM $MnCl_2$, 90.1 μM $CaCl_2$, 32.9 μM $FeSO_4$]. *Escherichia coli* strains were grown at 37°C in LB. *E. coli* DH5α was used for gene cloning. Media for propagation of *E. coli* β2163 was supplemented with 300 μM 2,6-diaminopimelic acid. Where appropriate, antibiotics were used at the following final concentrations (in μg mL^{-1}): ampicillin, 100; kanamycin, 25 (*E. coli*), 75 (*Serratia*); streptomycin, 50; tetracycline, 10. Sucrose was added to a final concentration of 10% (w/v) to select for derivatives of a second crossover event during marker exchange mutagenesis. *C. elegans* was maintained at 15°C using standard methods (Brenner, 1974). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were grown at 30°C in yeast peptone dextrose (YPD, per liter: 10 g yeast extract, 20 g peptone, 20 g glucose).

DNA TECHNIQUES, DNA MANIPULATION, AND BIOINFORMATICS ANALYSES

Plasmid DNA was isolated using the Anachem Keyprep plasmid DNA kit. Manufacturer's instructions were followed for DNA digestion (New England Biolabs). The Anachem gel recovery kit was used to recover DNA from agarose gels. Ligation reactions and total DNA extraction were performed using standard protocols (Sambrook et al., 1989). Competent cells were prepared using calcium chloride and transformations were performed using standard protocols (Sambrook et al., 1989). PCR fragments for cloning were amplified using *Phusion*® high fidelity DNA polymerase (New England Biolabs), and all sequences were confirmed. DNA sequencing was performed at the University of Cambridge DNA Sequencing Facility on an Applied Biosystems 3730xl DNA analyzer. Genome comparison analyses were performed using the wgVISTA on-line tool (Frazer et al., 2004). Open reading frames (ORFs) in the zeamine (*zmn*) gene cluster were predicted using Glimmer 3.0 (Delcher et al., 1999). BLAST was used for functional gene assignment. Protein domain organization was identified using the NCBI conserved domains database (Marchler-Bauer et al., 2011).

Table 1 | Strains, phages, and plasmids used in this study.

Strain	Genotype or relevant characteristic[a]	Reference or source
***Serratia plymuthica* A153 strains**		
A153	Wild-type, rhizosphere isolate (Zea$^+$)	Åström and Gerhardson (1988)
A153L[b]	*lac-* derivative of A153	Matilla and Salmond (unpublished results)
A153A	*lac-*,Δ*andR,* made by marker exchange mutagenesis	Matilla and Salmond (unpublished results)
A153C	*lac-*,Δ*csrB*	Matilla and Salmond (unpublished results)
A153H	*lac-*,Δ*hfq,* made by marker exchange mutagenesis	Matilla and Salmond (unpublished results)
A153AH	*lac-*,Δ*andR*Δ*hfq,* made by marker exchange mutagenesis	Matilla and Salmond (unpublished results)
A153P	*lac-*,Δ*pigP*::Km, Km^r	Matilla and Salmond (unpublished results)
A153R	*lac-*,Δ*rpoS*::Km, Km^r	Matilla and Salmond (unpublished results)
A153T6	*lac-*; 1.259 bp deletion of the promoter region of the two predicted transcriptional units of the T6SS of A153; made by marker exchange mutagenesis	Matilla and Salmond (unpublished results)
A153Ce10	*lac-, zmn16*::mini-Tn*5*Sm/Sp, Zea$^-$, Sm^r	This study
A153Ce10A	*lac-*,Δ*andR, zmn16*::mini-Tn*5*Sm/Sp, Zea$^-$, Sm^r; derivative of A153A following transduction using ΦMAM1 grown on strain A153Ce10	This study
A153JH1	*lac-*, Δ*andR, zmn16*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH5	*lac-*, Δ*andR, zmn18/19*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH6	*lac-*, Δ*andR, zmn13*::Tn-KRCPN1*lacZ*, Zea$^-$; Km^r	This study
A153JH6H	*lac-*, Δ*andR*, Δ*hfq, zmn13*::Tn-KRCPN1*lacZ*, Zea$^-$, Km^r; derivative of A153AH following transduction using ΦMAM1 grown on strain A153JH6	This study
A153JH8	*lac-*, Δ*andR, ydhI*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH9[c]	*lac-*, Δ*andR mpg*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH10	*lac-*, Δ*andR, ydhJ*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH11[c]	*lac-*, Δ*andR, far*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH14	*lac-*, Δ*andR ydhI*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH23	*lac-*, Δ*andR ydhI*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH24	*lac-*, Δ*andR zmn10*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH27	*lac-*, Δ*andR zmn9*::Tn-KRCPN1, Zea$^-$; Km^r	This study
A153JH28	*lac-*, Δ*andR zmn10*::Tn-KRCPN1, Zea$^-$; Km^r	This study
***Serratia* strains**		
S. plymuthica AS9	Wild-type	Neupane et al. (2012a)
S. plymuthica AS12	Wild-type	Neupane et al. (2012b)
S. plymuthica AS13	Wild-type	Neupane et al. (2012c)
***Escherichia coli* strains**		
OP50	uracil auxotroph	Brenner (1974)
β2163	F$^-$ RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*), Km^r	Demarre et al. (2005)
***Bacillus subtilis* strains**		
JH642	*pheA1 trpC2*	Perego et al. (1989)
***Dickeya* strains**		
Dickeya sp. MK7	Wild-type	Pritchard et al. (2013b)
Dickeya solani MK10	Wild-type	Pritchard et al. (2013a)
Dickeya solani MK16	Wild-type	Pritchard et al. (2013a)
Dickeya solani IPO 2222	Wild-type	Pritchard et al. (2013a)

(Continued)

Table 1 | Continued

Strain	Genotype or relevant characteristic[a]	Reference or source
Dickeya sp. NCPPB 3274	Wild-type	Pritchard et al. (2013b)
Dickeya sp. CSL RW 240	Wild-type	Pritchard et al. (2013a)
Fungal strains		
Verticillium dahliae 5368	Wild-type, plant pathogen	J. Cooper
Saccharomyces cerevisiae	Wild-type	S. Oliver
Schizosaccharomyces pombe	Wild-type	J. Mata
Caenorhabditis elegans		
DH26	*fer-15*(*b26*)	*Caenorhabditis* genetics center
Bacteriophage		
ΦMAM1	Generalized transducing phage for *S. plymuthica* A153	Matilla and Salmond (2014)
Plasmids		
pKRCPN1	Km^r, Tc^r; Derivative of pDS1028*uidA* with the *uidA* and *cat* genes replaced with ´*lacZ* and *km* genes	Roberts (2010)
pUT-mini-Tn*5*-Sm/Sp	Delivery plasmid for mini-Tn*5*Sm/Sp, Ap^r, Sm^r	de Lorenzo et al. (1990)
pTRB30	pQE-80L (Quiagen)-based expression vector, Ap^r replaced by Km^r, Km^r	T. Blower
pJEEUH13	*hfq* expression vector, Km^r	This study

[a] *Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Zmn, zeamine.* [b] *Strain A153L is considered the A153 wild-type precursor of derivatives used in this study and is referred to as such throughout.* [c] *Transposon insertions in these strains were in previously unlabelled genes, and have been named as follows: A153JH9, mannose-1-phosphate guanyltransferase (mpg); A153JH11, ferric aerobactin receptor (far).*

IN VITRO GROWTH KINETICS

The kinetics of bacterial growth (OD_{600}) were measured with a Unicam Heλios spectrophotometer at 600 nm, using bacterial cultures grown in LB or OMM at 215 r.p.m. at 25°C, unless otherwise indicated. Three replicates were used for each condition and strain.

RANDOM TRANSPOSON MUTAGENESIS, MUTANT SCREENING, AND GENERALIZED TRANSDUCTION

Random transposon mutagenesis of A153 was performed by conjugation with *E. coli* β2163 harboring plasmids containing transposons Tn-KRCPN1 or mini-Tn*5*Sm/Sp, using a previously published protocol (Matilla et al., 2012). Mutant libraries for phenotypic screening were collected on antibiotic-selective plates before screening. Screening for mutants with reduced nematicidal activity was performed as described in Kurz et al. (2003), and screening for mutants with reduced antibacterial activity was performed as described by Zhou et al. (2011). To confirm that mutant phenotypes were associated with single insertions and to ensure association between mutation and phenotype, the mutations were transduced into the parent genetic background using the generalized transducing bacteriophage ΦMAM1, as described in Matilla and Salmond (2014). Random-primed PCR and amplicon sequencing were used to identify transposon insertion points, as previously described Matilla et al. (2012). The genome of *S. plymuthica* A153 has been recently sequenced (Matilla and Salmond, unpublished results).

PHENOTYPIC AND VIRULENCE ASSAYS

Antibacterial and antifungal assays against *Bacillus subtilis* JH642 and *Verticillium dahliae* 5368, respectively, were performed as described in Matilla et al. (2012), at 25°C unless stated otherwise. Assays for fungicidal activity against yeast were performed in the same manner as the antibacterial assays, but using overnight cultures *S. cerevisiae* and *S. pombe* grown in YPD. *C. elegans* virulence assays were performed as previously described Matilla et al. (2012), with the exception that synchronized worms were obtained by egg-lay, as described by Gems et al. (1998), with synchronized populations allowed to develop at 25°C to induce the *fer-15* sterility phenotype. Large-scale phenotypic screens for nematicidal activity were performed using around 50 synchronized L1-stage worms per well in 24-well plates (Kurz et al., 2003), where each well was inoculated with a transposon mutant for testing. Plates were incubated at 25°C and the survival of the worms was scored after 24 and 48 h. A Mantel-Cox log-rank test was used for statistical analysis of worm survival curves and was performed using Prism 5.0 (GraphPad Software). *P* values of 0.05 and below were considered statistically significant.

MEASUREMENT OF β-GALACTOSIDASE ACTIVITY

Transcriptional fusion assays to detect expression of the *lacZ* reporter gene were performed as described previously (Ramsay, 2013), using the fluorogenic substrate 4-methylumbelliferyl β-D-galactoside (MUG2). Gene transcription values were expressed as relative fluorescence units (*r.f.u.*) s^{-1} $OD_{600}{}^{-1}$.

QUANTIFICATION OF ZEAMINE ACTIVITY

Quantification of A153 supernatant bioactivity was performed using cultures of the andrimid-negative mutant, A153A. Bacterial cells were grow at 25°C, for 72 h, in MM supplemented with one of the 15 different carbon sources tested. Cultures were harvested after 72 h and culture supernatants were sterile-filtered (0.2 μm). Supernatant bioactivity (given as zeamine activity, Z_A)

was determined using a *B. subtilis* antibacterial bioassay (Matilla et al., 2012), and given as the fraction of the diameter of the resultant halo (H) and the diameter of the well (W) over the maximum OD_{600} attained by the bacteria in the relevant media.

GENETIC COMPLEMENTATION OF Δhfq

For single-copy complementation of the in-frame *hfq* deletion mutant A153H, an inducible plasmid construct was first generated, using wild-type *hfq*. For this, the A153 *hfq* ORF was amplified using primers hfq-BamHI (5′-TAATTCCGCTAAGGGGCAATCTTTGCA-3′) and hfq-PstI (5′-TAATCTGCAGCTCGCAACGCGCTTTATTC-3′). The PCR product was digested with PstI and BamHI and inserted at the same sites in the vector pTRB30. The resulting plasmid, pJEEUH13, was introduced into A153H by electroporation. Gene expression was induced with 0.1 mM IPTG.

RESULTS

CHARACTERIZATION OF A153 FAST-KILLING OF *Caenorhabditis elegans*

It has been reported previously that *Serratia* spp. such as *S. marcescens* (Kurz and Ewbank, 2000) and *Serratia* sp. ATCC 39006 (Coulthurst et al., 2004) are capable of killing *C. elegans* over the span of 3–5 days by establishing an infection in the nematode intestine. We observed that *S. plymuthica* A153 is capable of killing worms quickly, within hours (**Figure 1**). To better understand this interaction, the A153 killing dynamics of *C. elegans* were subjected to an initial characterization, whereby *C. elegans* L4 larvae were transferred from the standard *C. elegans* food-source *E. coli* OP50 onto lawns of A153. After transfer, the worms succumbed in stages. Initially, they were strongly repulsed by the bacteria and showed strong avoidance of the bacterial lawn. The immediacy of this effect suggests that the bacteria may produce odorants or surfactants that deter nematode grazing (Pradel et al., 2007; Burlinson et al., 2013). Worms that remained on the bacterial lawn quickly become immobilized, within hours of transfer, and remained alive for a latency period of about 6 h, before they started dying. While immobilized, but before death, worms showed little to no spontaneous movement, although the pharynx and body wall muscles could be induced to contract by the gentle touch of an instrument. The majority of worms were dead within 24 h of transfer. The speed of A153 killing of *C. elegans* suggested that A153 produces a fast-acting nematicide that functions as a potent virulence factor in the nematode infection model.

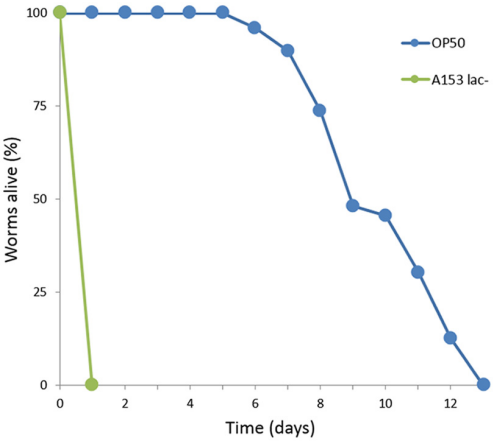

FIGURE 1 | *Serratia plymuthica A153 antagonizes Caenorhabditis elegans.* Survival of *C. elegans* when cultured on *S. plymuthica* A153. The results of a representative trial with at least 50 worms under each condition is shown.

Caenorhabditis elegans SHOW AGE-DEPENDENT SENSITIVITY TO A153 FAST-KILLING

The age of individual *C. elegans* has been shown previously to determine worm susceptibility to bacterial pathogens. Some fast-killing bacterial toxins such as pyocyanin have been shown to be more toxic to younger worms (Mahajan-Miklos et al., 1999). In contrast, under slow-killing infection models, older worms are generally more sensitive (Laws et al., 2004; Portal-Celhay et al., 2012). To determine if there were any age-dependent susceptibility effects to A153 fast-killing of *C. elegans*, worms from each of the four *C. elegans* larval stages (L1–L4) and day-1 and day-2 adults were transferred onto lawns of A153 and nematode survival was assayed hourly. The results revealed that *C. elegans* show significant age-dependent differential susceptibility to the A153 nematicide, with early larvae being more sensitive than late larvae (**Figure 2**; L1 vs. L4: $P << 0.05$), and with larvae being more sensitive than adult worms (**Figure 2**; L4 vs. d1: $P << 0.05$). This shows that A153 fast-killing and *C. elegans* susceptibility to the A153 nematicide is inversely correlated with the developmental stage and age of the worm.

ISOLATION OF A153 MUTANTS WITH REDUCED VIRULENCE AGAINST *C. elegans*

To identify the genes involved in A153 fast-killing of *C. elegans*, a mutant library was generated using random transposon mutagenesis. In an initial screen, the library was screened looking for mutants with reduced nematicidal activity against L1-stage larvae.

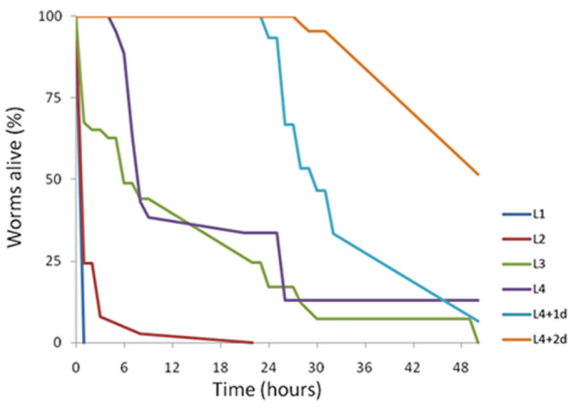

FIGURE 2 | *Caenorhabditis elegans shows age-dependent sensitivity to the A153 nematicide.* Survival of different larval stages and adult *C. elegans* worms when cultured on *S. plymuthica* A153. The results of a representative trial with at least 50 worms under each condition is shown.

This approach yielded one mutant, A153Ce10, which showed significantly reduced 'fast-killing' of worms. This mutant still showed wild-type antibacterial (**Figure 3A**) and antifungal (**Figure 3B**) activities, suggesting that A153Ce10 is specifically deficient in worm-killing. Random-primed PCR confirmed that the transposon in A153Ce10 was in the gene *zmn16*, encoding a putative thioester reductase, and forming part of a hybrid FAS/PKS/NRPS gene cluster responsible for the biosynthesis of the broad-spectrum antibacterial antibiotic, zeamine (zmn), which was first described by Masschelein et al. (2013).

Zeamine was first identified in the plant pathogen *Dickeya zeae* EC1 as a phytotoxic virulence factor with antibacterial properties (Wu et al., 2010; Zhou et al., 2011), and our results suggest that zeamine also has nematicidal activity. In A153, the antibacterial activity of zeamine was masked by the production of another potent antibiotic, andrimid (cf. **Figures 3A,C**; Matilla and Salmond, unpublished results). Using a mutant defective in the production of this antibacterial compound, A153A, a random transposon mutant library was constructed and screened for mutants lacking the small halo associated with the production of zeamine by A153 (**Figure 3C**). Six mutants showing no, or reduced, antibacterial activity (A153JH1, A135JH5, A153JH6, A153JH24, A153JH27, and A153JH28) and with transposon insertions in the *zmn* gene cluster (**Figure 4A**) were isolated. These mutants showed significantly reduced nematicidal activity compared to that of the A153A parent strain ($P << 0.05$; **Figure 5A**, Figure S1), confirming that zeamine is indeed the A153 nematicide and responsible of the observed A153 fast-killing of *C. elegans*.

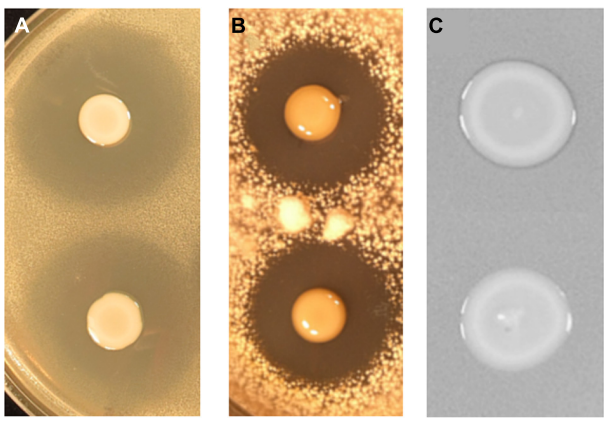

FIGURE 3 | *Serratia plymuthica A153 mutants that show reduced virulence in C. elegans are specifically attenuated for zeamine production.* Plate-based bioassays showing andrimid **(A)** oocydin A **(B)** and zeamine **(C)** production. **(A,B)** The upper culture spot shows the A153 wild-type whereas the lower culture spot shows the derived mutant A153Ce10. **(C)** The upper culture spot shows the A153 Δ*andR* mutant A153A whereas the lower culture spot shows the derived mutant A153Ce10A, which is representative of all mutants characterized in this study. In all cases, plates were incubated at 25°C either overnight **(A,C)** or for 5 days **(B)**.

The A153 *zmn* gene cluster spans over 54 kb and contains 22 genes (*zmn1-22*), organized into three putative operons (**Figure 4A**; Table S1). As previously described in *S. plymuthica* RVH1, the A153 *zmn* gene cluster contains the genes for three multidomain PKSs (*zmn10, zmn11, zmn18*) and two multidomain NRPSs (*zmn16, zmn17*). In addition to these biosynthetic genes, the cluster also contains genes encoding modifying enzymes (*zmn3, zmn12, zmn14, zmn15, zmn22*) and transport-related proteins (*zmn7, zmn8, zmn9, zmn20, zmn21*) – the latter with a proposed role in conferring innate resistance to the zeamine antibiotic (Masschelein et al., 2013). A putative integrase-encoding gene marks the downstream end of the cluster, suggesting that it could have been acquired by horizontal gene transfer.

THE *zmn* GENE CLUSTER IS WIDELY DISPERSED WITHIN *Serratia* AND *Dickeya* GENERA

Genome comparison analyses revealed that the *zmn* gene cluster is present in *S. plymuthica* strains AS9, AS12, AS13, A30, S13, and V4 (Table S2). Additionally, we also identified the biosynthetic gene cluster in several phytopathogenic strains belonging to the *Dickeya* genus, including *D. solani* MK10, MK16, IPO222; *D. zeae* DZ2Q and ZJU1202; and *Dickeya* spp. MK7 and NCPPB 3274 (Table S2). The *Serratia* and *Dickeya zmn* clusters span between 50.64- and 54.02-Kbp and are between 59.7 and 96.4%

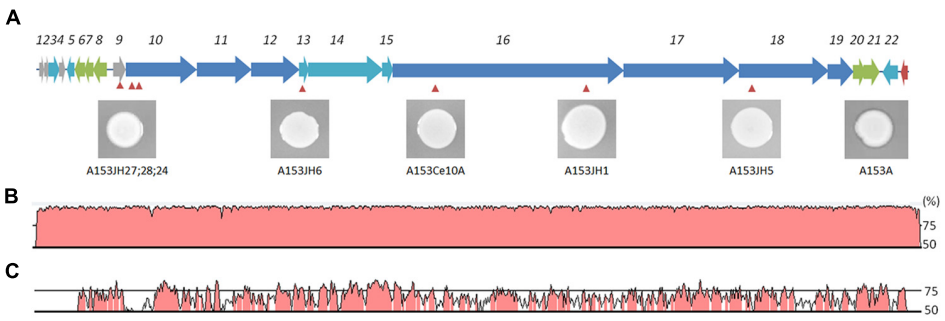

FIGURE 4 | The zeamine gene cluster is present in *Serratia* and *Dickeya* strains. Genetic organization of the *zmn* gene cluster sequence in *S. plymuthica* A153 **(A)**. Location of the Tn-KRCPN1 transposon insertions are indicated by red arrowheads. Inserts show mutant antibacterial phenotypes against *B. subtilis* after an overnight incubation at 25°C. Note the panel depicting the phenotype of the A153 Δ*andR* parent strain to the far right. DNA homology (%) between the *zmn* gene cluster of A153 and those of *S. plymuthica* RVH1 **(B)** and *D. solani* MK10 **(C)** is presented. Sequence comparisons were performed using wgVISTA and show regions of >50% homology.

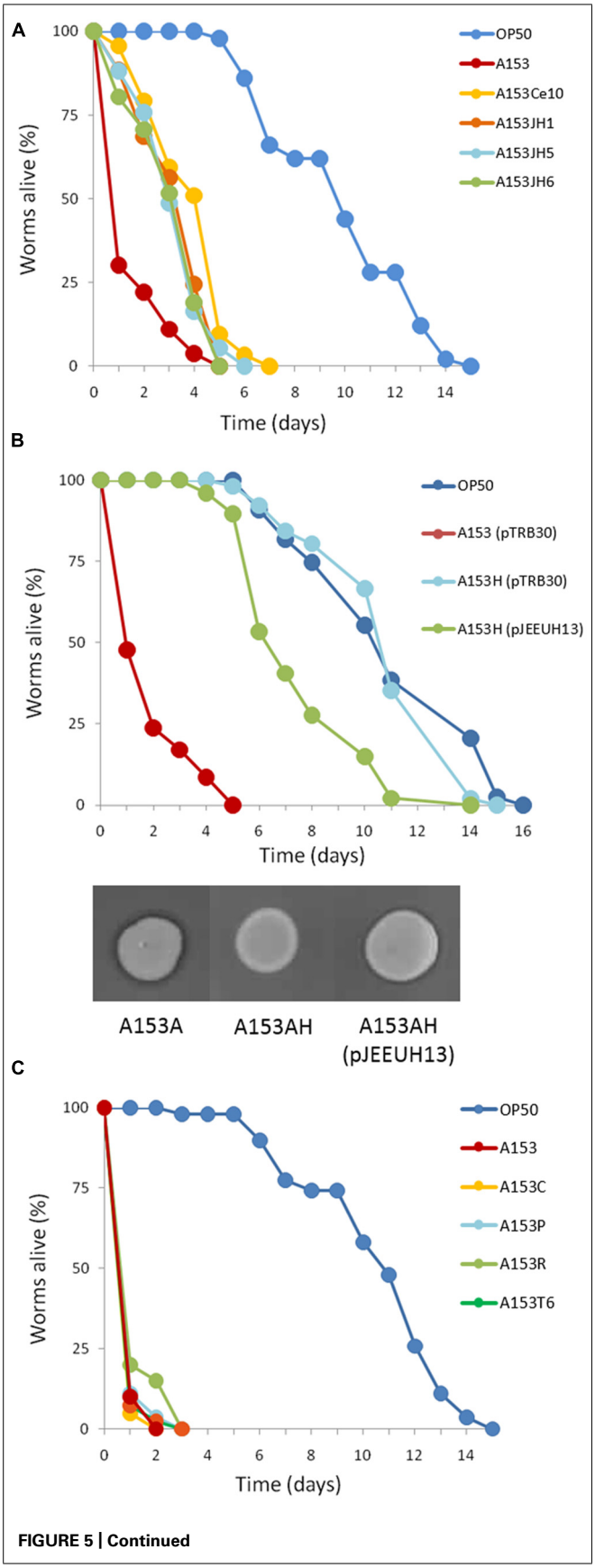

FIGURE 5 | Continued

FIGURE 5 | Continued
Kinetics of the nematicidal properties of *Serratia plymuthica* A153 strains. (A) Mutation of the *zmn* gene cluster attenuates *S. plymuthica* A153 fast killing of *C. elegans*. Compared with the A153A parent strain, mutants A153JH1, A153JH5, A153JH6 and A153Ce10A, show reduced virulence in *C. elegans* ($P < < 0.05$). **(B)** Mutation of the chaperone Hfq eliminates A153 virulence in *C. elegans*. The virulence could be partially restored by the *in trans* expression of *hfq* (using plasmid pJEEUH13). Gene expression was induced using 0.1 mM IPTG. Inserts show antibacterial phenotypes of the parent and complemented strains after an overnight incubation at 25°C. **(C)** Mutation of the regulators *rpoS*, *pigP,* and *csrB* had no effect on A153 fast–killing of *C. elegans,* and neither did deletion of the A153 T6SS. For each experiment, worms were transferred at the L4 stage and the results of one representative trial with at least 50 worms under each condition is shown.

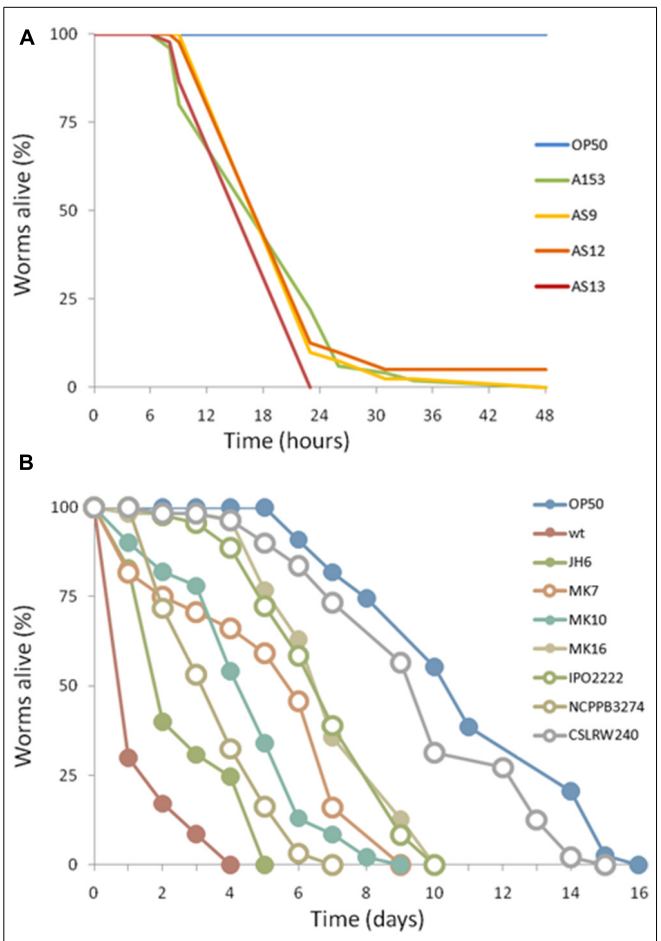

FIGURE 6 | Virulence of enterobacterial strains harboring the *zmn* gene cluster. The pigmented *S. plymuthica* strains AS9, AS12 and AS13 show fast killing of *C. elegans* **(A)**. In contrast, *D. solani* strains MK10, MK16, IPO2222 and *Dickeya* spp. MK7 and NCPPB3274 showed no fast killing of *C. elegans* **(B)**. For each experiment, worms were transferred at the L4 stage and the results of a representative trial with at least 50 worms under each condition is shown.

identical at the DNA level with the A153 *zmn* gene cluster (Table S3).

In silico analyses showed that the A153 and RVH1 *zmn* gene clusters have the same gene and domain organization (**Figure 4B**).

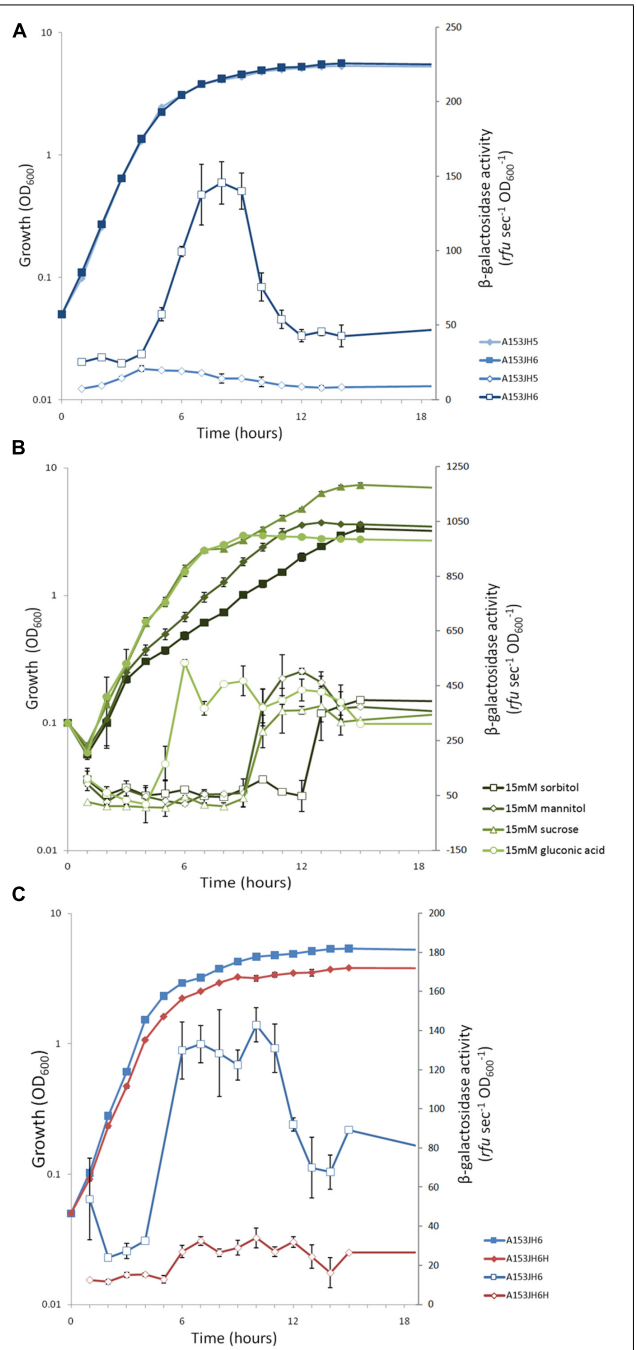

FIGURE 7 | Continued

FIGURE 7 | Continued
The expression of the *S. plymuthica* A153 *zmn* gene cluster is growth phase-dependent and post-transcriptionally regulated by the RNA chaperone Hfq. (A) Transcription of the *zmn* gene cluster throughout growth in *Serratia plymuthica* A153. The dip in β−galactosidase activity post−peak suggests the enzyme is subject to proteolytic turnover, which has been observed previously in A153 (Matilla and Salmond, unpublished results). **(B)** Different carbon sources have different effects on the correlation between *zmn* gene transcription and the bioactivity of A153A−derived supernatants. β−galactosidase activity was measured in strain A153JH6 grown in 1C−OMM supplemented with 15 mM sorbitol, mannitol, sucrose, or gluconic acid. **(C)** Expression of the *zmn* gene cluster is regulated by the RNA chaperone Hfq. β−galactosidase activity was measured in strain A153JH6 in the presence (red) or absence (blue) of a chromosomal *hfq* gene deletion. **(A,B)** The strains were grown in LB at 25°C and β-galactosidase activities were measured in strains expressing chromosomal *zmn13::lacZ* fusions. In all panels solid symbols represent growth of the corresponding strains whereas open symbols represent β−galactosidase activity. Data shown are the average values ±SD of at least three experiments.

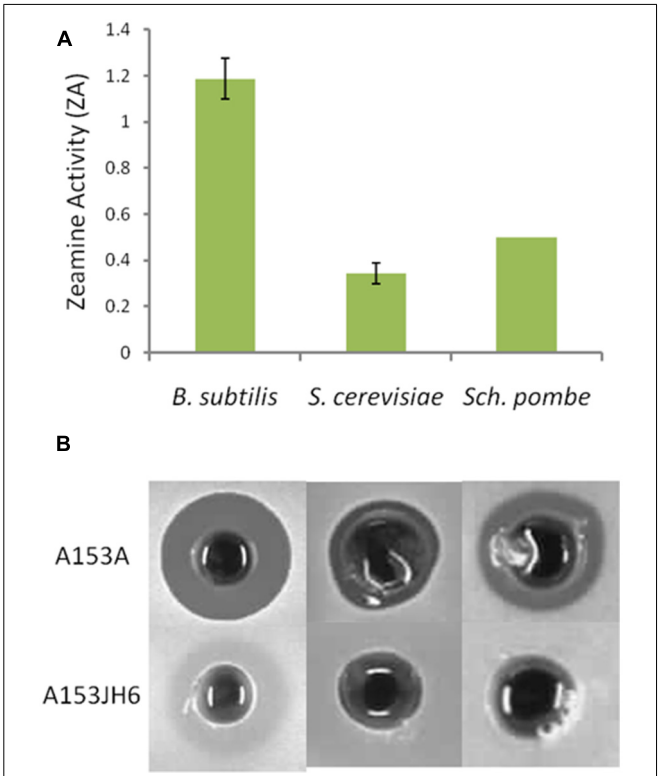

FIGURE 8 | Zeamine is toxic to the ascomycete yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. (A) Bioactivity of cell-free supernatants of A153A show bioactivity against *Bacillus subtilis* and the ascomycete yeasts *S. cerevisiae* and *S. pombe*. A153 strains were grown in OMM at 25°C for 48 h and the bioactivities were quantified from bioassay plates incubated overnight at 30°C. Data shown are average values ±SD from at least three experiments. **(B)** Supernatants from stationary phase cultures of A153JH6 grown under the same conditions produce no antibiotic halos on bioassay plates, confirming that the halos are produced by zeamine.

However, we found that the A153 PKS Zmn10 contains a previously unidentified dehydratase (DH) domain. Based on the high sequence identity (around 95%) between *zmn* gene clusters in A153 and related *S. plymuthica* strains, we hypothesized that all these strains could possess high nematicidal activities. Thus, using L4-stage larvae, we showed that *S. plymuthica* strains AS9, AS12, and AS13 are highly virulent and capable of killing *C. elegans* at similar levels to those of A153, with 50% of worms dying within 18 h of transfer (**Figure 6A**).

Importantly, pairwise comparisons *in silico* indicate that genes *zmn1-4* are not present in strains of *Dickeya* and that the *zmn5* homolog constitutes the first gene of these *zmn* gene clusters

(**Figure 4C**). In addition, whereas the *zmn* gene clusters in different strains of *Dickeya* show around 60% sequence identity with the A153 cluster, the putative permease Zmn9 is only about 50% conserved between the genera. The NCBI conserved domains database predicts that the *Serratia* Zmn9 contains a zinc-dependent phospholipase domain, whereas Zmn9 in *Dickeya* is predicted to contain a CDP-alcohol phosphatidyltransferase domain. To investigate if strains of *Dickeya* show similar nematicidal differences to *zmn*-carrying strains of *Serratia* despite these differences, a subset of *Dickeya* strains that carry the *zmn* gene cluster (MK7, MK10, MK16, IPO2222, and NCPPB 3274) were chosen for *C. elegans* virulence assays. Interestingly, although the tested *Dickeya* strains had variable pathogenic capacity against L4-stage *C. elegans* (**Figure 6B**), none of them was found to show the fast-killing phenotype observed in strains of *S. plymuthica* that carry the *zmn* gene cluster.

THE *zmn* GENE CLUSTER IS TRANSCRIBED IN A GROWTH PHASE-DEPENDENT MANNER

To investigate the transcription of the *zmn* gene cluster, β-galactosidase activity was measured in a chromosomal *zmn13*::*lacZ* fusion strain (strain A153JH6). Transcription of *zmn* biosynthetic genes started in mid-logarithmic phase of growth and reached an apparent maximum in early stationary phase of growth (**Figure 7A**). The sharp decrease in β-galactosidase levels beyond this point may be explained by proteolytic turnover of β-galactosidase (Matilla and Salmond, unpublished results).

THE PRODUCTION OF ZEAMINE IS CARBON SOURCE-DEPENDENT

Zeamine production has been shown previously to differ between growth media, being higher in *D. zeae* EC1 when grown in an OMM, compared to the standard LB culture medium (Zhou et al., 2011). Thus, we investigated zeamine production in strains grown in different carbon sources by determining the bioactivity of cell-free supernatants against *B. subtilis,* which is sensitive to zeamine (**Figure 3C**). Our results showed that the production of the bioactive molecule is carbon source-dependent, with some carbon sources favoring high levels of zeamine biosynthesis (e.g., sorbitol and mannitol) whereas others do not support the production of zeamine at all (e.g., gluconic acid; Table S4). Expression of the *zmn* gene cluster, reported using β-galactosidase assays, was examined in a modified OMM with different carbon sources (1C-OMM). Unexpectedly, no correlation between *zmn* gene transcription and zeamine production was observed (**Figure 7B**; Table S4).

THE RNA CHAPERONE Hfq REGULATES THE PRODUCTION OF ZEAMINE AND THE EXPRESSION OF *zmn* BIOSYNTHETIC GENES

The RNA chaperone Hfq acts as a regulator of gene expression by interacting with small regulatory RNAs to stabilize the interaction between these and their target mRNAs through the formation of regulatory RNA-RNA complexes (Vogel and Luisi, 2011). Mutants defective in *hfq* are highly pleiotropic and can be attenuated in both virulence and the production of secondary metabolites in *Serratia* sp. ATCC39006 (Wilf et al., 2012). To investigate whether Hfq is involved in regulating the production of zeamine in *S. plymuthica* A153, *C. elegans* virulence assays were performed. The results showed that deletion of *hfq* in strain A153H strongly attenuated virulence to *C. elegans* L4 larvae ($P << 0.05$; **Figure 5B**). The virulence of A153H could be partially restored by expressing *hfq in trans* (**Figure 5B**). β-galactosidase assays showed that deletion of *hfq* abolished the transcription of the *zmn* gene cluster (**Figure 7C**), confirming that Hfq positively regulates the production of zeamine. It is well known that Hfq regulates the translation of the stationary-phase sigma factor RpoS (Vogel and Luisi, 2011). To investigate whether Hfq regulation is dependent on RpoS, we phenotypically characterized an *rpoS* mutant in A153. However, this mutant showed the same antibacterial and nematicidal activities as the wild-type strain (**Figure 5C**). Mutants defective in the non-coding small RNA *csrB* (Babitzke and Romeo, 2007) and the transcriptional regulator PigP (Fineran et al., 2005) were also unaffected in their virulence against *C. elegans* (**Figure 5C**).

ZEAMINE IS TOXIC TO ASCOMYCETE YEASTS

Zeamine shows some structural similarity to another family of hybrid polyamino-polyketides: fabclavines. These natural products have been shown to have broad-spectrum antibiotic activity against a diverse set of organisms including bacteria and ascomycete fungi (Fuchs et al., 2014). Using cell-free supernatants of A153A and A153JH6, we showed that zeamine is bioactive against the ascomycete yeasts *S. cerevisiae* and *S. pombe* (**Figure 8**).

THE TYPE VI SECRETION SYSTEM OF *S. plymuthica* A153 IS NOT INVOLVED IN VIRULENCE

The bacterial Type VI secretion system (T6SS) is the most recently described secretion system in Gram-negative bacteria and has been found to promote bacterial virulence against both prokaryotic competitors and eukaryote hosts (Coulthurst, 2013). *In silico* analyses revealed that a T6SS gene cluster highly homologous to that present in *Citrobacter rodentium* ICC168 is present in the genome of A153 (Matilla and Salmond, unpublished results). The role of the T6SS in virulence against nematodes remains largely uncharacterized but Sana et al. (2012) showed that it plays a role in *P. aeruginosa* "slow killing" of *C. elegans*. To investigate if the *S. plymuthica* A153 T6SS is involved in virulence, an A153 T6SS mutant was constructed (A153T6) and characterized. However, no difference in the virulence was observed between A153T6 and the A153 wild-type (**Figure 5C**).

DISCUSSION

Although zeamine was first described as a phytotoxin with broad-spectrum antibacterial properties (Wu et al., 2010; Zhou et al., 2011), this study showed that zeamine is also a potent nematicide. Furthermore, it is a characteristic of some bacterial toxins that younger hosts are more susceptible than older ones (Mahajan-Miklos et al., 1999), and consistent with this, *C. elegans* showed age-dependent sensitivity to the A153 nematicide. In addition, we have shown that zeamine is capable of killing *S. cerevisiae* and *S. pombe*, and therefore zeamine, by definition, is also a fungicide. This leads us to conclude that zeamine is a very broad-spectrum antibiotic that is capable of antagonizing a phylogenetically diverse set of organisms, making it unsuitable, in an unmodified form, for application as a therapeutic antibiotic.

Like the fabclavines (Fuchs et al., 2014), the zeamine molecule has a polyaminated fatty acid backbone that is derived from modified secondary lipid metabolism (Wu et al., 2010; Masschelein et al., 2013). Zeamine also shows some structural similarity with compounds such as the phytotoxin syringomycin, which has been shown to antagonize plant cells by forming ion channels in the plant cell membrane (Hutchison and Gross, 1997). Altogether, it is possible that zeamine has lipophilic properties and might interact with the lipids of cell membranes through a mechanism analogous to that of cationic antimicrobial peptides (Hancock, 2001). A membranal target would be entirely consistent with the observed strong susceptibility of very diverse organisms to zeamine.

Fatty acids have been shown previously to function as nematicides against various phytopathogenic nematodes, and have been hypothesized to disrupt plasma membranes to facilitate solubilization of the nematode cuticle or hypodermis (Anke et al., 1995; Davis et al., 1997). Similarly, cationic peptides are capable of interacting with and disrupting cell membranes owing to a three-dimensional amphiphilic structure (Joondan et al., 2014), and various classes thereof have been shown effective against fungi, protozoa and mammalian cells (Hancock, 2001). Intriguingly, cationic peptides share many of these properties with zeamine, and have been shown previously to function as nematicides (Colgrave et al., 2008), and are capable of causing severe damage to the intestine of lepidopteran larvae (Barbeta et al., 2008). It is therefore possible that the nematicidal cytotoxicity of zeamine involves damage to, and vacuolisation of, the cells that line the *C. elegans* intestine.

Our results raise questions about the role of zeamine in nature. The majority of sequenced *S. plymuthica* strains that carry the *zmn* gene cluster were isolated from agricultural contexts, such as the plant antho- and rhizosphere (e.g., Åström and Gerhardson, 1988; Fürnkranz et al., 2012; Neupane et al., 2012a,b,c). Plant root exudates are rich in sugars and other organic nutrients that favor root colonization by soil-borne bacteria (Bais et al., 2006), and this study has shown that different sugars have different effects on zeamine production by A153 – with some repressing the production of the antibiotic whilst others favor it. In this regard, it is notable that A153 was initially isolated on the basis that it antagonized plant growth (Åström and Gerhardson, 1988), and that zeamine has been found to be a potent phytotoxin, capable of antagonizing both shoot and root development in rice seedlings (Zhou et al., 2011). Together with our results showing that zeamine is also a potent nematicide, these observations raise the question: are there any possible large-scale effects of zeamine production by rhizosphere-associated soil bacteria?

Various *Dickeya* spp. are pathogens of plants, and as some of these have been found to contain the *zmn* gene cluster, their nematicidal properties were investigated. However, contrary to expectation, none of the selected strains were found to show fast-killing of *C. elegans*. The absence of genes *zmn1-4* in strains of *Dickeya* suggests that the cryptic *zmn* gene clusters of the assayed *Dickeya* isolates may not be effectively or functionally expressed, correlating with a lack of rapid nematicidal activity. Alternatively, and considering the low sequence conservation between *Serratia* and *Dickeya zmn9*, it is possible that the final biosynthetic products of the *zmn* gene clusters of the *Dickeya* strains analyzed in this study do not possess the same biological properties as zeamine. For example, Masschelein et al. (2013) showed that *S. plymuthica* RVH1 is capable of producing three different zeamine molecules (zeamine, zeamine I, and zeamine II), of which zeamine I is the predominant molecule produced by *D. zeae* EC1 (Wu et al., 2010). The individual contributions of these molecules to the antibiotic activity of zeamine, *sensu lato*, is unclear, although further work on this topic might reveal differential toxic activities between different zeamines and derived molecules. If different zeamine species are found to show differential specific toxicities, this would raise the possibility that these may be used as more-specific antibiotics or pesticides. It is also possible that the *zmn* gene clusters of the *Dickeya* isolates tested in this study are simply cryptic under the conditions tested – as is known for gene clusters encoding other secondary metabolites (Osbourn, 2010). It is, however, clear that various *Dickeya* spp. are virulent in the *C. elegans* model, consistent with previous work that found that the plant pathogens *D. dadantii* 3937c, *Agrobacterium tumefaciens* CFBP2413, and *Pectobacterium carotovorum* CFBP 2141, are capable of killing *C. elegans* through infection (Couillault and Ewbank, 2002).

The expression pattern of the A153 *zmn* gene cluster is characteristic for secondary metabolite antibiotics, which are typically produced during conditions of nutrient limitation and reduced growth (Bibb, 2005). As is common with secondary metabolites, transcription of *zmn* biosynthetic genes is sensitive to environmental conditions. In contrast to findings in other bacteria such as *S. plymuthica* RVH1 (Masschelein et al., 2013), *zmn* gene transcription in A153 does not appear to be thermoregulated (Figure S2). Zeamine production does, however, show media-dependent effects in A153, as has previously been reported for *D. zeae* EC1 (Zhou et al., 2011). The basis of this effect appears to be post-transcriptional, as we did not observe a correlation between *zmn* gene transcription and zeamine bioactivity between different carbon sources (cf. **Figure 7B**; Table S4).

We observed a correlation between *zmn* gene transcription and the activity of the RNA chaperone Hfq – with the A153 *zmn* gene cluster being silent in a Δ*hfq* background – consistent with its role as a regulator of secondary metabolism in other species of *Serratia*. Mutation of *hfq* has been shown previously to reduce the transcription of genes involved in iron uptake in *E. coli* (Večerek et al., 2003), and intriguingly, as part of our mutagenesis program, we isolated a mutant (A153JH11) with a transposon insertion in the A153 homolog of the ferric aerobactin receptor (Figure S3A), which showed increased production of zeamine (Figure S3B), suggesting that production of the antibiotic is increased during conditions that might mimic iron limitation.

In summary, the work presented in this study has shown that *S. plymuthica* A153 produces the very broad-spectrum antibiotic, zeamine. Although zeamine was initially described as a phytotoxin with antibacterial activity, our results show that zeamine is also a potent nematicidal compound and antifungal. That zeamine antagonizes such a phylogenetically diverse set of organisms suggests that it targets a highly conserved cellular process, which

would make it unsuitable as a specific antibiotic. That target is likely to be the cell membranes of diverse hosts. However, the research presented here may help toward the development of zeamine analogs with enhanced host specificity in nematodes and fungi.

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REFERENCES

Aballay, A., Yorgey, P., and Ausubel, F. M. (2000). *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr. Biol.* 10, 1539–1542. doi: 10.1016/S0960-9822(00)00830-7

Anke, H., Stadler, M., Mayer, A., and Sterner, O. (1995). Secondary metabolites with nematicidal and antimicrobial activity from nematophagous fungi and Ascomycetes. *Can. J. Bot.* 73, S932–S939. doi: 10.1139/b95-341

Åström, B., and Gerhardson, B. (1988). Differential reactions of wheat and pea genotypes to root inoculation with growth-affecting rhizosphere bacteria. *Plant Soil* 109, 263–269. doi: 10.1007/BF02202093

Babitzke, P., and Romeo, T. (2007). CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr. Opin. Microbiol.* 10, 156–163. doi: 10.1016/j.mib.2007.03.007

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Barbeta, B. L., Marshall, A. T., Gillon, A. D., Craik, D. J., and Anderson, M. A. (2008). Plant cyclotides disrupt epithelial cells in the midgut of lepidopteran larvae. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1221–1225. doi: 10.1073/pnas.0710338104

Bibb, M. I. (2005). Regulation of secondary metabolism in *Streptomyces*. *Curr. Opin. Microbiol.* 8, 208–215. doi: 10.1016/j.mib.2005.02.016

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.

Burlinson, P., Studholme, D., Cambray-Young, J., Heavens, D., Rathjen, J., Hodgkin, J., et al. (2013). *Pseudomonas fluorescens* NZI7 repels grazing by *C. elegans*, a natural predator. *ISME J.* 7, 1126–1138. doi: 10.1038/ismej.2013.9

Cezairliyan, B., Vinayavekhin, N., Grenfell-Lee, D., Yuen, G. J., Saghatelian, A., and Ausubel, F. M. (2013). Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. *PLOS Pathog.* 9:e1003101. doi: 10.1371/journal.ppat.1003101

Challis, G. L., and Hopwood, D. A. (2003). Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci. U.S.A.* 100, 14555–14561. doi: 10.1073/pnas.1934677100

Chitwood, D. J. (2002). Phytochemical based strategies for nematode control. *Annu. Rev. Phytopathol.* 40, 221–249. doi: 10.1146/annurev.phyto.40.032602.130045

Colgrave, M. L., Kotze, A. C., Huang, Y. H., O'Grady, J., Simonsen, S. M., and Craik, D. J. (2008). Cyclotides: natural, circular plant peptides that possess significant activity against gastrointestinal nematode parasites of sheep. *Biochemistry* 47, 5581–5589. doi: 10.1021/bi800223y

Couillault, C., and Ewbank, J. J. (2002). Diverse bacteria are pathogens of *Caenorhabditis elegans*. *Infect. Immun.* 70, 4705–4707. doi: 10.1128/IAI.70.8.4705-4707.2002

Coulthurst, S. J. (2013). The Type VI secretion system–a widespread and versatile cell targeting system. *Res. Microbiol.* 164, 640–654. doi: 10.1016/j.resmic.2013.03.017

Coulthurst, S. J., Kurz, C. L., and Salmond, G. P. C. (2004). luxS mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapanem and prodigiosin. *Microbiology* 150, 1901–1910. doi: 10.1099/mic.0.26946-0

Czajkowski, R., De Boer, W. J., Van Veen, J. A., and Van der Wolf, J. M. (2012). Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. biovar 3 in vitro and in planta. *Plant Pathol.* 61, 169–182. doi: 10.1111/j.1365-3059.2011.02486.x

Davis, E. L., Meyers, D. M., Dullum, C. J., and Feitelson, J. S. (1997). Nematicidal activity of fatty acid esters on soybean cyst and root-knot nematodes. *J. Nematol.* 29, 677–684.

Delcher, A. L., Harmon, D., Kasif, S., White, O., and Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. *Nucleic Acids Res.* 27, 4636–4641. doi: 10.1093/nar/27.23.4636

de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram negative eubacteria. *J. Bacteriol.* 172, 6568–6572.

Demarre, G., Guerout, A. M., Matsumoto-Mashimo, C., Rowe-Magnus, D. A., Marliere, P., and Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPα) conjugative machineries and their conjugant *Escherichia coli* host strains. *Res. Microbiol.* 156, 245–255. doi: 10.1016/j.resmic.2004.09.007

De Vleesschauwer, D., and Höfte, M. (2003). Using *Serratia plymuthica* to control fungal pathogens of plants. *CAB Rev.* 2:046. doi: 10.1079/PAVSNNR20072046

Fineran, P. C., Slater, H., Everson, L., Hughes, K., and Salmond, G. P. (2005). Biosynthesis of tripyrrole and β-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. *Mol. Microbiol.* 56, 1495–1517. doi: 10.1111/j.1365-2958.2005.04660.x

Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M., and Dubchak, I. (2004). VISTA. Computational tools for comparative genomics. *Nucleic Acids Res.* 32, W273–W279. doi: 10.1093/nar/gkh458

Fuchs, S. W., Grundmann, F., Kurz, M., Kaiser, M., and Bode, H. B. (2014). Fabclavines: bioactive peptide–polyketide-polyamino hybrids from *Xenorhabdus*. *ChemBioChem* 15, 512–516. doi: 10.1002/cbic.201300802

Fürnkranz, M., Lukesch, B., Müller, H., Huss, H., Grube, M., and Berg, G. (2012). Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens. *Microb. Ecol.* 63, 418–428. doi: 10.1007/s00248-011-9942-4

Gems, D., Sutton, A. J., Sundermeyer, M. L., Albert, P. S., King, K. V., Edgley, M. L., et al. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. *Genetics* 150, 129–155.

Gould, M., Nelson, L. M., Waterer, D., and Hynes, R. K. (2008). Biocontrol of *Fusarium sambucinum*, dry rot of potato, by *Serratia plymuthica* 5-6. *Biocontrol Sci. Technol.* 18, 1005–1016. doi: 10.1080/09583150802478189

Grimont, P. A., and Grimont, F. (1978). The genus *Serratia*. *Annu. Rev. Microbiol.* 32, 221–248. doi: 10.1146/annurev.mi.32.100178.001253

Hancock, R. E. W. (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* 1, 156–164. doi: 10.1016/S1473-3099(01)00092-5

Hedges, S. B., Dudley, J., and Kumar, S. (2006). TimeTree: a public knowledge-base of divergence times among organisms. *Bioinformatics* 22, 2971–2972. doi: 10.1093/bioinformatics/btl505

Hutchison, M. L., and Gross, D. C. (1997). Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. syringae: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Mol. Plant Microbe Interact.* 10, 347–354. doi: 10.1094/MPMI.1997.10.3.347

Jones, J. T., Haegeman, A., Danchin, E. G., Gaur, H. S., Helder, J., Jones, M. G., et al. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol.* 14, 946–961. doi: 10.1111/mpp.12057

Joondan, N., Jhaumeer-Laulloo, S., and Caumul, P. (2014). A study of the antibacterial activity of L-Phenylalanine and L-Tyrosine esters in relation to their CMCs and their interactions with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC as model membrane. *Microbiol. Res.* 169, 675–685. doi: 10.1016/j.micres.2014.02.010

Kamensky, M., Ovadis, M., Chet, I., and Chernin, L. (2003). Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biol. Biochem.* 35, 323–331. doi: 10.1016/S0038-0717(02)00283-3

Kurz, C. L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., and Michel, G. P., et al. (2003). Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *EMBO J.* 22, 1451–1460. doi: 10.1093/emboj/cdg159

Kurz, C. L., and Ewbank, J. J. (2000). *Caenorhabditis elegans* for the study of host-pathogen interactions. *Trends Microbiol.* 8, 142–144. doi: 10.1016/S0966-842X(99)01691-1

Laws, T. R., Harding, S. V., Smith, M. P., Atkins, T. P., and Titball, R. W. (2004). Age influences resistance of *Caenorhabditis elegans* to killing by pathogenic bacteria. *FEMS Microbiol. Lett.* 234, 281–287. doi: 10.1111/j.1574-6968.2004.tb09545.x

Levenfors, J. J., Hedman, R., Thaning, C., Gerhardson, B., and Welch, C. J. (2004). Broad-spectrum antifungal metabolites produced by the soil bacterium *Serratia plymuthica* A 153. *Soil Biol. Biochem.* 36, 677–685. doi: 10.1016/j.soilbio.2003.12.008

Liu, X., Bimerew, M., Ma, Y., Müller, H., Ovadis, M., Eberl, L., et al. (2007). Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of Serratia plymuthica. *FEMS Microbiol. Lett.* 270, 299–305. doi: 10.1111/j.1574-6968.2007.00681.x

Mahajan-Miklos, S., Tan, M.-W., Rahme, L. G., and Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogensis model. *Cell* 96, 47–56. doi: 10.1016/S0092-8674(00)80958-7

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., et al. (2011). CDD: a conserved domain database for the functional annotation of proteins. *Nucleic Acids Res.* 39, D225–D229. doi: 10.1093/nar/gkq1189

Masschelein, J., Mattheus, W., Gao, L. J., Moons, P., Van Houdt, R., Uyterhoeven, B., et al. (2013). A PKS/NRPS/FAS hybrid gene cluster from *Serratia plymuthica* RVH1 encoding the biosynthesis of three broad spectrum, zeamine-related antibiotics. *PLoS ONE* 8:e54143. doi: 10.1371/journal.pone.0054143

Matilla, M. A., and Salmond, G. P. (2014). The viunalikevirus, bacteriophage ϕMAM1, is a broad host-range, high efficiency generalised transducing phage that infects environmental and clinical isolates of the enterobacteria, *Serratia* and *Kluyvera*. *Appl. Environ. Microbiol.* 80, 6446–6457. doi: 10.1128/AEM.01546-14

Matilla, M. A., Stöckmann, H., Leeper, F. J., and Salmond, G. P. (2012). Bacterial biosynthetic gene clusters encoding the anti-cancer haterumalide class of molecules: biogenesis of the broad spectrum antifungal and anti-oomycete compound, oocydin A. *J. Biol. Chem.* 287, 39125–39138. doi: 10.1074/jbc.M112.401026

Mazzola, M., Cook, R. J., Thomashow, L. S., Weller, D. M., and Pierson, L. S. (1992). Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* 58, 2616–2624.

Meziane, H., Gavriel, S., Ismailov, Z., Chet, I., Chernin, L., and Höfte, M. (2006). Control of green and blue mould on orange fruit by *Serratia plymuthica* strains IC14 and IC1270 and putative modes of action. *Postharvest Biol. Technol.* 39, 125–133. doi: 10.1016/j.postharvbio.2005.10.007

Neupane, S., Högberg, N., Ahlström, S., Lucas, S., Han, J., Lapidus, A., et al. (2012a). Complete genome sequence of the rapseed plant-growth promoting *Serratia plymuthica* strain AS9. *Stand. Genomic Sci.* 6, 54–62. doi: 10.4056/sigs.2595762

Neupane, S., Finlay, R. D., Alström, S., Goodwin, L., Kyrpides, N. C., Lucas, S., et al. (2012b). Complete genome sequence of *Serratia plymuthica* strain AS12. *Stand. Genomic Sci.* 6, 165–173. doi: 10.4056/sigs.2705996

Neupane, S., Finlay, R. D., Kyrpides, N. C., Goodwin, L., Ahlström, S., Lucas, S., et al. (2012c). Complete genome sequence of the plast-associated *Serratia plymuthica* strain AS13. *Stand. Genomic Sci.* 7, 22–30. doi: 10.4056/sigs.2966299

Osbourn, A. (2010). Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends Genet.* 26, 449–457. doi: 10.1016/j.tig.2010.07.001

Perego, M., Cole, S. P., Burbulys, D., Trach, K., and Hoch, J. A. (1989). Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *J. Bacteriol.* 171, 6187–6196.

Portal-Celhay, C., Bradley, E. R., and Blaser, M. J. (2012). Control of bacterial proliferation in regulation of lifespan in *Caenorahbditis elegans*. *BMC Microbiol.* 12:49. doi: 10.1186/1471-2180-12-49

Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C. I., and Ewbank, J. J. (2007). Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marscences* by *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2295–2300. doi: 10.1073/pnas.0610281104

Price-Whelan, A., Dietrich, L. E., and Newman, D. K. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nature Chem. Biol.* 2, 71–78. doi: 10.1038/nchembio764

Pritchard, L., Humphris, S., Baeyen, S., Maes, M., Van Vaerenbergh, J., Elphinstone, J., et al. (2013a). Draft genome sequences of four *Dickeya dianthicola* and four *Dickeya solani* strains. *Genome Announc.* 1, e00087–e00112. doi: 10.1128/genomeA.00087-12

Pritchard, L., Humphris, S., Saddler, G. S., Elphinstone, J. G., Pirhonen, M., and Toth, I. K. (2013b). Draft genome sequences of 17 isolates of the plant pathogenic bacterium *Dickeya*. *Genome Announc.* 1, e00978–e00913. doi: 10.1128/genomeA.00978-13

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., et al. (2000). Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8815–8821. doi: 10.1073/pnas.97.16.8815

Ramsay, J. P. (2013). High-throughput β-galactosidase and β-glucuronidase assays using fluorogenic substrates. *Bio-Protocol.* 3:e827.

Roberts, K. J. (2010). *Quorum Sensing in the Mouse Intestinal Pathogen Citrobacter rodentium*. Ph.D. thesis, University of Cambridge, Cambridge.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning*, Vol. 2. New York: Cold Spring Harbor Laboratory Press, 14–29.

Sana, T. G., Hachani, A., Bucior, I., Soscia, C., Garvis, S., Termine, E., et al. (2012). The second type VI secretion system of *Pseudomonas aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. *J. Biol. Chem.* 287, 27095–27105. doi: 10.1074/jbc.M112.376368

Sattely, E. S., Fischbach, M. A., and Walsh, C. T. (2008). Total biosynthesis: in vitro reconstitution of polyketide and nonribosomal peptide pathways. *Nat. Prod. Rep.* 25, 757–793. doi: 10.1039/b801747f

Sifri, C. D., Begun, J., and Ausubel, F. M. (2005). The worm has turned – microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol.* 13, 119–127. doi: 10.1016/j.tim.2005.01.003

Thaning, C., Welch, C. J., Borowicz, J. J., Hedman, R., and Gerhardson, B. (2001). Suppression of *Sclerotinia sclerotiorum* apothecial formation by the soil bacterium *Serratia plymuthica*: identification of a chlorinated macrolide as one of the causal agents. *Soil Biol. Biochem.* 33, 1817–1826. doi: 10.1016/S0038-0717(01)00109-2

Večerek, B., Moll, I., Afonyushkin, T., Kaberdin, V., and Bläsi, U. (2003). Interaction of the RNA chaperone Hfq with mRNAs: direct and indirect roles of Hfq in iron metabolism of *Escherichia coli*. *Mol. Microbiol.* 50, 897–909. doi: 10.1046/j.1365-2958.2003.03727.x

Vogel, J., and Luisi, B. F. (2011). Hfq and its constellation of RNA. *Nat. Rev. Microbiol.* 9, 578–589. doi: 10.1038/nrmicro2615

Wei, J. Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S. C., et al. (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2760–2765. doi: 10.1073/pnas.0538072100

Wilf, N. M., Williamson, N. R., Ramsay, J. P., Poulter, S., Bandyra, K. J., and Salmond, G. P. C. (2012). The RNA chaperone, Hfq, controls two luxR-type regulators and plays a key role in pathogenesis and production of antibiotics in

Serratia sp. ATCC39006. *Environ. Microbiol.* 13, 2649–2666. doi: 10.1111/j.1462-2920.2011.02532.x

Wu, J., Zhang, H. B., Xu, J. L., Cox, R. J., Simpson, T. J., and Zhang, L. H. (2010). 13C labeling reveals multiple amination reactions in the biosynthesis of a novel polyketide polyamine antibiotic zeamine from *Dickeya zeae*. *Chem. Commun.* 46, 333–335. doi: 10.1039/b916307g

Zhou, J., Zhang, H., Wu, J., Liu, Q., Xi, P., Lee, J., et al. (2011). A novel multidomain polyketide synthase is essential for zeamine production and the virulence of *Dickeya zeae*. *Mol. Plant Microbe Interact.* 24, 1156–1164. doi: 10.1094/MPMI-04-11-0087

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Prodigiosin Induces Autolysins in Actively Grown *Bacillus subtilis* Cells

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Prodigiosin produced by marine bacterium *Vibrio ruber* DSM 14379 exhibits a potent antimicrobial activity against a broad range of Gram positive and Gram negative bacteria. The mechanism of prodigiosin antimicrobial action, however, is not known. In this work, the effect of prodigiosin on *Bacillus subtilis* growth, cell membrane leakage, and induction of autolysins was studied. Treating *B. subtilis* with prodigiosin resulted in rapid decline of optical density and increased cell membrane leakage measured by β-galactosidase activity. Cell lysis was initiated immediately after treatment with prodigiosin in the middle exponential phase and was completed within 2 h. Lytic activity of prodigiosin in mutant strains with impaired autolysin genes *lytABCD* decreased for 80% compared to the wild type strain, while in *lytABCDEF* mutant strain prodigiosin had no bacteriolytic but only bacteriostatic effect. Fast prodigiosin lytic activity on individual *B. subtilis* cells was confirmed by a modified comet assay. The results indicate that prodigiosin autolysin induction in *B. subtilis* is growth phase dependent.

Keywords: prodigiosin, autolysis, *Bacillus subtilis*, antimicrobial, mechanism, autolysin, lytic rate, comet assay

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INTRODUCTION

Prodigiosin, a secondary metabolite, is produced by several bacterial genera including *Serratia, Streptomyces, Vibrio*, *Hahella*, *Zooshikella,* and *Pseudoalteromonas* (Farmer et al., 1988; Sawabe et al., 1998; Shieh et al., 2003; Williamson et al., 2006; Kumar and Nair, 2007; Fehér et al., 2010; Starič et al., 2010; Lee et al., 2011; Stankovic et al., 2012). Prodigiosin provides numerous ecophysiological benefits for the producing cell and has a good potential for biotechnological as well as medical applications (Burger and Bennett, 1985; Hood et al., 1992; Ryazantseva et al., 1995; Seganish and Davis, 2005; Haddix et al., 2008; Starič et al., 2010; Borić et al., 2011). Prodigiosin has been used in control of plant diseases caused by bacteria and fungi (Okamoto et al., 1998; Someya et al., 2001; Meschke et al., 2012). It has affinity to DNA (Melvin et al., 2000), but shows no *in vitro* or *in vivo* genotoxic effect on *Salmonella typhimurium* cells (Guryanov et al., 2013). It modulates H^+/Cl^- symport activity (Konno et al., 1998). When used as a coloring agent, it retains its antibiotic activity (Alihosseini et al., 2008). Prodigiosin has anticancer, antimalarial and immunosuppressant properties (Pérez-Tomás et al., 2003; Williamson et al., 2007). It inhibits growth of a wide range of Gram positive bacteria including *Bacillus subtilis* and *Staphylococcus aureus*, as well as Gram negative *Escherichia coli*, *Salmonella enterica,* and *Erwinia carotovora* (reviewed in Stankovic et al., 2014). The mechanism of its antibacterial action, however, is not understood on the molecular level. In this work, we study the molecular targets of prodigiosin in *B. subtilis*.

Many antibacterial agents target cell membrane and cell wall (Falk et al., 2010; Lacriola et al., 2013). In *B. subtilis* induction of autolysins and subsequent cell wall degradation is a major mechanism of antimicrobial action of several different compounds (Falk et al., 2010; Lacriola et al., 2013). Autolysins are enzymes involved in hydrolysis and remodeling of the peptidoglycan in the bacterial cell wall (Smith et al., 2000). It is absolutely critical to maintain the regulation of potentially suicidal activity of autolysins. The regulation of autolysin activity is mainly due to the local pH in respiring cells. In respiring cells, protons are extruded across the cytoplasmic membrane and bind to cell wall constituents (Jolliffe et al., 1981; Kemper et al., 1993; Calamita and Doyle, 2002). The protonation of the cell wall constituents create a wall matrix of low pH. Proton motive force sustains this local low pH and, in turn, autolytic activity is inhibited because *N*-acetylmuramyl-L-alanine amidase cannot function below pH 5.5. There are 36 autolysins present in the *B. subtilis* genome (Smith et al., 2000). Among different autolysins in *B. subtilis*, two autolysins of vegetative growth named LytC and LytD are responsible for about 95% of the bacterial cell autolysis during active growth (reviewed in Smith et al., 2000). It has been shown previously that β-galactosidase release correlates with activation of pathways leading to autolysis (Falk et al., 2007). Apart from β-galactosidase release several other methods (e.g., optical density, cell count) can be used to determine bacterial cell lysis. The comet assay is a method primarily used for the detection of DNA damage and cell lysis in a single cell (Ostling and Johanson, 1984; Singh et al., 1988). Recently, comet assay was applied to detect bacterial lysis after bacteriophage treatment (Khairnar et al., 2014). Using comet assay to detect single cell lysis provides an advantage over enzyme activity or optical density measurements of cell lysis which probe a population response.

In this work, the mechanism of antibacterial activity of prodigiosin on *B. subtilis* was studied. The ability of prodigiosin to affect bacterial population structure was determined in simple co-culture experiments. The effect of prodigiosin on the bacterial growth, cell wall integrity, autolysis, and DNA damage was determined on the wild type strain and several autolysin mutants. This study is the first attempt to determine the mechanism of antibacterial activity of prodigiosin in *B. subtilis in vivo*. The results indicate that prodigiosin activates rapid autolysis in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains, Mutant Strain Construction and Growth Conditions

Bacterial strains used in this study were *B. amyloliquefaciens* FZB42, *B. licheniformis* ATCC9445A, *B. subtilis* NCIB3610 all obtained from BGSC, *B. subtilis* ATCC6051, *B. mycoides* DSM2048 obtained from DSMZ, *B. subtilis* PS-216 *wt* (Stefanic and Mandic-Mulec, 2009), *B. subtilis* PS-216 *amyE*::$P_{hyperspank}$-*mKate2 cat* (Stefanic et al., 2015), *Escherichia coli* MG1655 and *Vibrio ruber* DSM14379 (Stopar et al., 2004; Borić et al., 2011). In addition mutant strains of *B. subtilis* PS-216 Δ*lytABC::neo*, *B. subtilis* PS-216 Δ*lytABC::neo* Δ*lytD::tet*, *B. subtilis* PS-216 Δ*lytABC::neo* Δ*lytD::tet* Δ*lytE::cam*, *B. subtilis* PS-216 Δ*lytABC::neo* Δ*lytD::tet* Δ*lytE::cam* Δ*lytF::spc* and *B. subtilis* PS-216 *srfA-lacZ neo* were prepared. The *lyt* mutants were constructed by transforming the *B. subtilis* PS-216 *wt* strain with the DNA isolated from *B. subtilis* L16611 and *B. subtilis* L16628 obtained from BGSC (Margot and Karamata, 1992). The *srfA-lacZ* construct was made by transforming *B. subtilis* PS-216 *wt* strain with the DNA isolated from *B. subtilis* KTB308 (Bacon Schneider et al., 2002). *Bacillus* strains were grown at 37°C (except *B. mycoides* at 28°C) and 200 rpm in LB medium or CM medium (Albano et al., 1987) or at 28°C and 200 rpm in PYE medium with 3% (w/V) NaCl (Danevčič et al., 2005).

Prodigiosin Extraction

Vibrio ruber DSM14379 was cultured overnight in PYE medium with 3% (w/V) NaCl at 28°C and 200 rpm (Danevčič et al., 2005). Overnight culture was diluted 100 times in 400 mL of fresh M9 medium supplied with 5 g/L glucose (Starič et al., 2010) and incubated for 16 h at the same growth conditions. Cells were harvested with centrifugation at 10397 *g* for 10 min. Prodigiosin was extracted from cells with an equal volume of acetone at 28°C and 200 rpm for 2 h. After centrifugation for 15 min at 10397 *g* to remove cell debris, the solvent was evaporated. Dry biomass was resuspended in sterile 96% (V/V) ethanol and the pigment extract was filtered through 0.20 μm filter. Prodigiosin concentration in the extract was determined as described previously by Starič et al. (2010). The prodigiosin purity in the extract was confirmed by HPLC using Kinetex C18 column (250 mm × 4.6 mm, 5 μm, Phenomenex, USA) according to the modified method described previously (Nakashima et al., 2006). The separation was achieved using 0.1% trifluoroacetic acid (A) and methanol (B) mobile phases, and a gradient elution program at 1 mL^{-1} min with the following parameters: 0–5 min 100% A, 5–25 min 0–100% B (linear gradient), 25–30 min 100% B (isocratic), and 30–50 min 100% A (isocratic) to re-equilibrate the column, monitored by UV-VIS detection. The purity was determined by HPLC at different wavelengths in the range from 400 to 600 nm. The HPLC elugram at 535 nm is given in supplementary materials (Supplementary Figure S1). In the tested range the extract contained more than 98% prodigiosin of both isomers α and β.

Minimal Inhibitory Concentration Determination

Minimal inhibitory concentration (MIC) values were determined in 96-well microtiter plate assay according to CLSI standards (Clinical and Laboratory Standards Institute, 2009). Briefly, bacterial strains were grown overnight in LB medium at 28°C or 37°C. Inoculum was prepared by diluting overnight cultures in 0.9% NaCl to 0.5 McFarland (∼10^7 cells per mL). Wells of microtiter plates were filled with 0.1 mL of the appropriate concentration of the prodigiosin, ethanol or water and 0.01 mL of the inoculum to have the approximate number of cells in each inoculated well 10^5. The dilutions of prodigiosin, ethanol and water were made in LB medium. Prodigiosin was diluted in the final concentration range from 0.6 mg L^{-1} to 367.1 mg

L^{-1}, while ethanol was diluted in the final concentration range from 0.02 to 12.03% (V/V). For positive controls LB medium was diluted with the same amounts of sterile distilled water as ethanol. Microtiter plates were incubated without shaking at 37°C for 20 h and the optical density at 650 nm (OD_{650}) was measured spectrophotometrically at the beginning and the end of incubation. The lowest prodigiosin concentration in the well with no growth after 20 h of incubation, was taken as the MIC value.

Co-culture Experiments

Bacillus subtilis PS-216 *wt* was grown either in co-culture with *V. ruber* DSM14379 or in pure culture. For co-culture experiments, strains were separately grown in PYE medium with 3% (w/V) NaCl (*V. ruber*) or in LB medium (*B. subtilis*) until the stationary phase. Co-cultures were inoculated into PYE medium with 3% (w/V) NaCl with *V. ruber* DSM14379: *B. subtilis* PS-216 in the ratio 1:2 (V:V), the overall inoculum being 1% (V/V). CFU was determined at the beginning and 20 h after incubation at 28°C and 200 rpm. CFU values were determined on PYE plates without NaCl (for *B. subtilis* PS-216 *wt*) and PYE plates with 3% (w/V) NaCl (for both strains). Due to the red pigmentation of *V. ruber* DSM14379 the colonies were clearly distinguished. Malthusian fitness was calculated based on CFU counts according to Ahn et al. (2006). Prodigiosin concentration in co-cultures was determined as described previously in Borić et al. (2011).

Cell Treatment with Prodigiosin in Different Growth Phases

Bacillus subtilis PS-216 *wt* cells were cultured in liquid LB medium at 37°C and 200 rpm overnight. Overnight culture was diluted 100 times in 20 mL of fresh LB medium and incubated further at the same growth conditions. OD_{650} was measured in regular intervals during the culture incubation. At appropriate OD_{650} in different growth phases [i.e., middle exponential ($OD_{650} \sim 0.7$), late exponential ($OD_{650} \sim 1.1$) and stationary phase $OD_{650} \sim 1.3$], the strains were treated with 5.9 mg L^{-1} of prodigiosin. As a control, an equivalent amount of sterile 96% (V/V) ethanol was added to the LB medium in the final concentration 0.19% (V/V).

Treatment of Different Bacterial Strains with Prodigiosin

Bacillus mycoides DSM2048 cells were cultured in liquid LB medium at 28°C and 200 rpm overnight, while *B. licheniformis* ATCC9445A cells were cultured in liquid LB medium at 37°C and 200 rpm overnight. Overnight cultures were diluted 100 times in 20 mL of fresh LB medium and incubated further at the same growth conditions. Cultures were treated with prodigiosin (1.2 mg L^{-1} *B. mycoides* or 6.9 mg L^{-1} *B. licheniformis*) or ethanol as a control [0.04% (V/V) *B. mycoides* or 0.23 % (V/V) *B. licheniformis*] in the middle exponential phase at OD_{650} between 0.4 and 0.5. Treated cells were then incubated further and OD_{650} was measured in regular intervals during 6 h of incubation. To determine the influence of prodigiosin on different strains, the rate of lysis was calculated from measured decrease of OD_{650} after prodigiosin treatment.

Cell Morphology and Viability

Bacillus subtilis PS-216 *wt* and *B. subtilis* PS-216 *amyE*::$P_{hyperspank}$-*mKate2* strains were grown in LB medium as described above and treated with 5.9 mg L^{-1} of prodigiosin or 0.19% (V/V) of ethanol in early exponential phase at OD_{650} between 0.4 and 0.5. Cell morphology and viability were inspected by Differential Interference Contrast (DIC) and fluorescence microscopy using appropriate filter set under the inverted microscope Axio Observer Z1 (Carl Zeiss, Germany) at 0, 1, and 5 h after the prodigiosin or ethanol treatment.

Effect of Prodigiosin on Inactive Cells

Bacillus subtilis PS-216 *wt* cells were grown in LB medium as described above. When cells reached OD_{650} between 0.4 and 0.5, bacterial culture was autoclaved at 121°C and 1.03 bar for 20 min to inactivate cells. After autoclaving, cell suspension was cooled down to room temperature and 5.9 mg L^{-1} of prodigiosin was added. Cell suspension was incubated further at 37°C and 200 rpm and OD_{650} was measured every 30 min for 5 h.

Cytoplasmic Membrane Leakage

Cytoplasmic membrane leakage was determined as β-galactosidase activity according to the modified method by Mensa et al. (2011). Briefly, *B. subtilis* PS-216 *srfA-lacZ* (*neo)* cells were grown overnight in LB medium with appropriate antibiotic at 37°C and 200 rpm and diluted 100 times in 20 mL of the fresh CM medium. Cells were incubated at 37°C and 200 rpm until they reach $OD_{650} \sim 0.6$. At that point 70 μL of cells were transferred in a microtiter plate and mixed with 20 μL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM $MgSO_4$, pH 7) supplemented with 4 mg mL^{-1} ONPG (*o*-nitrophenyl-β-galactoside) and 40 μM β-mercaptoethanol. Cells were then treated with 10 μL of prodigiosin to a final concentration of 2.9 mg L^{-1}. Microtiter plates were incubated in a spectrometer for 2 h at 37°C and absorbance at 420 nm was measured every 2 min to determine the rate of ONPG hydrolysis. The addition of 10 μL of 0.1% (w/V) sodium dodecyl sulfate (SDS) detergent [final concentration 0.01% (w/V)] represented a positive control, while the addition of 10 μL of 96% (V/V) ethanol [final concentration 9.6% (V/V)] represented a negative control. To calculate specific enzyme activity, the rate of ONPG hydrolysis was normalized to the cell biomass.

Bacterial Response to Different Autolysin Inducing Agents

Bacillus subtilis PS-216 *wt* strain was grown in LB medium as described above and treated with 0.0125% (V/V) Triton X-100, 0.01% (w/V) SDS , 0.01% (w/V) cetyl trimethylammonium bromide (CTAB), 100 μg mL^{-1} ampicillin or 100 μg mL^{-1} kanamycin in the middle exponential phase at OD_{650} between 0.4 and 0.5. Ampicillin was used as a positive control known to induce cell lysis, whereas kanamycin was used as a negative control (Chung et al., 2009; Falk et al., 2010; Lacriola et al., 2013).

Cell suspension was incubated further at 37°C and 200 rpm and OD_{650} was measured every 30 min for 5 h. To determine the impact of different detergents on bacterial strain, the rate of lysis was calculated from measured decrease of OD_{650} after treatment with antimicrobial agents.

Autolytic Response of Bacterial Strains to Prodigiosin

Bacillus subtilis strains PS-216 *wt*, PS-216 Δ*lytABC*, PS-216 Δ*lytABCD*, PS-216 Δ*lytABCDE* and PS-216 Δ*lytABCDEF* were grown in LB medium as described above and treated with 5.9 mg L^{-1} of prodigiosin or 0.19 % (V/V) of ethanol as a negative control at OD_{650} between 0.4 and 0.7. Cells were then incubated further and OD_{650} was measured in regular intervals during 6 h of incubation at 37°C and 200 rpm. To determine the influence of prodigiosin on different mutant strains, the rate of lysis was calculated from measured decrease of OD_{650} after prodigiosin treatment.

Modified Comet Assay

Bacillus subtilis strains PS-216 *wt*, PS-216 Δ*lytABC*, PS-216 Δ*lytABCD*, PS-216 Δ*lytABCDE* and PS-216 Δ*lytABCDEF* were grown in LB medium as described above. At OD_{650} between 0.4 and 0.6 cells were treated with 5.9 mg L^{-1} of prodigiosin or 0.19% (V/V) of ethanol. Cells were then incubated further for 1 h at 37°C and 200 rpm to lyse. Minigels were prepared on frosted-end microscopic slides. Slides were first dipped into 1% (w/V) normal melting point agarose (NMP) and one side was wiped clean. After drying on a hot plate (1 min, 100°C), the second layer of 300 μL 1% (w/V) NMP agarose was applied and solidified at 4°C for 5 min. For the third layer treated or non-treated cells were resuspended in 1% (w/V) low melting agarose (ratio 1:10) and left to solidify for 5 min. Minigels were immediately electrophoresed in TBE (Tris/borate/EDTA buffer, pH 8.3) at 1 V/cm and run for 30 min. Afterward minigels were neutralized in 400 mM Tris buffer (pH 7.4). Gels were stained with 30x solution of GelRed™ (Biotium, USA) and observed under epifluorescent microscope (Olympus BX-50, Japan) equipped with EMCCD camera (Luca^{EM}r, Andor Technology Ltd., United Kingdom) and the appropriate filter set. Images were captured by Komet 7.0 software (Andor Technology Ltd., UK).

Data Analysis

All the values presented are averages with standard deviations or standard errors. Results were statistically evaluated using one-way ANOVA. Samples with *p*-values equal or lower than 0.05 were taken as statistically significant.

RESULTS AND DISCUSSION

Prodigiosin isolated from *V. ruber* DSM14379 inhibits growth of different bacterial species. The MIC value for *Escherichia coli* is 103.4 ± 6.3 mg L^{-1}, while different *Bacillus* sp. strains tested have significantly lower MIC values. The MIC values for different *Bacillus* sp. strains were in the range between 5 and 7 mg L^{-1} (MIC values for *B. amyloliquefaciens* FZB42 was

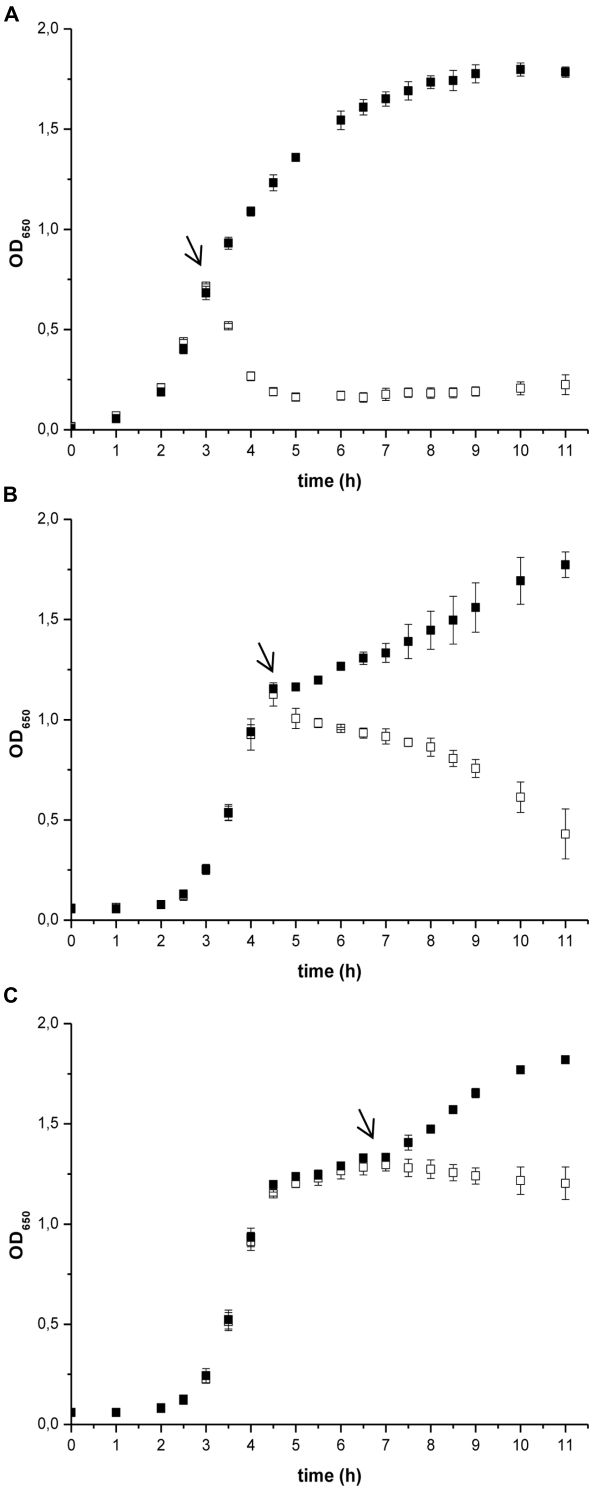

FIGURE 1 | The effect of prodigiosin addition on *Bacillus subtilis* PS-216 *wt* growth in LB medium at 37°C and 200 rpm at different growth phases. The arrows represent the time of addition of 5.9 mg L^{-1} prodigiosin (opened squares) or 0.19% (V/V) ethanol in the control samples (filled squares). **(A)** cells treated in the middle of the exponential phase; **(B)** cells treated in the late exponential phase; **(C)** cells treated in the stationary phase. Data are presented as averages and standard deviations ($n = 3$).

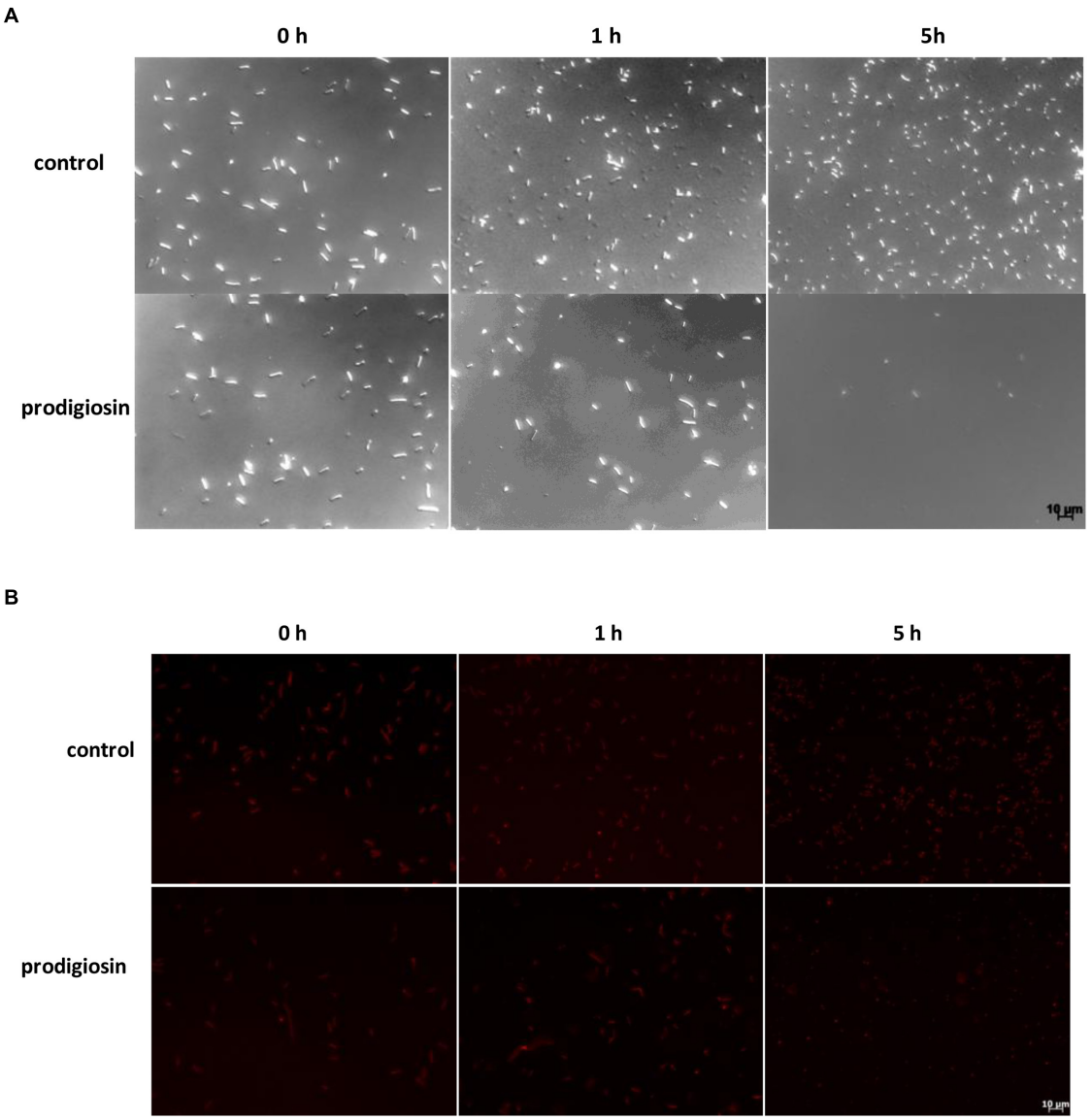

FIGURE 2 | *Bacillus subtilis* PS-216 *wt* cells (A) and *B. subtilis* PS-216 *amyE*::$P_{hyperspank}$-*mKate2* (B) grown in LB medium at 37°C and 200 rpm observed by DIC and fluorescent microscopy at 0, 1, and 5 h after the addition of either 5.9 mg L^{-1} of prodigiosin or 0.19% (V/V) of ethanol in control samples in the middle of the exponential phase. The scale bar represents 10 µm.

6.1 ± 1.2 mg L^{-1}, *B. licheniformis* ATTC9445A was 6.9 ± 2.2 mg L^{-1}, *B. mycoides* was 1.2 mg L^{-1}, *B. subtilis* NCIB3610 was 5.2 ± 1.4 mg L^{-1}, *B. subtilis* ATCC6051 was 5.9 mg L^{-1} and *B. subtilis* PS-216 was 5.9 mg L^{-1}) and were lower as previously reported (Stankovic et al., 2012; Priya et al., 2013). Approximately the same MIC values for different *Bacillus* species may suggest that prodigiosin has the same mode of action on all tested *Bacillus sp.* Only undomesticated natural isolate *B. subtilis* PS-216 *wt* was used for further analysis with MIC value 5.9 mg L^{-1}. To test the *in vivo* activity of prodigiosin on *B. subtilis* PS-216 *wt* simple co-cultures with prodigiosin producing *V. ruber* DSM14379 were made. There was a significant decrease in the number of *B. subtilis* cells after incubation in co-culture. The Malthusian fitness of *B. subtilis* grown in a monoculture was 5.7 ± 0.2, but was significantly lower −6.8 ± 1 in co-culture experiments. The concentration of prodigiosin in co-culture at the end of the experiment was 16.3 ± 0.4 mg L^{-1} which is 2.7 fold higher than the MIC required for growth inhibition of *B. subtilis* PS-216 *wt*. The results indicate that prodigiosin has *in vivo* antimicrobial activity.

The effect of prodigiosin on *B. subtilis* was dependent on the growth phase as shown in **Figure 1**. Prodigiosin treatment in the middle of the exponential phase (**Figure 1A**) caused instantaneous termination of growth and immediate decrease of optical density. Cell lysis was completed in 2 h after prodigiosin addition. As shown in **Figure 2** cell number significantly

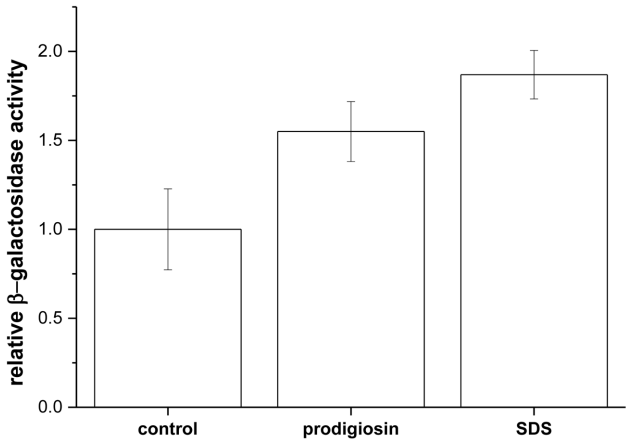

FIGURE 3 | Relative β-galactosidase activity upon addition of 5.9 mg L^{-1} prodigiosin and 0.01% (w/V) SDS in *B. subtilis* PS-216 *srfA-lacZ* (*neo*) cells compared with control [addition of 0.19% (V/V) ethanol]. Cells were grown in LB medium at 37°C and 200 rpm. Data are presented as averages and standard errors ($n = 3$–6).

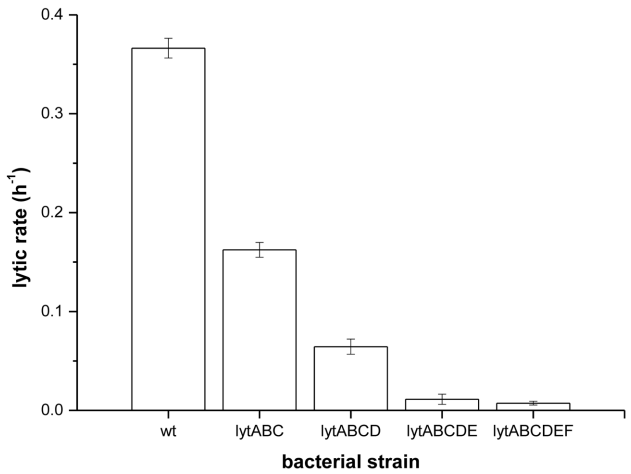

FIGURE 4 | Lytic rates of different *lyt* mutants measured as a rate of OD_{650} decrease after treatment with prodigiosin (5.9 mg L^{-1}) in LB medium at 37°C and 200 rpm. Data are presented as averages and standard deviations ($n = 3$).

decreased after prodigiosin treatment. After 5 h of incubation with prodigiosin the bacterial solution was clear and only a few bacteria were observed. Cells treated with prodigiosin in the late exponential phase (**Figure 1B**) lysed as well. However, the rate of lysis was three times lower than in the middle of the exponential phase. After 5 h of prodigiosin treatment, optical density decreased for approximately 50%. When cells were treated with prodigiosin in the stationary phase (**Figure 1C**) their growth ceased, but cells did not lyse. When those cells were re-inoculated into a fresh medium and incubated at optimal growth conditions, they resumed grow immediately. This implies that the mode of action of prodigiosin on *B. subtilis* cells depends on the growth phase. During the exponential growth prodigiosin has a bacteriolytic activity, whereas in the stationary phase it has bacteriostatic activity. This finding is not surprising, as it is generally accepted that effectiveness of antibiotic is greatly dependent on bacterial growth rate (Hobby et al., 1942; Greulich et al., 2015). For example, β-lactam antibiotics that target cell wall have their maximum effect during fast bacterial growth and are not effective toward stationary cells (Eng et al., 1991). The requirement of cell growth for prodigiosin susceptibilty was further tested with inactivated cells from the middle of the exponential phase. There was no significant change in optical density during 5 h of the incubation with prodigiosin, suggesting that the inactive cells did not lyse (data not shown). After the addition of the control containing ethanol (**Figure 1**), the growth of *B. subtilis* continued. Although the culture was not significantly diluted (the total volume increased only by 0.2%), the added ethanol may have had a positive effect on the growth of *B. subtilis* as demonstrated previously (Gomaa, 2014).

For cell lysis one expects both cell wall and membrane disintegration. Prodigiosin is a small molecule that can diffuse through the cell wall and due to its amphiphilic character it can incorporate in the membrane (Diaz et al., 2007). Membrane loaded with prodigiosin may be compromised and allow leakage of cell constituents. To test cytoplasmic membrane permeability ONPG hydrolysis by β-galactosidase was used. ONPG can enter the cell and reach cytoplasmatic β-galactosidase only if cytoplasmic membrane is compromised. As given in **Figure 3** prodigiosin was able to permeabilize cytoplasmic membrane. Similar permeabilization was observed with SDS detergent, which is a known cytoplasmic membrane solubilization agent in *B. subtilis* and *Streptococcus faecalis* (Bishop et al., 1967; Cornett and Shockman, 1978). There were no statistically significant differences between prodigiosin and SDS treatment ($p = 0.4$). The results indicate that prodigiosin interacts with cytoplasmic membrane and increases its permeability. Increased cytoplasmic permeability was associated with autolysis in *B. subtilis* (Falk et al., 2007). It has been shown that proton-motive force influences regulation of autolysins in *B. subtilis* cells (Calamita and Doyle, 2002). Prodigiosin functions in an energy-spilling process as a tightly regulated uncoupler of proton transport and ATP synthesis by oxidative phosphorylation (Haddix et al., 2008).

When cells were treated with prodigiosin in the middle of the exponential phase, approximately half of the cells lysed in 45 min. This lytic rate of prodigiosin ($0.37 \pm 0.01\ h^{-1}$) is comparable to cell lysis induced by Triton X-100 ($0.36 \pm 0.03\ h^{-1}$) and ampicillin ($0.49 \pm 0.06\ h^{-1}$), which is a known autolysin induction agent (Lacriola et al., 2013). On the other hand, the other known agents that induce autolysins (i.e., SDS and CTAB) had lower lytic rates. SDS had lytic rate $0.13 \pm 0.01\ h^{-1}$, while CTAB had lytic rate $0.15 \pm 0.01\ h^{-1}$. Kanamycin known as a non-autolysin agent did not lyse *B. subtilis*, the lytic rate was $-0.12 \pm 0.03\ h^{-1}$. The results indicate that there was a significant difference between lytic rate of prodigiosin and SDS, which is different from the β-galactosidase results (**Figure 3**). Whereas β-galactosidase release assay shows the cytopasmic membrane permeabilisation, the lytic rate shows autolysin induction. The

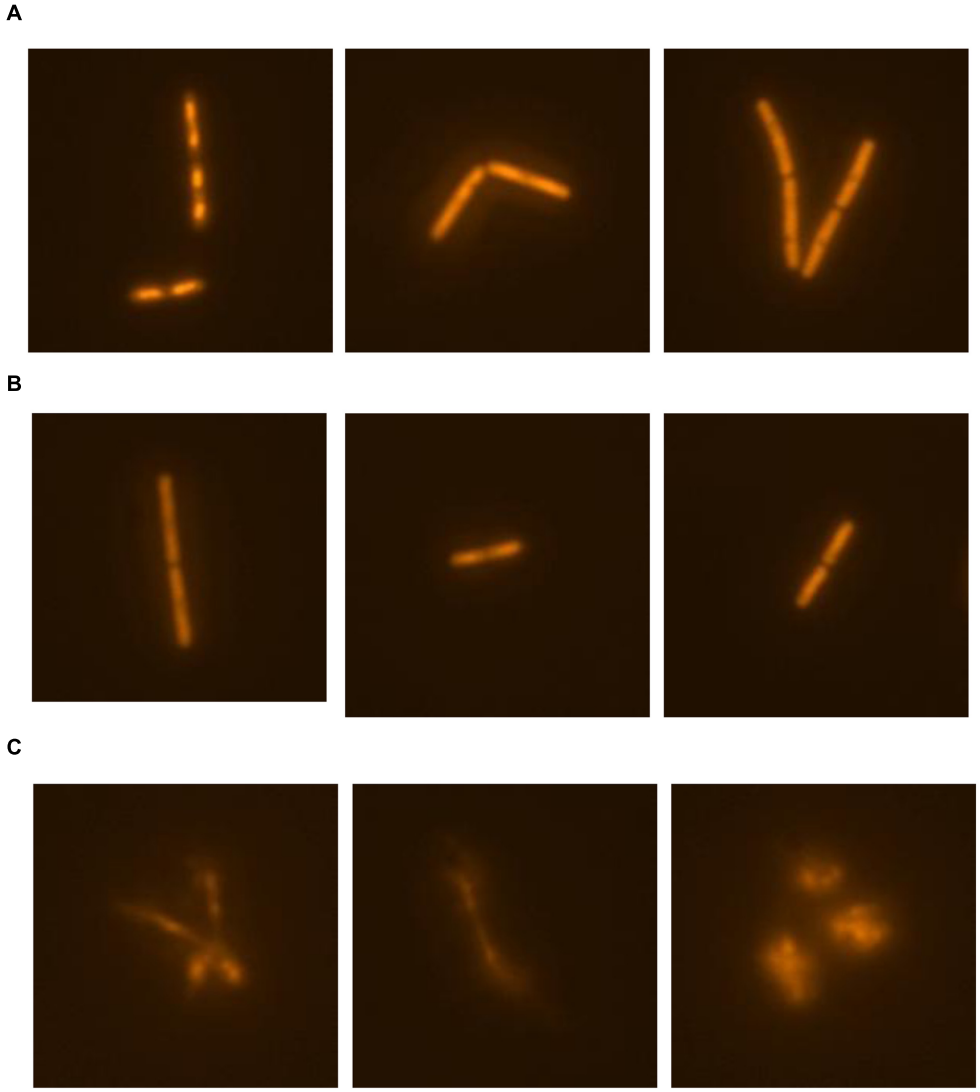

FIGURE 5 | The modified comet assay for studying prodigiosin mediated cell lysis of *B. subtilis* PS-216 *wt* cells grown in LB medium at 37°C and 200 rpm. (A) non-treated bacterial cells; **(B)** control cells treated with 0.19% (V/V) of ethanol; **(C)** cells treated with 5.9 mg L^{-1} of prodigiosin. Cells were treated in the middle exponential phase and were inspected 1 h after the treatment. The gels were stained with GelRed^{TM}. The images were observed with epifluorescent microscopy.

two are not necessarily coupled. For example, SDS is a typical solubilizing agent of cytoplasmic membrane proteins in *B. subtilis* (Bishop et al., 1967), but is usually not used as an autolysin inducer due to its low lytic effectiveness. It has been demonstrated in *Enterobacteriacea* that the SDS-grown cells underwent rapid lysis only when they ran out of energy (Kramer et al., 1980). The energy dependence reflects a requirement for ATP rather than for a proton gradient or membrane potential (Aspedon and Nickerson, 1994). Prodigiosin, on the other hand, acts as a potent lytic agent for *B. subtilis* that uncouples proton transport in the cytoplasmic membrane (Haddix et al., 2008), thereby activating autolysins. Prodigiosin lytic activity was not limited to *B. subtilis* cells only. For example, treating *B. mycoides* and *B. licheniformis* with prodigiosin at MIC concentrations induces cell lysis and the cell suspension cleared after 20 h of treatment. The corresponding lytic rates were 0.014 ± 0.002 h^{-1} for *B. mycoides* and 0.009 ± 0.001 h^{-1} for *B. licheniformis*, which indicates that prodigiosin lytic activity could be a more general mechanism in *Bacillus* species.

To prove that prodigiosin induces autolysis several mutant strains of *B. subtilis* PS-216 deficient in major autolysis genes (*lyt* genes) were produced. The lytic rates after treatment with prodigiosin of mutant strains deficient to a different extent in autolytic response are given in **Figure 4**. In the *lytABC* deficient strain the cell lysis was significantly reduced and the lytic rate was approximately two fold lower compared to the wild type strain. When additionally *lytD* gen was deleted a further decrease in the lytic rate was observed. The *lytABCD* deficient strain

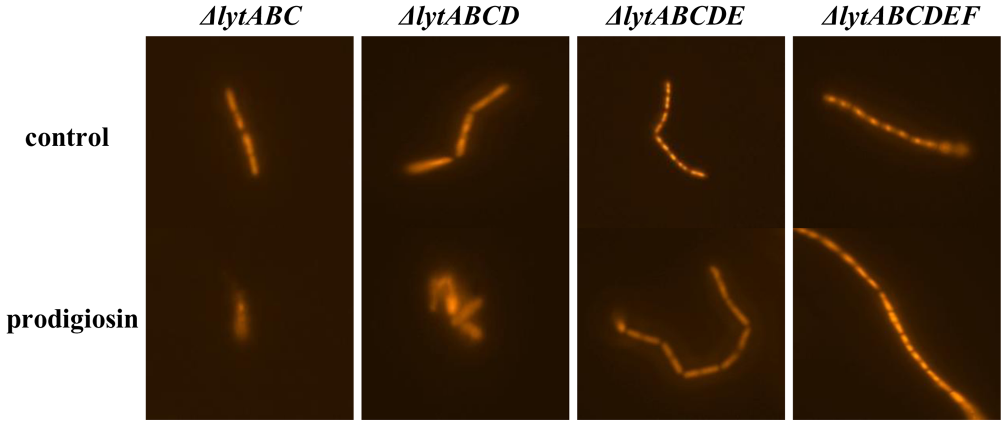

FIGURE 6 | The modified comet assay on different autolysin deficient strains of *B. subtilis* PS-216 cells grown in LB medium at 37°C and 200 rpm treated with 0.19% (V/V) of ethanol (control) or treated with 5.9 mg L^{-1} of prodigiosin. Cells were treated in the middle exponential phase and were inspected 1 h after the treatment. The gels were stained with GelRed™. The images were observed with epifluorescent microscopy.

showed approximately 80% reduction of lysis compared to the wild type strain. This is consistent with previous reports of a key role of LytC and LytD in autolysis (Smith et al., 2000). In addition, it has been shown previously that *lytE* mutant may significantly reduce autolysis (Margot et al., 1998). Consistently in *lytABCDE* mutant there was almost no lysis after prodigiosin addition. Finally, in *lytABCDEF* mutant the lytic rate upon addition of prodigiosin was 50 times lower compared to the wild type strain. Although no lytic response was observed in *lytABCDEF* mutant the addition of prodigiosin in the middle of exponential phase had a bacteriostatic effect. The results obtained by different *lyt* deficient strains strongly suggest that prodigiosin induces autolysin dependent bacteriolysis in *B. subtilis*. For a complete lysis of *B. subtilis* by prodigiosin a complement of different autolysins is needed. Since autolysins can be induced also by other amphiphatic molecules such as short- and medium–chain fatty acids (Tsuchido et al., 1985) this further supports the hypothesis that prodigiosin is a potent autolysin modulator.

The release of nucleic material during cell lysis was followed with modified comet assay (**Figure 5**). Non-treated cells retained cell integrity through the experiment, displaying clearly defined shape without tail or halo (**Figure 5A**). Similary, in control cultures treated with 0.19% (V/V) ethanol, the same concentration as used for prodigiosin solubilization, cells were intact (**Figure 5B**). On the other hand, treatment with prodigiosin induced instant lysis in almost all examined cells (**Figure 5C**). Cells that lysed appeared blurred with rapid decline of fluorescence indicating DNA diffusing out of disrupted cells. The comet assay was also applied to *B. subtilis* mutant strains deficient in major autolysin genes. *Lyt* mutants treated with prodigiosin responded differently. The comet assay suggests that lysis was most intense in *lytABC* mutant. The degree of cell lysis decreased from *lytABC* to *lytABCDEF* deficient strain (**Figure 6**). Negligible cell lysis was observed in *lytABCDEF* mutant strain after prodigiosin treatment. The results obtained with the modified comet assay confirm that prodigiosin induces rapid autolysis in *B. subtilis* cells.

Finally, we note that upon entry into the stationary phase prodigiosin acts as a bacteriostatic agent. During transition to the stationary phase *B. subtilis* cells begin to lose their ability to move and start to produce biofilm. Biofilms and motility are mutually exclusive lifestyles of *B. subtilis* (Diethmaier et al., 2011) and are controlled by a common regulator, the transcription factor SinR. During biofilm formation SinR interacts with SinI and de-represses synthesis of extracellular matrix important in biofilm formation. One of the components of extracellular matrix EpsE interacts with flagellar motor switch protein FliG and prevents rotation of the flagellum (Blair et al., 2008). In addition, upon the entry into a stationary phase cells shut down synthesis of flagella components. The synthesis of flagella and autolysin are under the same regulation control. The synthesis of flagella proteins requires genes which are under the control of alternative RNA polymerase sigma factor, σ^D (Shahbabian et al., 2009). σ^D is a pleiotrophic transcription regulator during the exponential growth and has a positive effect on autolysin synthesis (Fein, 1979). Upon entry into the stationary phase σ^H decreases the expression of σ^D (Britton et al., 2002), which in turn down-regulates autolysin production. In the absence of autolysins, as demonstrated in this work, the lytic activity of prodigiosin is abolished. However, as shown with *lytABCDEF* mutant prodigiosin may still act as a bacteriostatic agent preventing further growth of *B. subtilis*.

CONCLUSION

The application of prodigiosin as an antibacterial agent is hampered by the lack of knowledge of its molecular targets. The results of this study demonstrate that prodigiosin exhibits a potent antimicrobial activity against *B. subtilis*. It acts as a bacteriolytic agent during the exponential growth and as a bacteriostatic agent during the stationary growth. Prodigiosin interferes with cytoplasmic membrane function and increases its permeability. The obtained results indicate that prodigiosin's

bacteriolytic activity is due to the induction of autolysins. Prodigiosin proves to be a strong autolysin inducer in *B. subtilis* cells comparable in its activity to Triton X-100. Since prodigiosin functions as an uncoupler of proton transport and ATP synthesis, it is ideally suited for autolysins induction and killing of different *Bacillus* species.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TD, MV, and DS. Performed the experiments: TD, MV, MT, and MZ. Analyzed the data: TD, MV, MT, and MZ. Wrote the paper: TD, MV and DS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00027

REFERENCES

Ahn, H. J., La, H. J., and Forney, L. J. (2006). System for determining the relative fitness of multiple bacterial populations without using selective markers. *Appl. Environ. Microbiol.* 72, 7383–7385. doi: 10.1128/AEM.01246-06

Albano, M., Hahn, J., and Dubnau, D. (1987). Expression of competence genes in *Bacillus subtilis*. *J. Bacteriol.* 169, 3110–3117.

Alihosseini, F., Ju, K. S., Lango, J., Hammock, B. D., and Sun, G. (2008). Antibacterial colorants: characterization of prodiginines and their applications on textile materials. *Biotechnol. Prog.* 24, 742–747. doi: 10.1021/bp070481r

Aspedon, A., and Nickerson, K. W. (1994). The energy dependence of detergent resistance in *Enterobacter cloacae*: a likely requirement for ATP rather than a proton gradient or a membrane potential. *Can. J. Microbiol.* 40, 184–191. doi: 10.1139/m94-031

Bacon Schneider, K., Palmer, T. M., and Grossman, A. D. (2002). Characterization of comQ and comX, two genes required for production of ComX pheromone in *Bacillus subtilis*. *J. Bacteriol.* 184, 410–419. doi: 10.1128/JB.184.2.410-419.2002

Bishop, D. G., Rutberg, L., and Samulsson, B. (1967). The solubilization of the cytoplasmic membrane of *Bacillus subtilis* by sodium dodecyl sulphate. *Eur. J. Biochem.* 2, 454–459. doi: 10.1111/j.1432-1033.1967.tb00159.x

Blair, K. M., Turner, L., Winkelman, J. T., Berg, H. C., and Kearns, D. B. (2008). A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* 320, 1636–1638. doi: 10.1126/science.1157877

Borić, M., Danevčič, T., and Stopar, D. (2011). Prodigiosin from *Vibrio* sp. DSM 14379; a new UV-protective pigment. *Microb. Ecol.* 62, 528–536. doi: 10.1007/s00248-011-9857-0

Britton, R. A., Eichenberger, P., Gonzalez-Pastor, J. E., Fawcett, P., Monson, R., Losick, R., et al. (2002). Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J. Bacteriol.* 184, 4881–4890. doi: 10.1128/JB.184.17.4881-4890

Burger, S. R., and Bennett, J. W. (1985). Droplet enrichment factors of pigmented and nonpigmented *Serratia marcescens*: possible selective function for prodigiosin. *Appl. Environ. Microbiol.* 50, 487–490.

Calamita, H. G., and Doyle, R. J. (2002). Regulation of autolysins in teichuronic acid-containing *Bacillus subtilis* cells. *Mol. Microbiol.* 44, 601–606. doi: 10.1046/j.1365-2958.2002.02872.x

Chung, J. K., Yoon, H. E., Shin, H. C., Choi, E. Y., and Byeon, W. H. (2009). Induction of growth phase-specific autolysis in *Bacillus subtilis* 168 by growth inhibitors. *J. Microbiol.* 47, 50–59. doi: 10.1007/s12275-008-0256-2

Clinical and Laboratory Standards Institute (2009). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard*, 8th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Cornett, J. B., and Shockman, G. D. (1978). Cellular lysis of *Streptococcus faecalis* induced with Triton X-100. *J. Bacteriol.* 135, 153–160.

Danevčič, T., Rilfors, L., Štrancar, J., Lindblom, G., and Stopar, D. (2005). Effects of lipid composition on the membrane activity and lipid phase behaviour of *Vibrio* sp. DSM14379 cells grown at various NaCl concentrations. *Biochim. Biophys. Acta* 1712, 1–8. doi: 10.1016/j.bbamem.2005.03.013

Diaz, R. I. S., Regourd, J., Santacroce, P. V., Davis, J. T., Jakeman, D. L., and Thompson, A. (2007). Chloride anion transport and copper – mediated DNA cleavage by C-ring functionalized prodigiosenes. *Chem. Commun.* 26, 2701–2703. doi: 10.1039/B701919J

Diethmaier, C., Pietack, N., Gunka, K., Wrede, C., Lehnik-Habrink, M., Herzberg, C., et al. (2011). A novel factor controlling bistability in *Bacillus subtilis*: the YmdB protein affects flagellin expression and biofilm formation. *J. Bacteriol.* 193, 5997–6007. doi: 10.1128/JB.05360-11

Eng, R. H., Padberg, F. T., Smith, S. M., Tan, E. N., and Cherubin, C. E. (1991). Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob. Agents Chemother.* 35, 1824–1828. doi: 10.1128/AAC.35.9.1824

Falk, S. P., Noah, J. W., and Weisblum, B. (2010). Screen for inducers of autolysis in *Bacillus subtilis*. *Antimicrob. Agents Chemother.* 54, 3723–3729. doi: 10.1128/AAC.01597-09

Falk, S. P., Ulijasz, A. T., and Weisblum, B. (2007). Differential assay for high-throughput screening of antibacterial compounds. *J. Biomol. Screen.* 12, 1102–1108. doi: 10.1177/1087057107308161

Farmer, J. J., Hickman-Brenner, F. W., Fanning, G. R., Gordon, C. M., and Brenner, D. J. (1988). Characterization of *Vibrio metschnikovii* and *Vibrio gazogenes* by DNA-DNA hybridization and phenotype. *J. Clin. Microbiol.* 26, 1993–2000.

Fehér, D., Barlow, R. S., Lorenzo, P. S., and Hemscheidt, T. K. (2010). A 2-Substituted Prodiginine, 2-(p-Hydroxybenzyl)prodigiosin, from *Pseudoalteromonas rubra*. *J. Nat. Prod.* 71, 1970–1972. doi: 10.1021/np800493p

Fein, J. E. (1979). Possible involvement of bacterial autolytic enzymes in flagellar morphogenesis. *J. Bacteriol.* 137, 933–946.

Gomaa, E. Z. (2014). Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol. *Braz. Arch. Biol. Technol.* 57, 145–154. doi: 10.1590/S1516-89132014000100020

Greulich, P., Scott, M., Evans, M. R., and Allen, R. J. (2015). Growth – dependent bacterial susceptibility to ribosome – targeting antibiotics. *Mol. Syst. Biol.* 11, 796. doi: 10.15252/msb.20145949

Guryanov, I. D., Karamova, N. S., Yusupova, D. V., Gnezdilov, O. I., and Koshkarova, L. A. (2013). Bacterial pigment prodigiosin and its genotoxic effect. *Russ. J. Bioorg. Chem.* 39, 106–111. doi: 10.1134/S1068162012060040

Haddix, P. L., Jones, S., Patel, P., Burnham, S., Knights, K., Powell, J. N., et al. (2008). Kinetic analysis of growth rate, ATP, and pigmentation suggests an energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. *J. Bacteriol.* 190, 7453–7463. doi: 10.1128/JB.00909-08

Hobby, G. L., Meyer, K., and Chaffee, E. (1942). Observation on the mechanism of penicillin. *Proc. Soc. Exp. Biol. Med.* 50, 281–285. doi: 10.3181/00379727-50-13773

Hood, D. W., Heidstra, R., Swoboda, U. K., and Hodgson, D. A. (1992). Molecular genetic analysis of proline and tryptophan biosynthesis in *Streptomyces coelicolor* A3(2): interaction between primary and secondary metabolism – a review. *Gene* 115, 5–12. doi: 10.1016/0378-1119(92)90533-U

Jolliffe, L. K., Doyle, R. J., and Streips, U. N. (1981). The energized membrane and cellular autolysis in *Bacillus subtilis*. *Cell* 25, 753–763. doi: 10.1016/0092-8674(81)90183-5

Kemper, M. A., Urrutia, M. M., Beveridge, T. J., Koch, A. L., and Doyle, R. J. (1993). Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. *J. Bacteriol.* 175, 5690–5696.

Khairnar, K., Sanmukh, S., Chandekar, R., and Paunikar, W. (2014). A simple and novel modification of comet assay for determination of bacteriophage mediated bacterial cell lysis. *J. Virol. Methods* 203, 33–38. doi: 10.1016/j.jviromet.2014.02.028

Konno, H., Matsuya, H., Okamoto, M., Sato, T., Tanaka, Y., Yokoyama, K., et al. (1998). Prodigiosins uncouple mitochondrial and bacterial F-ATPases: evidence for their H+/Cl- symport activity. *J. Biochem.* 124, 547–556. doi: 10.1093/oxfordjournals.jbchem.a022147

Kramer, V. C., Calabrese, D. M., and Nickerson, K. W. (1980). Growth of *Enterobacter cloacae* in the presence of 25% sodium dodecyl sulfate. *Appl. Environ. Microbiol.* 40, 973–976.

Kumar, N. R., and Nair, S. (2007). *Vibrio rhizosphaerae* sp. nov.; a red-pigmented bacterium that antagonizes phytopathogenic bacteria. *Int. J. Syst. Evol. Microbiol.* 57, 2241–2246. doi: 10.1099/ijs.0.65017-0

Lacriola, C. J., Falk, S. P., and Weisblum, B. (2013). Screen for agents that induce autolysis in *Bacillus subtilis*. *Antimicrob. Agents Chemother.* 57, 229–234. doi: 10.1128/AAC.00741-12

Lee, J. S., Kim, Y. S., Park, S., Kim, J., Kang, S. J., Lee, M. H., et al. (2011). Exceptional production of both prodigiosin and cycloprodigiosin as major metabolic constituents by a novel marine bacterium, *Zooshikella rubidus* S1-1. *Appl. Environ. Microbiol.* 77, 4967–4973. doi: 10.1128/AEM.01986-10

Margot, P., and Karamata, D. (1992). Identification of the structural genes for N-acetylmuramoyl-L-alanine amidase and its modifier in *Bacillus subtilis* 168: inactivation of these genes by insertional mutagenesis has no effect on growth or cell separation. *Mol. Gen. Genet.* 232, 359–366. doi: 10.1007/BF00266238

Margot, P., Whalen, M., Ghdamhoseinian, A., Piggot, P., and Karamata, D. (1998). The lytE gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. *J. Bacteriol.* 180, 749–752.

Melvin, M. S., Tomlinson, J. T., Saluta, G. R., Kucera, G. L., Lindquist, N., and Manderville, R. A. (2000). Double-strand DNA cleavage by copper-prodigiosin. *J. Am. Chem. Soc.* 122, 6333–6334. doi: 10.1021/ja0000798

Mensa, B., Kim, Y. H., Choi, S., Scott, R., Caputo, G. A., and DeGrado, W. F. (2011). Antibacterial mechanism of action of arylamide foldamers. *Antimicrob. Agents Chemother.* 55, 5043–5053. doi: 10.1128/AAC.05009-11

Meschke, H., Walter, S., and Schrempf, H. (2012). Characterization and localization of prodiginines from *Streptomyces lividans* suppressing *Verticillium dahliae* in the absence or presence of *Arabidopsis thaliana*. *Environ. Microbiol.* 14, 940–952. doi: 10.1111/j.1462-2920.2011.02665.x

Nakashima, T., Miyazaki, Y., Matsuyama, Y., Muraoka, W., Yamaguchi, K., and Oda, T. (2006). Producing mechanism of an algicidal compound against red tide phytoplankton in a marine bacterium γ-proteobacterium. *Appl. Microbiol. Biotechnol.* 73, 684–690. doi: 10.1007/s00253-006-0507-2

Okamoto, H., Sato, Z., Sato, M., Koiso, Y., Iwasaki, S., and Isaka, M. (1998). Identification of antibiotic red pigments of *Serratia marcescens* F-1-1, a biocontrol agent of damping-off of cucumber, and antimicrobial activity against other plant pathogens. *Ann. Phytopathol. Soc. Jpn.* 64, 294–298. doi: 10.3186/jjphytopath.64.294

Ostling, O., and Johanson, K. J. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* 123, 291–298. doi: 10.1016/0006-291X(84)90411-X

Pérez-Tomás, R., Montaner, B., Llagostera, E., and Soto-Cerrato, V. (2003). The prodigiosins, proapoptotic drugs with anticancer properties. *Biochem. Pharmacol.* 66, 1447–1452. doi: 10.1016/S0006-2952(03)00496-9

Priya, K. A., Satheesh, S., Ashokkumar, B., Varalakshmi, P., Selvakumar, G., and Sivakumar, N. (2013). "Antifouling activity of prodigiosin from estuarine isolate of *Serratia marcescens* CMST 07," in *Microbiological Research in Agroecosystem Management*, Vol. XVI, ed. R. K. Velu (New Delhi: Springer), 11–21. doi: 10.1007/978-81-322-1087-0_2

Ryazantseva, I. N., Andreyeva, I. N., Klementyeva, G. S., Ogorodnikova, T. I., and Petrov, V. Y. (1995). Pigment-dependent light influence on the energetics of *Serratia marcescens*. *Thermochim. Acta* 251, 63–67. doi: 10.1016/0040-6031(94)02148-H

Sawabe, T., Makino, H., Tatsumi, M., Nakano, K., Tajima, K., Iqbal, M. M., et al. (1998). *Pseudoalteromonas bacteriolytica* sp. nov.; a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*. *Int. J. Syst. Bacteriol.* 48, 769–774. doi: 10.1099/00207713-48-3-769

Seganish, J. L., and Davis, J. T. (2005). Prodigiosin is a chloride carrier that can function as an anion exchanger. *Chem. Commun.* 46, 5781–5783. doi: 10.1039/B511847F

Shahbabian, K., Jamalli, A., Zig, L., and Putzer, H. (2009). Rnase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *EMBO J.* 28, 3523–3533. doi: 10.1038/emboj.2009.283

Shieh, W. Y., Chen, Y. W., Chaw, S. M., and Chiu, H. H. (2003). *Vibrio ruber* sp. nov.; a red, facultatively anaerobic, marine bacterium isolated from sea water. *Int. J. Syst. Evol. Microbiol.* 53, 479–484. doi: 10.1099/ijs.0.02307-0

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175, 184–191. doi: 10.1016/0014-4827(88)90265-0

Smith, T. J., Blackman, S. A., and Foster, S. J. (2000). Autolysis of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology* 146, 249–262. doi: 10.1099/00221287-146-2-249

Someya, N., Nakajima, M., Hirayae, K., Hibi, T., and Akutsu, K. (2001). Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produces by biocontrol bacterium, *Serratia marcescens* strain B2 against gray mold pathogen, *Botrytis cinerea*. *J. Gen. Plant Pathol.* 67, 312–317. doi: 10.1007/PL00013038

Stankovic, N., Radulovic, V., Petkovic, M., Vuckovic, I., Jadranin, M., Vasiljevic, B., et al. (2012). *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Appl. Microbiol. Biotechnol.* 96, 1217–1231. doi: 10.1007/s00253-012-4237-3

Stankovic, N., Senerovic, L., Ilic-Tomic, T., Vasiljevic, B., and Nikodinovic-Runic, J. (2014). Properties and applications of undecylprodigiosin and other bacterial prodigiosins. *Appl. Microbiol. Biotechnol.* 98, 3841–3858. doi: 10.1007/s00253-014-5590-1

Starič, N., Danevčič, T., and Stopar, D. (2010). *Vibrio* sp. DSM14379 pigment production – a competitive advantage in the environment? *Microb. Ecol.* 60, 592–598. doi: 10.1007/s00248-010-9671-0

Stefanic, P., Kraigher, B., Lyons, N. A., Kolter, R., and Mandic-Mulec, I. (2015). Kin discrimination between sympatric *Bacillus subtilis* isolates. *Proc. Natl. Acad. Sci. U.S.A.* 112, 14042–14047. doi: 10.1073/pnas.1512671112

Stefanic, P., and Mandic-Mulec, I. (2009). Social interactions and distribution of *Bacillus subtilis* pherotypes at microscale. *J. Bacteriol.* 191, 1756–1764. doi: 10.1128/JB.01290-08

Stopar, D., Cerne, A., Zigman, M., Poljsak-Prijatelj, M., and Turk, V. (2004). Viral abundance and a high proportion of lysogens suggest that viruses are important members of the microbial community in the Gulf of Trieste. *Microb. Ecol.* 47, 1–8. doi: 10.1007/s00248-002-3009-5

Tsuchido, T., Hiraoka, T., Takano, M., and Shibasaki, I. (1985). Involvement of autolysin in *Bacillus subtilis* induced by short– and medium-chain fatty acids. *J. Bacteriol.* 162, 42–46.

Williamson, N. R., Fineran, P. C., Gristwood, T., Chawrai, S. R., Leeper, F. J., and Salmond, G. P. C. (2006). The biosynthesis and regulation of bacterial prodiginines. *Nat. Rev. Microbiol.* 4, 887–899. doi: 10.1038/nrmicro1531

Williamson, N. R., Fineran, P. C., Gristwood, T., Chawrai, S. R., Leeper, F. J., and Salmond, G. P. C. (2007). Anticancer and immunosuppressive properties of bacterial prodiginines. *Future Microbiol.* 2, 605–618. doi: 10.2217/17460913.2.6.605

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frontiers in Microbiology

MINI REVIEW
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Bioengineering Lantibiotics for Therapeutic Success

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Several examples of highly modified antimicrobial peptides have been described. While many such peptides are non-ribosomally synthesized, ribosomally synthesized equivalents are being discovered with increased frequency. Of the latter group, the lantibiotics continue to attract most attention. In the present review, we discuss the implementation of *in vivo* and *in vitro* engineering systems to alter, and even enhance, the antimicrobial activity, antibacterial spectrum and physico-chemical properties, including heat stability, solubility, diffusion and protease resistance, of these compounds. Additionally, we discuss the potential applications of these lantibiotics for use as therapeutics.

Keywords: antimicrobial peptide, nisin, mutagenesis, lantibiotic, post-translational modification, bacteriocin, bacterial resistance

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INTRODUCTION

Given that antibiotic resistance has now reached a crisis point, novel compounds and innovative methods are urgently required to arrest the spread and development of drug-resistant infections in both the nosocomial and community environments. Ideally, such novel substances should exhibit distinctly different mechanisms of action to currently used chemotherapeutics in order to decrease resistance development. Ribosomally synthesized antimicrobial peptides produced by bacteria (bacteriocins) constitute an emerging class of natural products that have attracted considerable interest as promising alternatives to existing antibiotics (Sahl and Bierbaum, 2008). Within this diverse group of peptides, the lantibiotics, i.e., class I bacteriocins which contain the post-translationally modified amino acids lanthionine and methyllanthionine, have become the focus of many biomedical and pharmaceutical research groups due to their demonstrable high potency *in vitro*, multiple modes of action and ability to destroy target cells rapidly (Cotter et al., 2005; Cavera et al., 2015). In general, lantibiotics exhibit activity against Gram positive bacteria. Importantly, this includes many drug resistant targets including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin intermediate *S. aureus* (VISA), vancomycin resistant enterococci (VRE), *Streptococcus pneumoniae* and *Clostridium difficile*, amongst others (Cotter et al., 2013). Furthermore, several lantibiotic peptides have demonstrated excellent *in vivo* activities and have progressed toward clinical evaluation for the treatment of life-threatening diseases (Dawson and Scott, 2012; Sandiford, 2015). Indeed, these and a range of other desirable features make them suitable for use in human and veterinary medicine and also in the pharmaceutical industry (Dischinger et al., 2014). However, despite these promising attributes, there are a number of limitations that has prevented their more widespread use, including instability and/or insolubility at physiological pH, low production levels and susceptibility to proteolytic digestion. The implementation of multiple technologies, including genome mining as well as high-throughput screening strategies in combination with *in vivo* and *in vitro* expression systems has provided a wealth of information relating to the widespread existence, structural diversity and functionality of lantibiotics while facilitating the identification

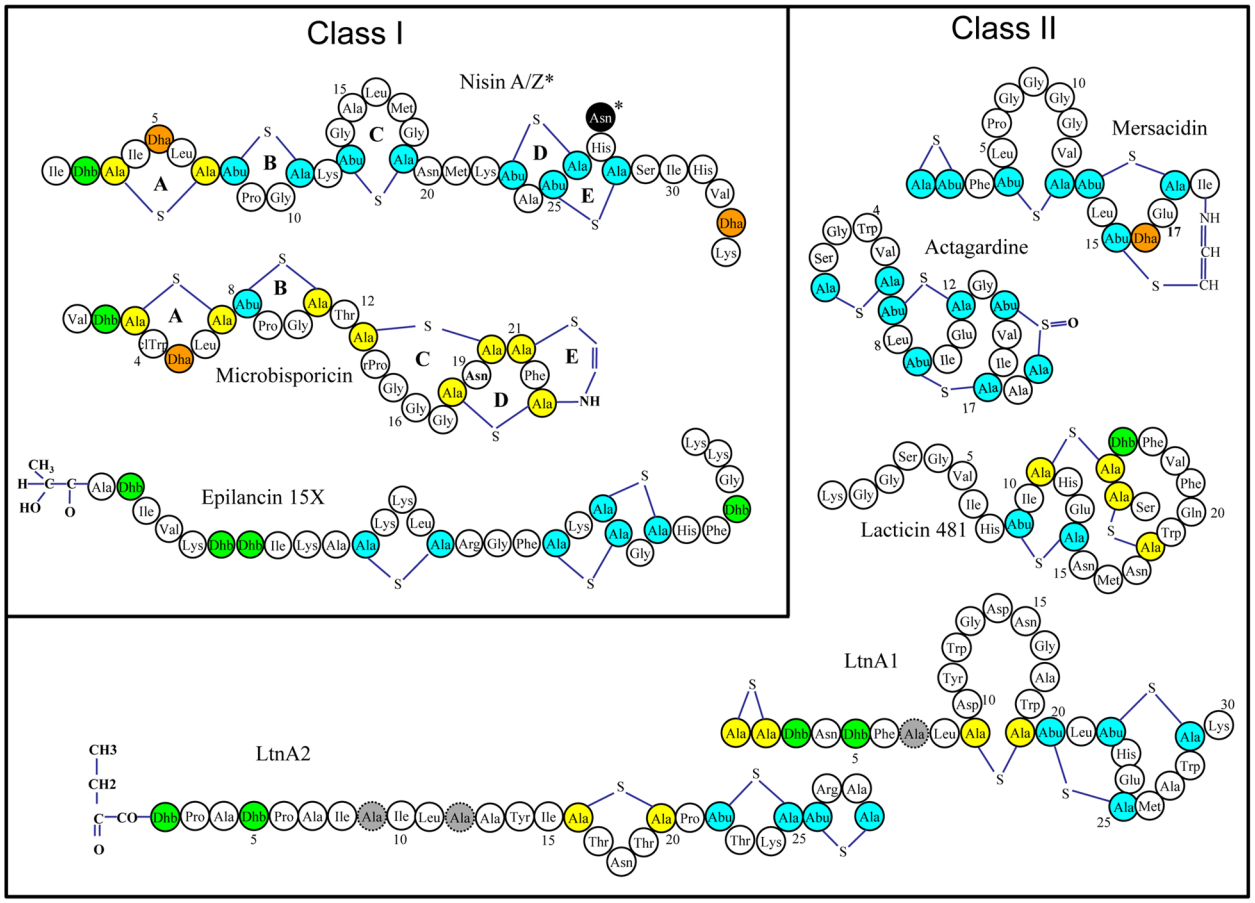

FIGURE 1 | Representative structures of various single and two-component lantibiotics. Post translational modifications are indicated as follows: Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine, Dha: dehydroalanine, Dhb: dehydrobutyrine. D-Ala residues (in lacticin 3147) are shaded gray.

of structural regions that can be targeted to enhance their biological and physicochemical properties. The present review will focus on recent developments with regard to these achievements.

LANTIBIOTICS: THE CASE FOR THERAPEUTIC USE (*IN VITRO* AND *IN VIVO* POTENCY)

New antimicrobials that possess novel modes of action, particularly against drug resistant organisms so that they can be specifically targeted for clinical applications, are required as a matter of urgency. In this regard lantibiotics hold considerable potential as a consequence of their unusual structure, unique mechanisms of action and their potency against multi-drug resistant bacteria. Today, close to 100 of these bioactive peptides have been described, the majority of which are produced by Gram-positive bacteria (Dischinger et al., 2014). The common feature that links all lantibiotics is the presence of a number of distinctive amino acids which result from enzymatically mediated post-translational modifications, including dehydration and cyclisation, leading to the formation of the eponymous (methyl)lanthionine bridges. These bridges convert the linear peptide chain into a polycyclic form giving structure and function to the peptide. It should be noted that only those peptides that display antimicrobial activity within the larger family of lanthionine-containing peptides or lanthipeptides are termed lantibiotics.

Many lantibiotics exert their antimicrobial action through complexation with lipid II, an essential precursor of the bacterial cell wall, either by inhibiting cell wall synthesis through sequestration of lipid II and/or by disruption of membrane integrity and pore formation (Breukink and de Kruijff, 2006). Indeed, the prototypical and best studied lantibiotic nisin performs both of these functions as a consequence of two distinct structural domains located at the N- and C-termini (**Figure 1**). It has been established that the A, B, and C rings form a "cage-like" enclosure that facilitates binding of the pyrophosphate moiety of lipid II, thus inhibiting cell wall synthesis (Hsu et al., 2004) This binding enhances the ability of the C-terminal segment, containing rings D and E, to form pores in the cell membrane, resulting in the rapid efflux of ions and cytoplasmic solutes (Wiedemann et al., 2001). This mechanism of action is not common to all lantibiotics, and some of them lack the

TABLE 1 | A Selection of Lantibiotics and their Potential Therapeutic Applications.

Lantibiotic	Commercially relevant targets	*In vivo* tests	Potential applications	Reference
Nisin	Gram positive bacteria	✓	Treatment of staphylococcal (including MRSA) and enterococcal infections. Treatment of bacterial mastitis. Oral hygiene, deodorants. Anti-cancer	Mota-Meira et al. (2000); Brumfitt et al. (2002); Cotter et al. (2005); Piper et al. (2009b); Joo et al. (2012); Kamarajan et al. (2015)
Mersacidin	MRSA VRE, *C. difficile*	✓	Treatment of staphylococcal (including MRSA) and enterococcal infections. Treatment of CDAD	Niu and Neu (1991); Hoffmann et al. (2002); Appleyard et al. (2009)
Actagardine	MRSA, VRE, *C. difficile*	✓	Treatment of staphylococcal (including MRSA) and enterococcal infections. Treatment of CDAD	Hoffmann et al. (2002)
Deoxyactagardine/NVB302	*C. difficile*	✓	Treatment of *C. difficile* infections	Dawson and Scott (2012)
Gallidermin/Epidermin	*Propionibacteria*, Staphylococci, Streptococci	✓	Skin disorders including acne, eczema, folliculitis and impetigo	Bonelli et al. (2006)
Pinensins	Yeast/fungi		Antifungal/yeast	Mohr et al. (2015)
Planosporicin	MRSA, VRE, Streptococci	✓	Treatment of staphylococcal (including MRSA) and enterococcal infections including VRE	Castiglione et al. (2007)
Microbisporicin	MRSA, VISA, VRE, *C. difficile*	✓	Treatment of staphylococcal (including MRSA and VISA) and enterococcal infections including VRE. Acne	Castiglione et al. (2008)
Mutacin B-Ny266	Multi-drug resistant bacteria	✓	Treatment of multi-drug resistant bacteria including MRSA and VRE	Mota-Meira et al. (2000)
Lacticin 3147	Gram positive bacteria	✓	Treatment of bacterial mastitis. staphylococcal and enterococcal infections including VRE. Acne	Galvin et al. (1999); Lawton et al. (2007); Piper et al. (2009b)
Salivaricin B	Streptococci including *S. pyogenes* and *S. sobrinus*	✓	Treatment of streptococcal infections with emphasis on the causative agents of sore throats (caused mainly by *S. pyogenes*) and dental caries (caused in part by *S. sobrinus*).	Tagg (2004); Wescombe et al. (2009)
Duramycin	Increase chloride transport and fluid secretions	✓	Treatment of Cystic Fibrosis, ocular diseases and disorders	Grasemann et al. (2007); Oliynyk et al. (2010)

ability to elicit pores or to bind lipid II or both, but can still exhibit antimicrobial activity (Pag and Sahl, 2002). The poor activity of lantibiotics toward Gram negative bacteria is due to the outer membrane (OM) of the Gram negative cell wall which acts as a barrier for the cell, restricting the access of the peptides to the cytoplasmic membrane (Nikaido and Vaara, 1985).

Lantibiotics have been classified on the basis of their biosynthetic pathways (Willey and van der Donk, 2007). According to this scheme, class I lantibiotics are those modified by two separate enzymes, a LanB (dehydratase) and LanC (cyclase); class II are modified by a single LanM enzyme with both dehydratase and cyclase activity. The third and fourth classes of lanthipeptides are also modified by a single enzyme (general nomenclature LanKC for class III and LanL for class IV; van der Donk and Nair, 2014). Most of the class III lanthipeptides reported thus far have no or weak antimicrobial activities, but some have been shown to possess anti-allodynic/antinociceptive activity (Meindl et al., 2010; Iorio et al., 2014), antiviral activity (Férir et al., 2013) or morphogenetic activities (Willey and Gaskell, 2011). The designation Lan is used generically to refer to proteins associated with the biosynthesis of, or immunity to, lantibiotics. A typical lantibiotic operon will also contain genes encoding enzymes to carry out transport/processing (LanT), immunity (LanI and LanFEG), proteolytic processing (LanP) as well as the structural gene (LanA). Other enzymes, responsible for the formation of less common residues may also be present. Importantly, individual components of the lantibiotic biosynthetic machinery show even greater flexibility as demonstrated by their activity *in vitro* (Li et al., 2009).

ACTIVITY OF LANTIBIOTICS *IN VITRO*

Although lantibiotics such as nisin have been in use for decades as safe and natural food preservatives (Delves-Broughton, 2005), the continued escalation of multi-drug resistant bacterial infections has led to a re-appraisal of their capacity for use against life-threatening infections. A multitude of studies have highlighted the *in vitro* potency of lantibiotics against nosocomial pathogens (the reader is directed to a comprehensive review: Piper et al., 2009a). Many lantibiotics, including lacticin 3147, mutacins B-Ny266 and 1140, nisin, mersacidin, epidermin, Pep5, and planosporicin exhibit activity against clinically-relevant targets (**Table 1**) such as MRSA, VRE, *Propionibacterium acne*, *Streptococcus mutans*, *Streptococcus pyogenes*, *S. pneumoniae*, *C. difficile*, *Listeria*, and *Bacillus* species (Severina et al., 1998; Galvin et al., 1999; Mota-Meira et al., 2000; Brumfitt et al., 2002; Rea et al., 2007; Ghobrial et al., 2009; Piper et al., 2009b). Notably, both Pep 5 and epidermin successfully inhibit the adhesion of staphylococcal cells to the surfaces of siliconized catheters (Fontana et al., 2006). Although it is the general view that lantibiotics exhibit less potential as chemotherapeutics to combat infections with Gram-negative organisms, lantibiotics including mutacin B-Ny266 are selectively active against a few strains of *Neisseria* and *Helicobacter*

(Mota-Meira et al., 2000), while purified nisin displays activity against *Escherichia coli* (Kuwano et al., 2005).

Nisin has also been shown to effectively inhibit spore outgrowth including spores of *Bacillus anthracis* (Gut et al., 2008) and those of *C. difficile* (Nerandzic and Donskey, 2010). Additionally, studies have revealed that the lantibiotic gallidermin efficiently prevents biofilm formation in both *S. aureus* and *S. epidermidis* species (Saising et al., 2012).

Recently, an intriguing and novel (sub-)class of lantibiotics termed pinensins were found to be highly active against many filamentous fungi and yeasts but displayed only weak antibacterial activity. Not only do pinensin A and pinensin B represent the first examples of a lantibiotic fungicide, they are also the first lantibiotics to be isolated from a Gram-negative native producer (Mohr et al., 2015).

LANTIBIOTICS DEMONSTRATE *IN VIVO* POTENCY

While the *in vitro* success of a chemotherapeutic agent does not always necessarily translate to *in vivo* efficacy, there have been a number of encouraging studies to suggest that this may not be a major shortcoming of lantibiotics. For instance, mutacin B-Ny266 was shown to be as active as vancomycin against MRSA *in vivo* (Mota-Meira et al., 2005), mersacidin was able to effectively eradicate an MRSA infection in a mouse rhinitis model (Kruszewska et al., 2004) and Nisin F, a natural nisin variant, was also found to successfully control *S. aureus* infection in rats (De Kwaadsteniet et al., 2009). Similarly, microbsporicin (**Figure 1**) (NAI-107) was evaluated for its therapeutic potential in nosocomial infection and demonstrated efficacy against MRSA in a rat endocarditis model (Jabes et al., 2011). The efficacy of MU1140 (mutacin 1140) has also been investigated *in vivo* (Ghobrial et al., 2009) and is currently in pre-clinical development for the treatment of Gram positive infections. NVB302, a derivative of deoxyactagardine B, is currently undergoing phase I clinical trials as a therapeutic for the treatment of *C. difficile* infections due to its selective targeting of this organism over the predominantly Gram negative normal gut flora (Dawson and Scott, 2012). Investigations into the use of lantibiotics to control the microorganisms responsible for dental plaque, halitosis and "sore throat" infections have also yielded promising results (Hillman, 2002; Burton et al., 2006; Dierksen et al., 2007).

Some lantibiotics possess additional bioactivities that hold promise for therapeutic application. A smaller subcategory of lantibiotics, such as cinnamycin and duramycin, have been found to influence eukaryotic metabolic functions by binding phosphatidylethanolamine in cell membranes and, in turn, inhibiting the enzyme phospholipase A2 (Marki et al., 1991). In addition to this activity, duramycin demonstrated efficacy in the treatment of cystic fibrosis by inhalation (Grasemann et al., 2007) as a result of its ability to stimulate chloride secretion in bronchial epithelial cells (Oliynyk et al., 2010).

Remarkably, the first instance of a lantibiotic, or indeed any bacteriocin, to prevent the growth of cancer cells has been confirmed. In a study by Joo and coworkers, nisin Z was shown to be effective in the treatment of head and neck squamous cell carcinoma (HNSCC; Joo et al., 2012). In subsequent mouse trials involving a highly purified form of nisin Z, reduced tumorigenesis *in vivo* was observed and long-term treatment with nisin Z extended survival. In addition, nisin treated mice exhibited normal organ histology with no evidence of inflammation, fibrosis or necrosis (Kamarajan et al., 2015).

BIOENGINEERING AND SYNTHETIC BIOLOGY- GENERATING MORE EFFECTIVE LANTIBIOTICS

Bioengineering (engineering within the cell) and the use of synthetic biology-based (*in vitro* engineering) approaches have been important for advancing our understanding of the fundamentals of bacteriocin activity and structure–function relationships (these approaches are the subject of a number of recent comprehensive reviews: Tabor, 2014; Escano and Smith, 2015). However, there is also a steadily growing number of engineered lantibiotic peptides that demonstrate enhanced functionalities (activity and/or stability) which make them more attractive from a clinical perspective (Cotter et al., 2013). The following provides some recent examples of bioengineered lantibiotics exhibiting enhanced pharmacological and physicochemical properties as well as developments in genetic systems to increase peptide yields.

Several bioengineered variants of the prototypical lantibiotic nisin have been generated that provide excellent examples of how lantibiotic functionality can be modulated by as little as one residue change. The nisin Z derivatives N20K, M21K, N27K, H31K generated by protein engineering displayed improved solubility, particularly at alkaline pH values where the solubility of the parent nisin is particularly reduced (Rollema et al., 1995; Yuan et al., 2004). Furthermore, the consequences of effecting single residue alterations at distinct locations in nisin has generated variants that exhibit not only improved antimicrobial activity against strains of clinical relevance (MRSA, VRE, VISA, MRSP, and *C. difficile*) but has also brought about the widening of its antimicrobial spectrum to include some Gram negative bacteria (Field et al., 2008, 2012, 2015; Molloy et al., 2013). More dramatic substitutions at the location of rings A and B at the N-terminal end of nisin A revealed that the various activities of nisin can be altered by changing the amino acid arrangement in this region of the peptide (Rink et al., 2007). The hinge-region of nisin has also been the subject of mutagenesis resulting in variants with enhanced antimicrobial activity (Field et al., 2008; Healy et al., 2013) as well as derivatives with an enhanced ability to diffuse through complex polymers (Rouse et al., 2012). In both mutacin 1140 and nukacin ISK-1 peptides, single residue changes brought about a significant increase in activity against several Gram positive strains(Islam et al., 2009; Chen et al., 2013). Similarly, mutagenesis of the mersacidin gene was ultimately successful in that several variants were identified which exhibited enhanced activity against a range of different targets including clinically relevant MRSA, VRE and *S. pneumoniae* (Appleyard et al., 2009).

Generating enhanced variants of two-component lantibiotics presents an even greater challenge given that two peptides are required to work jointly in synergy. However, a lacticin 3147

derivative with enhanced activity against a pathogenic strain of *S. aureus* was recently identified (Field et al., 2013), the first occasion such an increase in antibacterial properties has been observed for bioengineered two-component lantibiotics.

Synthetic biology approaches are another promising means to provide insights into structure-stability relationships and generate novel derivatives with improved function. Chemical synthesis enables the limitations of the modification machinery to be bypassed, extending the range of analogs that can be produced. For example, deoxyactagardine B is a single peptide lantibiotic that is rigid, compact and globular and differs from actagardine (**Figure 1**) by two amino acids and the absence of a sulfoxide bond (Boakes et al., 2010). A synthetically introduced C-terminal modification (1, 7 diaminoheptane) produced a variant, NVB302, that displayed greater solubility and activity compared to the parent molecule. NVB302 is now in phase I clinical trials for the treatment of *C. difficile* infections (Dawson and Scott, 2012). It has also been established that lantibiotics are susceptible to oxidation of the sulfur-containing lanthionine and this can lead to sharp decreases in antimicrobial activity. In the case of lactocin S, lanthionines were replaced with diaminopimelate to produce several analogs, one of which revealed greater stability whilst still retaining 100% biological activity (Ross et al., 2012).

Chemical synthesis methods were employed to produce enhanced analogs of the lantibiotic epilancin 15X (Knerr and van der Donk, 2012). A novel approach termed *in vitro* mutasynthesis has produced improved variants of the class II lantibiotic lacticin 481. Here, non-standard amino acids were introduced into the structural peptide by organic synthesis, and subsequently modified *in vitro* with purified LctM to generate derivatives with superior specific activity against a target strain (Levengood et al., 2009). Notably, synthetic chemistry approaches were employed to generate hybrids of nisin and vancomycin that demonstrated a 40-fold increase in potency compared to each of the components separately (Arnusch et al., 2008). Similarly, the nisin N-terminus (1–12) was synthetically modified by the coupling of simple membrane-active lipids to create biologically active and proteolytically stable hybrids (Koopmans et al., 2015).

Regardless of these bioengineering successes, one concern that remains to be tackled is that of production. Indeed, the discovery, study and application of lantibiotics is often compromised by limited, or the absence of, production of these peptides by the native producer, a problem which is further compounded when working with bioengineered derivatives. However, a number of instances have demonstrated that quite the opposite effect can be achieved in terms of production. In the case of mutacin 1140 and nukacin ISK-1, single residue alterations did not increase specific activity but instead increased peptide production by up to fourfold (Islam et al., 2009; Chen et al., 2013). Importantly, a recent study involving synthetic biology approaches describes the development of a genetic system that facilitates significant overproduction of nisin (Kong and Lu, 2014). Although heterologous expression of lantibiotic peptides (and their bioengineered derivatives) has been demonstrated in the Gram negative host *E. coli* on several occasions (Nagao et al., 2007; Caetano et al., 2011, 2014; Shi et al., 2012; Basi-Chipalu et al., 2015), a recent study describes a multigene assembly strategy for the overexpression of the two-component lantibiotic lichenicidin in *E. coli* (Kuthning et al., 2015). Such systems may also help in attaining higher yields to simplify isolation of and improve cost-efficiency of novel derivatives that are often compromised by limited production.

A major drawback that has yet to be overcome with respect to therapeutic use is the sensitivity of lantibiotics to proteolytic cleavage by intestinal enzymes. For example, nisin, pep5 and epidermin have been shown to be susceptible to the proteases trypsin and chymotrypsin (Jarvis and Mahoney, 1969; Bierbaum et al., 1996). Bioengineering strategies could be employed to replace the residues that serve as recognition sites by these and other digestive enzymes and potentially overcome the issue of vulnerability to proteolytic breakdown in the gastrointestinal tract. Indeed, the recent discovery of the class II lantibiotic pseudomycoicidin (which was found to be resistant to trypsin) provides the perfect example for this approach. A trypsin cleavage site which is located in the conserved lipid II binding motif, is protected by the presence of at least one thioether ring structure. This was confirmed by experiments with site-directed mutant peptides where the removal of thioether forming Cys residues resulted in the establishment of protease sensitivity (Basi-Chipalu et al., 2015).

Lastly, it should be remarked that the efficacy of individual lantibiotics could be further boosted through combination with other antimicrobials or membrane-active substances. For example, nisin displayed synergistic activity with the antibiotics colistin and clarithromycin against *Pseudomonas aeruginosa* (Giacometti et al., 2000) and with ramoplanin and other non-β-lactam antibiotics against many strains of MRSA and VRE (Brumfitt et al., 2002). Similarly, nisin-ceftriaxone and nisin-cefotaxime were found to be highly synergistic against clinical isolates of *Salmonella enterica* serovar Typhimurium as evident by checkerboard test and time-kill assay (Rishi et al., 2014).

CONCLUSION

Lantibiotics possess many of the attributes essential for the treatment of infections caused by multi-drug resistant bacteria and their potential for use as alternatives to traditional antibiotic therapies has been mooted for decades. While greater than 100 lantibiotic peptides have been described, not all of these have been characterized in great depth and so many may possess traits of commercial value. Indeed, as the number of microbial genome sequences has increased dramatically, an even larger collection of new lantibiotic biosynthetic gene clusters has been revealed. These clusters can be applied directly or, the information gained from their analysis, can be used indirectly to guide the bioengineering of new and existing peptide structures.

Finally, although nisin remains the only lantibiotic that is extensively exploited, its full use as a therapeutic entity has not yet been fulfilled, in part due to its low solubility and stability at physiological pH. It is thus notable that a broad range of technologies have been developed for the engineering of lantibiotics and the past decade has seen several bioengineering studies describe the generation of peptide derivatives including nisin with enhanced functionality in terms

of specific activity, spectrum of activity, solubility and/or temperature and pH stability. Critically, genetic systems are in continuous development to increase yields of peptide that may aid commercial viability. The further application of these systems to enhance nisin and other lantibiotics has the potential to lead to the development of novel derivatives for therapeutic use. Additionally, bioengineering in combination with semi-synthesis will expand structural diversity still further. It is thus likely that these peptides will be only the first of many generations of bioengineered lantibiotic and lantibiotic-like peptides. Given these recent developments and the fact that several lantibiotics are currently in clinical and preclinical trials reinforces our belief that bioengineered lantibiotics can contribute to a solution to antibiotic resistance across a broad range of bacterial pathogens.

AUTHOR CONTRIBUTIONS

DF drafted the manuscript. PC, CH, and RR revised and approved the final manuscript.

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REFERENCES

Appleyard, A. N., Choi, S., Read, D. M., Lightfoot, A., Boakes, S., Hoffmann, A., et al. (2009). Dissecting structural and functional diversity of the lantibiotic mersacidin. *Chem. Biol.* 16, 490–498. doi: 10.1016/j.chembiol.2009.03.011

Arnusch, C. J., Bonvin, A. M., Verel, A. M., Jansen, W. T., Liskamp, R. M., de Kruijff, B., et al. (2008). The vancomycin-nisin(1–12) hybrid restores activity against vancomycin resistant *Enterococci*. *Biochemistry* 47, 12661–12663. doi: 10.1021/bi801597b

Basi-Chipalu, S., Dischinger, J., Josten, M., Szekat, C., Zweynert, A., Sahl, H. G., et al. (2015). Pseudomycoicidin, a Class II Lantibiotic from *Bacillus pseudomycoides*. *Appl. Environ. Microbiol.* 81, 3419–3429. doi: 10.1128/AEM.00299-15

Bierbaum, G., Szekat, C., Josten, M., Heidrich, C., Kempter, C., Jung, G., et al. (1996). Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. *Appl. Environ. Microbiol.* 62, 385–392.

Boakes, S., Appleyard, A. N., Cortes, J., and Dawson, M. J. (2010). Organization of the biosynthetic genes encoding deoxyactagardine B (DAB), a new lantibiotic produced by Actinoplanes liguriae NCIMB41362. *J. Antibiot (Tokyo)* 63, 351–358. doi: 10.1038/ja.2010.48

Bonelli, R. R., Schneider, T., Sahl, H. G., and Wiedemann, I. (2006). Insights into *in vivo* activities of lantibiotics from gallidermin and epidermin mode-of-action studies. *Antimicrob. Agents Chemother.* 50, 1449–1457. doi: 10.1128/AAC.50.4.1449-1457.2006

Breukink, E., and de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nat. Rev. Drug Discov.* 5, 321–332. doi: 10.1038/nrd2004

Brumfitt, W., Salton, M. R., and Hamilton-Miller, J. M. (2002). Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. *J. Antimicrob. Chemother.* 50, 731–734. doi: 10.1093/jac/dkf190

Burton, J. P., Chilcott, C. N., Moore, C. J., Speiser, G., and Tagg, J. R. (2006). A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. *J. Appl. Microbiol.* 100, 754–764. doi: 10.1111/j.1365-2672.2006.02837.x

Caetano, T., Barbosa, J., Moesker, E., Sussmuth, R. D., and Mendo, S. (2014). Bioengineering of lanthipeptides in *Escherichia coli*: assessing the specificity of lichenicidin and haloduracin biosynthetic machinery. *Res. Microbiol.* 165, 600–604. doi: 10.1016/j.resmic.2014.07.006

Caetano, T., Krawczyk, J. M., Mosker, E., Sussmuth, R. D., and Mendo, S. (2011). Heterologous expression, biosynthesis, and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *Escherichia coli*. *Chem. Biol.* 18, 90–100. doi: 10.1016/j.chembiol.2010.11.010

Castiglione, F., Cavaletti, L., Losi, D., Lazzarini, A., Carrano, L., Feroggio, M., et al. (2007). A novel lantibiotic acting on bacterial cell wall synthesis produced by the uncommon actinomycete *Planomonospora* sp. *Biochemistry* 46, 5884–5895. doi: 10.1021/bi700131x

Castiglione, F., Lazzarini, A., Carrano, L., Corti, E., Ciciliato, I., Gastaldo, L., et al. (2008). Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. *Chem. Biol.* 15, 22–31. doi: 10.1016/j.chembiol.2007.11.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., and Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *Int. J. Antimicrob. Agents* 46, 494–501. doi: 10.1016/j.ijantimicag.2015.07.011

Chen, S., Wilson-Stanford, S., Cromwell, W., Hillman, J. D., Guerrero, A., Allen, C. A., et al. (2013). Site-directed mutations in the lanthipeptide mutacin 1140. *Appl. Environ. Microbiol.* 79, 4015–4023. doi: 10.1128/AEM.00704-13

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacterial lantibiotics: strategies to improve therapeutic potential. *Curr. Protein Pept. Sci.* 6, 61–75. doi: 10.2174/1389203053027584

Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins—a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11, 95–105. doi: 10.1038/nrmicro2937

Dawson, M. J., and Scott, R. W. (2012). New horizons for host defense peptides and lantibiotics. *Curr. Opin. Pharmacol.* 12, 545–550. doi: 10.1016/j.coph.2012.06.006

De Kwaadsteniet, M., Doeschate, K. T., and Dicks, L. M. (2009). Nisin F in the treatment of respiratory tract infections caused by *Staphylococcus aureus*. *Lett. Appl. Microbiol.* 48, 65–70. doi: 10.1111/j.1472-765X.2008.02488.x

Delves-Broughton, J. (2005). Nisin as a food preservative. *Food Australia* 57, 525–527.

Dierksen, K. P., Moore, C. J., Inglis, M., Wescombe, P. A., and Tagg, J. R. (2007). The effect of ingestion of milk supplemented with salivaricin A-producing *Streptococcus salivarius* on the bacteriocin-like inhibitory activity of streptococcal populations on the tongue. *FEMS Microbiol. Ecol.* 59, 584–591. doi: 10.1111/j.1574-6941.2006.00228.x

Dischinger, J., Basi Chipalu, S., and Bierbaum, G. (2014). Lantibiotics: promising candidates for future applications in health care. *Int. J. Med. Microbiol.* 304, 51–62. doi: 10.1016/j.ijmm.2013.09.003

Escano, J., and Smith, L. (2015). Multipronged approach for engineering novel peptide analogues of existing lantibiotics. *Expert. Opin. Drug Discov.* 10, 857–870. doi: 10.1517/17460441.2015.1049527

Férir, G., Petrova, M. I., Andrei, G., Huskens, D., Hoorelbeke, B., Snoeck, R., et al. (2013). The Lantibiotic peptide labyrinthopeptin A1 demonstrates broad Anti-HIV and Anti-HSV activity with potential for microbicidal applications. *PLoS ONE* 8:e64010. doi: 10.1371/journal.pone.0064010

Field, D., Begley, M., O'Connor, P. M., Daly, K. M., Hugenholtz, F., Cotter, P. D., et al. (2012). Bioengineered nisin a derivatives with enhanced activity against both gram positive and gram negative pathogens. *PLoS ONE* 7:e46884. doi: 10.1371/journal.pone.0046884

Field, D., Gaudin, N., Lyons, F., O'Connor, P. M., Cotter, P. D., Hill, C., et al. (2015). A bioengineered nisin derivative to control biofilms of *Staphylococcus pseudintermedius*. *PLoS ONE* 10:e0119684. doi: 10.1371/journal.pone.0119684

Field, D., Molloy, E. M., Iancu, C., Draper, L. A., PM, O. C., Cotter, P. D., et al. (2013). Saturation mutagenesis of selected residues of the alpha-peptide of the lantibiotic lacticin 3147 yields a derivative with enhanced antimicrobial activity. *Microb. Biotechnol.* 6, 564–575. doi: 10.1111/1751-7915.12041

Field, D., O'Connor, P. M., Cotter, P. D., Hill, C., and Ross, R. P. (2008). The generation of nisin variants with enhanced activity against specific gram-positive pathogens. *Mol. Microbiol.* 69, 218–230. doi: 10.1111/j.1365-2958.2008.06279.x

Fontana, M. B., de Bastos Mdo, C., and Brandelli, A. (2006). Bacteriocins Pep5 and epidermin inhibit *Staphylococcus epidermidis* adhesion to catheters. *Curr. Microbiol.* 52, 350–353. doi: 10.1007/s00284-005-0152-5

Galvin, M., Hill, C., and Ross, R. P. (1999). Lacticin 3147 displays activity in buffer against gram-positive bacterial pathogens which appear insensitive in standard plate assays. *Lett. Appl. Microbiol.* 28, 355–358. doi: 10.1046/j.1365-2672.1999.00550.x

Ghobrial, O. G., Derendorf, H., and Hillman, J. D. (2009). Pharmacodynamic activity of the lantibiotic MU1140. *Int. J. Antimicrob. Agents* 33, 70–74. doi: 10.1016/j.ijantimicag.2008.07.028

Giacometti, A., Cirioni, O., Barchiesi, F., and Scalise, G. (2000). *In vitro* activity and killing effect of polycationic peptides on methicillin-resistant *Staphylococcus aureus* and interactions with clinically used antibiotics. *Diagn. Microbiol. Infect. Dis.* 38, 115–118. doi: 10.1016/S0732-8893(00)00175-9

Grasemann, H., Stehling, F., Brunar, H., Widmann, R., Laliberte, T. W., Molina, L., et al. (2007). Inhalation of Moli1901 in patients with cystic fibrosis. *Chest* 131, 1461–1466. doi: 10.1378/chest.06-2085

Gut, I. M., Prouty, A. M., Ballard, J. D., van der Donk, W. A., and Blanke, S. R. (2008). Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrob. Agents Chemother.* 52, 4281–4288. doi: 10.1128/AAC.00625-08

Healy, B., Field, D., O'Connor, P. M., Hill, C., Cotter, P. D., and Ross, R. P. (2013). Intensive mutagenesis of the nisin hinge leads to the rational design of enhanced derivatives. *PLoS ONE* 8:e79563. doi: 10.1371/journal.pone.0079563

Hillman, J. D. (2002). Genetically modified *Streptococcus mutans* for the prevention of dental caries. *Antonie Van Leeuwenhoek* 82, 361–366. doi: 10.1023/A:1020695902160

Hoffmann, A., Pag, U., Wiedemann, I., and Sahl, H. G. (2002). Combination of antibiotic mechanisms in lantibiotics. *Farmaco* 57, 685–691. doi: 10.1016/S0014-827X(02)01208-9

Hsu, S. T., Breukink, E., Tischenko, E., Lutters, M. A., de Kruijff, B., Kaptein, R., et al. (2004). The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. *Nat. Struct. Mol. Biol.* 11, 963–967. doi: 10.1038/nsmb830

Iorio, M., Sasso, O., Maffioli, S. I., Bertorelli, R., Monciardini, P., Sosio, M., et al. (2014). A glycosylated, labionin-containing lanthipeptide with marked antinociceptive activity. *ACS Chem. Biol.* 9, 398–404. doi: 10.1021/cb400692w

Islam, M. R., Shioya, K., Nagao, J., Nishie, M., Jikuya, H., Zendo, T., et al. (2009). Evaluation of essential and variable residues of nukacin ISK-1 by NNK scanning. *Mol. Microbiol.* 72, 1438–1447. doi: 10.1111/j.1365-2958.2009.06733.x

Jabes, D., Brunati, C., Candiani, G., Riva, S., Romano', G., and Donadio, S. (2011). Efficacy of the new lantibiotic NAI-107 in experimental infections induced by MDR Gram positive pathogens. *Antimicrob. Agents Chemother.* 55, 1671–1676. doi: 10.1128/AAC.01288-10

Jarvis, B., and Mahoney, R. R. (1969). Inactivation of nisin by alpha-chymotrypsin. *J. Dairy Sci.* 52, 1448–1449. doi: 10.3168/jds.S0022-0302(69)86771-8

Joo, N. E., Ritchie, K., Kamarajan, P., Miao, D., and Kapila, Y. L. (2012). Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. *Cancer Med.* 1, 295–305. doi: 10.1002/cam4.35

Kamarajan, P., Hayami, T., Matte, B., Liu, Y., Danciu, T., Ramamoorthy, A., et al. (2015). Nisin ZP, a bacteriocin and food preservative, inhibits head and neck cancer tumorigenesis and prolongs survival. *PLoS ONE* 10:e0131008. doi: 10.1371/journal.pone.0131008

Knerr, P. J., and van der Donk, W. A. (2012). Chemical synthesis and biological activity of analogues of the lantibiotic epilancin 15X. *J. Am. Chem. Soc.* 134, 7648–7651. doi: 10.1021/ja302435y

Kong, W., and Lu, T. (2014). Cloning and optimization of a nisin biosynthesis pathway for bacteriocin harvest. *ACS Synth. Biol.* 3, 439–445. doi: 10.1021/sb500225r

Koopmans, T., Wood, T. M., t Hart, P., Kleijn, L. H., Hendrickx, A. P., Willems, R. J., et al. (2015). Semisynthetic lipopeptides derived from nisin display antibacterial activity and lipid II binding on par with that of the parent compound. *J. Am. Chem. Soc.* 137, 9382–9389. doi: 10.1021/jacs.5b04501

Kruszewska, D., Sahl, H. G., Bierbaum, G., Pag, U., Hynes, S. O., and Ljungh, A. (2004). Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. *J. Antimicrob. Chemother.* 54, 648–653. doi: 10.1093/jac/dkh387

Kuthning, A., Mosker, E., and Sussmuth, R. D. (2015). Engineering the heterologous expression of lanthipeptides in *Escherichia coli* by multigene assembly. *Appl. Microbiol. Biotechnol.* 99, 6351–6361. doi: 10.1007/s00253-015-6557-6

Kuwano, K., Tanaka, N., Shimizu, T., Nagatoshi, K., Nou, S., and Sonomoto, K. (2005). Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria. *Int. J. Antimicrob. Agents* 26, 396–402. doi: 10.1016/j.ijantimicag.2005.08.010

Lawton, E. M., Ross, R. P., Hill, C., and Cotter, P. D. (2007). Two-peptide lantibiotics: a medical perspective. *Mini Rev. Med. Chem.* 7, 1236–1247. doi: 10.2174/138955707782795638

Levengood, M. R., Knerr, P. J., Oman, T. J., and van der Donk, W. A. (2009). *In vitro* mutasynthesis of lantibiotic analogues containing non-proteinogenic amino acids. *J. Am. Chem. Soc.* 131, 12024–12025. doi: 10.1021/ja903239s

Li, B., Cooper, L. E., and van der Donk, W. A. (2009). *In vitro* studies of lantibiotic biosynthesis. *Methods Enzymol.* 458, 533–558. doi: 10.1016/S0076-6879(09)04821-6

Marki, F., Hanni, E., Fredenhagen, A., and van Oostrum, J. (1991). Mode of action of the lanthionine-containing peptide antibiotics duramycin, duramycin B and C, and cinnamycin as indirect inhibitors of phospholipase A2. *Biochem. Pharmacol.* 42, 2027–2035. doi: 10.1016/0006-2952(91)90604-4

Meindl, K., Schmiederer, T., Schneider, K., Reicke, A., Butz, D., Keller, S., et al. (2010). Labyrinthopeptins: a new class of carbacyclic lantibiotics. *Angew. Chem. Int. Ed. Engl.* 49, 1151–1154. doi: 10.1002/anie.200905773

Mohr, K. I., Volz, C., Jansen, R., Wray, V., Hoffmann, J., Bernecker, S., et al. (2015). Pinensins: the first antifungal lantibiotics. *Angew. Chem. Int. Ed.* 54, 11254–11258. doi: 10.1002/anie.201500927

Molloy, E. M., Field, D., Connor, P. M. O., Cotter, P. D., Hill, C., and Ross, R. P. (2013). Saturation mutagenesis of lysine 12 leads to the identification of derivatives of nisin a with enhanced antimicrobial activity. *PLoS ONE* 8:e58530. doi: 10.1371/journal.pone.0058530

Mota-Meira, M., LaPointe, G., Lacroix, C., and Lavoie, M. C. (2000). MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. *Antimicrob. Agents Chemother.* 44, 24–29. doi: 10.1128/AAC.44.1.24-29.2000

Mota-Meira, M., Morency, H., and Lavoie, M. C. (2005). *In vivo* activity of mutacin B-Ny266. *J. Antimicrob. Chemother.* 56, 869–871. doi: 10.1093/jac/dki295

Nagao, J., Aso, Y., Shioya, K., Nakayama, J., and Sonomoto, K. (2007). Lantibiotic engineering: molecular characterization and exploitation of lantibiotic-synthesizing enzymes for peptide engineering. *J. Mol. Microbiol. Biotechnol.* 13, 235–242. doi: 10.1159/000104749

Nerandzic, M. M., and Donskey, C. J. (2010). Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* Spores. *PLoS ONE* 5:e12285. doi: 10.1371/journal.pone.0012285

Nikaido, H., and Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* 49, 1–32.

Niu, W. W., and Neu, H. C. (1991). Activity of mersacidin, a novel peptide, compared with that of vancomycin, teicoplanin, and daptomycin. *Antimicrob. Agents Chemother.* 35, 998–1000. doi: 10.1128/AAC.35.5.998

Oliynyk, I., Varelogianni, G., Roomans, G. M., and Johannesson, M. (2010). Effect of duramycin on chloride transport and intracellular calcium concentration in cystic fibrosis and non-cystic fibrosis epithelia. *APMIS* 118, 982–990. doi: 10.1111/j.1600-0463.2010.02680.x

Pag, U., and Sahl, H. G. (2002). Multiple activities in lantibiotics–models for the design of novel antibiotics? *Curr. Pharm Des.* 8, 815–833. doi: 10.2174/1381612023395439

Piper, C., Cotter, P. D., Ross, R. P., and Hill, C. (2009a). Discovery of medically significant lantibiotics. *Curr. Drug Discov. Technol.* 6, 1–18. doi: 10.2174/157016309787581075

Piper, C., Draper, L. A., Cotter, P. D., Ross, R. P., and Hill, C. (2009b). A comparison of the activities of lacticin 3147 and nisin against drug-resistant *Staphylococcus aureus* and *Enterococcus* species. *J. Antimicrob. Chemother.* 64, 546–551. doi: 10.1093/jac/dkp221

Rea, M. C., Clayton, E., O'Connor, P. M., Shanahan, F., Kiely, B., Ross, R. P., et al. (2007). Antimicrobial activity of lacticin 3,147 against clinical *Clostridium difficile* strains. *J. Med. Microbiol.* 56, 940–946. doi: 10.1099/jmm.0.47085-0

Rink, R., Wierenga, J., Kuipers, A., Kluskens, L. D., Driessen, A. J., Kuipers, O. P., et al. (2007). Dissection and modulation of the four distinct activities of nisin by mutagenesis of rings A and B and by C-terminal truncation. *Appl. Environ. Microbiol.* 73, 5809–5816. doi: 10.1128/AEM.01104-07

Rishi, P., Preet Singh, A., Garg, N., and Rishi, M. (2014). Evaluation of nisin-beta-lactam antibiotics against clinical strains of *Salmonella enterica* serovar Typhi. *J Antibiot (Tokyo)* 67, 807–811. doi: 10.1038/ja.2014.75

Rollema, H. S., Kuipers, O. P., Both, P., de Vos, W. M., and Siezen, R. J. (1995). Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. *Appl. Environ. Microbiol.* 61, 2873–2878.

Ross, A. C., McKinnie, S. M., and Vederas, J. C. (2012). The synthesis of active and stable diaminopimelate analogues of the lantibiotic peptide lactocin S. *J. Am. Chem. Soc.* 134, 2008–2011. doi: 10.1021/ja211088m

Rouse, S., Field, D., Daly, K. M., O'Connor, P. M., Cotter, P. D., Hill, C., et al. (2012). Bioengineered nisin derivatives with enhanced activity in complex matrices. *Microb. Biotechnol.* 5, 501–508. doi: 10.1111/j.1751-7915.2011.00324.x

Sahl, H., and Bierbaum, G. (2008). Multiple activities of natural agents such as defensins and bacteriocins suggest a change in strategy when developing new antimicrobials. *Microbe* 3, 467–473.

Saising, J., Dube, L., Ziebandt, A.-K., Voravuthikunchai, S. P., Nega, M., and Götz, F. (2012). Activity of Gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms. *Antimicrob. Agents Chemother.* 56, 5804–5810. doi: 10.1128/AAC.01296-12

Sandiford, S. K. (2015). Perspectives on lantibiotic discovery—where have we failed and what improvements are required? *Expert. Opin. Drug Discov.* 10, 315–320. doi: 10.1517/17460441.2015.1016496

Severina, E., Severin, A., and Tomasz, A. (1998). Antibacterial efficacy of nisin against multidrug-resistant Gram-positive pathogens. *J. Antimicrob. Chemother.* 41, 341–347. doi: 10.1093/jac/41.3.341

Shi, Y., Bueno, A., and van der Donk, W. A. (2012). Heterologous production of the lantibiotic Ala(0)actagardine in *Escherichia coli*. *Chem. Commun. (Camb)* 48, 10966–10968. doi: 10.1039/c2cc36336d

Tabor, A. B. (2014). Recent advances in synthetic analogues of lantibiotics: what can we learn from these? *Bioorg. Chem.* 55, 39–50. doi: 10.1016/j.bioorg.2014.04.004

Tagg, J. R. (2004). Prevention of streptococcal pharyngitis by anti-*Streptococcus pyogenes* bacteriocin-like inhibitory substances (BLIS) produced by *Streptococcus salivarius*. *Indian J. Med. Res.* 119(Suppl.), 13–16.

van der Donk, W. A., and Nair, S. K. (2014). Structure and mechanism of lanthipeptide biosynthetic enzymes. *Curr. Opin. Struct. Biol.* 29, 58–66. doi: 10.1016/j.sbi.2014.09.006

Wescombe, P. A., Heng, N. C. K., Burton, J. P., Chilcott, C. N., and Tagg, J. R. (2009). Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. *Future Microbiol.* 4, 819–835. doi: 10.2217/fmb.09.61

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B., et al. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J. Biol. Chem.* 276, 1772–1779. doi: 10.1074/jbc.M006770200

Willey, J. M., and Gaskell, A. A. (2011). Morphogenetic signaling molecules of the streptomycetes. *Chem. Rev.* 111, 174–187. doi: 10.1021/cr1000404

Willey, J. M., and van der Donk, W. A. (2007). Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501. doi: 10.1146/annurev.micro.61.080706.093501

Yuan, J., Zhang, Z. Z., Chen, X. Z., Yang, W., and Huan, L. D. (2004). Site-directed mutagenesis of the hinge region of nisinZ and properties of nisinZ mutants. *Appl. Microbiol. Biotechnol.* 64, 806–815. doi: 10.1007/s00253-004-1599-1

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