NEW HORIZONS IN FOOD SCIENCE VIA AGRICULTURAL IMMUNITY

EDITED BY: Willem Van Eden, Tomonori Nochi and Corné M. J. Pieterse

PUBLISHED IN: Frontiers in Nutrition and Frontiers in Immunology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-646-4
DOI 10.3389/978-2-88963-646-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

NEW HORIZONS IN FOOD SCIENCE VIA AGRICULTURAL IMMUNITY

Topic Editors:
Willem Van Eden, Utrecht University, Netherlands
Tomonori Nochi, Tohoku University, Japan
Corné M. J. Pieterse, Utrecht University, Netherlands

Citation: Van Eden, W., Nochi, T., Pieterse, C. M. J., eds. (2020). New Horizons in Food Science via Agricultural Immunity. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-646-4

Table of Contents

frontiers in Nutrition

EDITORIAL
published: 28 February 2020
doi: 10.3389/fnut.2020.00019

Editorial: New Horizons in Food Science via Agricultural Immunity

Tomonori Nochi[1†], *Corné M. J. Pieterse*[2†] *and Willem Van Eden*[3*†]

[1] Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2] Department of Biology, Utrecht University, Utrecht, Netherlands, [3] Department of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

Keywords: agricultural immunology, innate immunity, plants, animals, fishes

Editorial on the Research Topic

New Horizons in Food Science via Agricultural Immunity

The excessive use of drugs such as antibiotics and pesticides in the fields of livestock and plant production has a significant impact on not only spreading the drugs in production sites of agriculture but also threatening human health. In particular, the use of antibiotics in agriculture has been considered to be a major factor in producing antibiotics-resistant bacteria that often infect humans. Although the evidence was firstly confirmed in the United Kingdom as "Swann Report" in 1969 (1), the issue had not yet been debated in depth for more than a quarter century. However, in recent years, the World Health Organization (WHO) has warned that infectious diseases caused by antibiotics-resistant bacteria will devastate us human beings. In addition, WHO recently adopted a proposal entitled "Global Action Plan on Antimicrobial Resistance" to avoid the crisis of such infectious diseases on a global scale (2). It should be noted that the number of people who die due to the infectious diseases caused by the antibiotics-resistant bacteria in 2050 has been estimated to be over 10 million, which exceeds the number of cancer deaths if no action is taken immediately (3).

Agricultural immunology is a novel research field that focuses on animal and plant immunology. Recent progress in agricultural immunological research has demonstrated that the innate immune system in animals and plants against pathogens that cause agricultural infection occasionally has common features regardless of the presence or absence of acquired immunity. In 2016 in Leiden, the Netherlands, a workshop entitled "Innate Immunity of Crops, Livestock and Fish: The Dawn of Agricultural Immunology" was organized by Leiden University Lorentz Center. Through the workshop, the current knowledge and recent findings regarding agricultural immunology in animals and plants were discussed interdisciplinary by participants who attended the workshop from the Netherlands, Japan and the US to create a knowledge platform necessary for future drug-independent agricultural production systems (4). Through the workshop, several possible approaches to reduce the amount of antibiotics and pesticides from the production sites of agriculture were considered. In addition, an idea of creating a Research Topic for Frontiers in Nutritional Immunology was proposed to precisely appreciate the importance of agricultural immunology.

In this Research Topic entitled "New Horizons in Food Science via Agricultural Immunity," 11 articles including 7 review articles, 3 original research papers, and 1 brief research report were published. Current knowledge of the innate immune function in the livestock intestine where multiple pathogens continuously infect was precisely summarized in a review article based on the results obtained using *in vitro* bovine intestinal epithelial cells (Villena et al.). Also, in one other review article an approach to stimulate immune functions in not only livestock but also chickens was comparatively discussed, proposing an effective vaccine strategy for preventing infectious diseases (Nochi et al.). A recent advance of metagenomics approach using a next generation sequencer has allowed identifying numerous micro-organisms cohabiting

OPEN ACCESS

Edited and reviewed by:
Josep Bassaganya-Riera, Virginia Tech, United States

***Correspondence:**
Willem Van Eden
w.vaneden@uu.nl

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *14 December 2019*
Accepted: *13 February 2020*
Published: *28 February 2020*

Citation:
Nochi T, Pieterse CMJ and Van Eden W (2020) Editorial: New Horizons in Food Science via Agricultural Immunity. Front. Nutr. 7:19. doi: 10.3389/fnut.2020.00019

in animals and plants. Since most of the micro-organisms identified by the new technology are anaerobic (thus cannot be cultured under general aerobic conditions), they had been a black box in bacteriology for a long time. Three review articles focused on understanding the role of such beneficial microorganisms that affect the innate immune function in the host (Ikeda-Ohtsubo et al.; Villena, Kitazawa et al.; Brugman et al.). A possible approach proposed by the review articles is to utilize the beneficial microorganisms as probiotics for animals and plants. In this regard, the two original research papers gained deeper insights into the understanding the function and characterization of such probiotic candidates (i.e., *Lactobacillus reuteri* and *Lactobacillus delbrueckii*) (Giri et al.; Kanmani et al.). Another important information obtained in this Research Topic were the presence of immune deviant phenotype observed in some cattle regardless of the vaccination status (Lutterberg et al.), the role of milk-derived extracellular vesicles including microRNA in the regulation of cell functions in offspring (van Herwijnen et al.), the effect of maillard reaction products and advanced glycation end-products on immunomodulation in animals (Teodorowicz et al.), and the function of bovine immunoglobulin in immune function, allergy and infection (Ulfman et al.). In conclusion, the topic provided multiple possibilities to strengthen the innate immune activity of animals and plants to reduce the use of antibiotics and pesticides in agriculture. Based on the knowledge updated in this topic, further additional studies should be carried out especially at the production sites of agriculture to establish the drug-independent food safety system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Core-to-Core Program (Advanced Research Networks) of the Japan Society for the Promotion of Science (establishment of international agricultural immunology research-core for a quantum improvement in food safety).

REFERENCES

1. Swann Report. *Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine*. Report. London: HMSO (1969).
2. WHO. *Global Action Plan on Antimicrobial Resistance*. Available online at: https://www.Who.int/antimicrobial-resistance/global-action-plan/en/
3. Jim O. *Antimicrobial Resistance; Tackling a Crisis for Health and Wealth of Nations*. Review on Antimicrobial Resistance, Wellcome Trust (2014). Available online at: https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf
4. Leiden University Lorentz Center Workshop. *Innate Immunity of Crops, Livestock and Fish: The Down of Agricultural Immunology*. (2016).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 02 March 2018
doi: 10.3389/fimmu.2018.00326

Immunobiotics for the Bovine Host: Their Interaction with Intestinal Epithelial Cells and Their Effect on Antiviral Immunity

Julio Villena[1,2,3], Hisashi Aso[4,5], Victor P. M. G. Rutten[6], Hideki Takahashi[7,8], Willem van Eden[6] and Haruki Kitazawa[3,5]**

[1] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [2] Immunobiotics Research Group, Tucuman, Argentina, [3] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Cell Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [5] Livestock Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [6] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [7] Laboratory of Plant Pathology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [8] Plant Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan*

OPEN ACCESS

Edited by:
Jia Sun,
Jiangnan University, China

Reviewed by:
Pinyi Lu,
Biotherapeutics, Inc., United States
Xu Zhang,
The Scripps Research Institute, United States

***Correspondence:**
Julio Villena
jcvillena@cerela.org.ar;
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *10 November 2017*
Accepted: *06 February 2018*
Published: *02 March 2018*

Citation:
Villena J, Aso H, Rutten VPMG, Takahashi H, van Eden W and Kitazawa H (2018) Immunobiotics for the Bovine Host: Their Interaction with Intestinal Epithelial Cells and Their Effect on Antiviral Immunity. Front. Immunol. 9:326. doi: 10.3389/fimmu.2018.00326

The scientific community has reported several cases of microbes that exhibit elevated rates of antibiotic resistance in different regions of the planet. Due to this emergence of antimicrobial resistant microorganisms, the use of antibiotics as promoters of livestock animals' growth is being banned in most countries around the world. One of the challenges of agricultural immunology therefore is to find alternatives by modulating the immune system of animals in drug-independent safe food production systems. In this regard, in an effort to supplant antibiotics from bovine feeds, several alternatives were proposed including the use of immunomodulatory probiotics (immunobiotics). The purpose of this review is to provide an update of the status of the modulation of intestinal antiviral innate immunity of the bovine host by immunobiotics, and the beneficial impact of immunobiotics on viral infections, focused on intestinal epithelial cells (IECs). The results of our group, which demonstrate the capacity of immunobiotic strains to beneficially modulate Toll-like receptor 3-triggered immune responses in bovine IECs and improve the resistance to viral infections, are highlighted. This review provides comprehensive information on the innate immune response of bovine IECs against virus, which can be further investigated for the development of strategies aimed to improve defenses in the bovine host.

Keywords: immunobiotics, antiviral immunity, beneficial microbes, bovine rotavirus, toll-like receptor 3 pathway, inflammation, agricultural immunology

INTRODUCTION

Over the past decades, the global bovine production has been subjected to intensification, in order to improve efficiency of production because of the demand from a growing human population. The intensification of bovine production involved the application of confinement methods characterized by the concentration of animals in large outdoor feedlots or in specialized indoor

environments. In confinement, the potential for transfer of pathogens among animals is higher, as there are more animals in a smaller space (1–3).

Severe gastrointestinal infectious diseases causing malabsorption and diarrhea are important causes of discomfort and death in young calves, resulting in important economic losses to bovine producers. Gastrointestinal infectious diseases are able to cause significant economic losses to the cattle industry in big cattle-producing countries and can impair the development of cattle industry in small cattle-producing countries (1–3). In particular, the neonatal gastroenteritis in the bovine host is a multifactorial disease. This disorder can be caused by different bacterial or viral pathogens, including bovine coronavirus (BCV), bovine rotavirus (BRV), and bovine viral diarrhea viruses (BVDV) (4, 5). Although these viral pathogens belong to distinct families and possess different physical characteristics, they are all able to infect intestinal epithelial cells (IECs), generate villous atrophy, and cause inflammatory intestinal tissue damage and diarrhea.

Probiotics are defined as live microorganisms with the capacity to confer a health benefit on the host when administered in adequate amounts. Among them, those that are able to impact on human and animal health by modulating the mucosal and systemic immune systems have been called immunobiotics. It has been reported that immunobiotic lactic acid bacteria are able to generate protection against viral pathogens by differentially modulating antiviral immune responses in humans and livestock animals like pigs (6, 7). It is also believed that immunobiotics could be used in cattle feeds to improve bovine health and produce safe animals (8–10).

The purpose of this review is to provide an update of the status of the modulation of intestinal antiviral innate immunity in the bovine host by immunobiotics, and their beneficial impact on viral infections. The results of our group, which demonstrate the capacity of immunobiotic strains to advantageously modulate Toll-like receptor (TLR)-3-triggered immune responses in bovine IECs and improve the resistance to viral infections, are particularly highlighted.

THE USE OF PROBIOTICS IN THE BOVINE HOST

Before weaning, dairy calves are highly susceptible to infectious diseases. For several years, antimicrobial compounds have been used to reduce the severity and mortality of infectious diseases and to improve economic benefits in terms of enhanced bovine performance and diminished medication expenses. However, the use of antibiotics in livestock animal management is in question because of the enhanced resistance of microbes to antimicrobial compounds. Therefore, there is an urgent need to reduce and finally eliminate the use of antibiotics in livestock and for this purpose many feed additives have been proposed including beneficial microbes (8–10). In fact, research from the past decade has provided evidence that probiotic bacteria and prebiotics can be effectively used to improve health and growth in calves and reduce the use of antibiotics (**Table 1**), although detailed mechanistic studies were not performed. The production of antimicrobial compounds, inhibition of adherence or aggregation with pathogens and the modulation of the microbiota were described as mechanisms of probiotics action in the bovine host [reviewed in Ref. (10)]. Immunomodulation was also proposed as a mechanism of bovine probiotics as mentioned later.

As described for other livestock animals, the most critical stage in the bovine life is the period from birth to weaning. During this stage factors like nutrition can directly affect the immune system development and function and impact later in bovine performance (26). However, nutritional interventions during this phase are often inappropriate due to the elevated costs of milk feeding. The administration of poor-quality milk or colostrum, and the additions of antibiotics are common practices. Poor nutrition during preweaning stage often conduces to low weaning weight and impaired immunity, thereby increasing losses related to disease. Therefore, the majority of research studying the influence of beneficial microbes in the bovine host has been performed during this period of life.

Early studies showed that orally administered *Bifidobacterium pseudolongum* M-602 or *Lactobacillus acidophilus* LAC-300 enhanced the gain of body weight and reduced diarrhea frequency in calves (11). Similarly, several subsequent studies reported that treatments with probiotic microorganisms beneficially influence body weight gain, body height, milk production, and the general health condition of calves (**Table 1**). Additionally, oral treatment with probiotics significantly reduced the incidence and the severity of gastrointestinal infections. Moreover, some studies have also shown that probiotic treatments are able to improve not only mucosal defenses in the bovine host but systemic immunity as well (20, 21).

Classical probiotic strains have been used to evaluate their beneficial effects on the bovine hosts including *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and the yeast *Saccharomyces* (**Table 1**). More recently, new species of beneficial bacteria have been also tested as next-generation probiotics. In this regard, the obligate anaerobic, Gram-positive microorganism *Faecalibacterium prausnitzii* that belongs to the phylum *Firmicutes* has been tested as a potential probiotic for the bovine host (27). Oikonomou et al. (27) reported that the high relative abundance of this bacterium in the first week of life of Holstein calves was associated with improved weight gain and diminished occurrence of diarrhea. Recently, Foditsch et al. (22) confirmed the safety and efficacy of *F. prausnitzii* in young dairy heifers. Researchers reported that the oral administration of viable *F. prausnitzii* reduced severe diarrhea incidence and its related mortality rate. Moreover, *F. prausnitzii* treatment significantly enhanced the weight gain.

The anti-inflammatory properties of *F. prausnitzii,* including its ability to synthetize butyrate, were proposed as factors involved in the beneficial effects observed in calves. These works demonstrated that this intestinal bacterium could be a novel approach to enhance the intestinal health in calves and improve their body weight gain (22).

These studies indubitably show the potential of beneficial microbes to differentially modulate weight gain, intestinal hemostasis, and immunocompetence in young calves.

TABLE 1 | Probiotics for the bovine host.

Strain	Viability	Route	Host	Effects	Reference
Lactobacillus acidophilus LAC-300	Viable	Oral	Holstein calves	Increase in body weight gain. Improvement in fecal scores	(11)
Bifidobacterium pseudolongum M-602	Viable	Oral	Holstein calves	Increase in body weight gain. Improvement in fecal scores	(11)
Bifidobacterium thermophilum S-501, *Lactobacillus acidophilus* LAC-300, and *Enterococcus faecium* FA-5	Viable	Oral	Holstein calves	No effect on body weight gain was observed. Reduction of diarrhea	(11)
Saccharomyces cerevisiae, Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus termophilus, and *Aspergillus niger*	Viable	Oral	Holstein calves	Improvement in daily live weight gain and feed efficiency ratio. Reduction of diarrhea	(12)
Lactobacillus rhamnosus GG	Viable	Oral	Holstein calves	The probiotic strain survived the gastrointestinal transit. No beneficial effect was recorded	(13, 14)
Saccharomyces boulardii	Viable	Oral	Holstein bull calves	Treated animals consumed more grain, had increased weight gain, and increased plasma glucose concentrations. Days with diarrhea were reduced	(15)
Lactobacillus acidophilus 15	Viable	Oral	buffalo calve (1 day to 31 weeks)	Weight gain was improved and feed: gain ratio was reduced	(16)
Multispecies probiotic preparation: *Lactobacillus acidophilus* W55, *Lactobacillus salivarius* W57, *Lactobacillus paracasei* W56, *Lactobacillus plantarum W59, Lactococcus lactis* W58, and *Enterococcus faecium* W54	Viable	Oral	Male Holstein-Friesian calves	Probiotics enhanced growth rate and average daily gain and feed efficiency were significantly improved. Modest effect on diarrhea	(17)
Calf-specific multistrain probiotic preparation (six lactobacilli strains)	Viable	Oral	Male Holstein-Friesian calves	Probiotics enhanced growth rate and average daily gain and feed efficiency were significantly improved. Treatment reduced the incidence of diarrhea and the fecal counts of coliforms	(17)
Lactobacillus casei subsp. *casei* JCM1134^T and Dextran	Viable	Oral	Holstein dairy calves	Increase the milk production	(18)
Lactobacillus *acidophillus, Bifidobacterium bifidum*, and *Enterococcus faecium*	Viable	Oral	Holstein calves	Significant reduction of diarrhea but no effect on mastitis	(19)
Bacillus subtilis natto	Viable	Oral	Male Holstein calves	Increased average daily gain and feed efficiency. No difference in serum IgE, IgA, and IgM, whereas serum IgG and IFN-γ were higher in probiotic-treated than in the controls.	(20)
Lactobacillus plantarum 220, *Enterococcus faecium* 26, and *Clostridium butyricum* Miyari	Viable	Oral	Holstein bull calves	Increased the numbers of CD282$^+$ monocytes, CD3$^+$ T cells and CD4$^+$, CD8$^+$, and WC1$^+$ $\gamma\delta$ T cell in blood. Increment of production of IL-6, INF-γ, and TNF-α were also observed	(21)
Faecalibacterium prausnitzii 34, *Faecalibacterium prausnitzii* 35, *Faecalibacterium prausnitzii* 1S, and *Faecalibacterium prausnitzii* 2S	Viable	Oral	Preweaned dairy Holstein heifer calves	Decreased the incidence of severe diarrhea and related mortality rate, while increasing weight gain	(22)
Lactobacillus plantarum GF103	Viable	Oral	Male Holstein calves	No significant differences were observed in dry matter intake or average daily gain, but the feed conversation ratio was improved. Treatment improved mitogen-induced lymphocyte proliferation	(23)
Kefir	Viable	Oral	Female Holstein calves calves	Kefir intake improved fecal scores and reduced days with diarrhea during the first 2 weeks of life. No effect on weight gain	(24)
Milk fortified with symbiotic complex containing prebiotics (mannan-oligossacharides) and probiotics (*Lactobacillus acidophilus, Enterococcus faecium, Bacillus subtilis, Saccharomyces cerevisiae*)	Viable	Oral	Female Holstein heifer calves	Symbiotic did not affect weight gain or feed efficiency of calves but it improved fecal scores	(25)

However, the cellular and molecular interactions of probiotics with the cells of the bovine host have not been studied in depth. A better molecular understanding of how the selected beneficial microbes improve resistance against infections by antagonizing pathogens and/or modulating the immune system is needed.

BOVINE IECs AS A MODEL TO STUDY ANTIVIRAL IMMUNITY

It is considered that an important step forward toward the understanding of the cellular and molecular interactions of pathogenic or probiotic microorganisms with the bovine host is the establishment of appropriate *in vitro* systems models. Therefore, the development of suitable bovine cell cultures such as IECs would be of great value to advance in this field of research. Those cell cultures should be minutely characterized with regard to their permissiveness for bacterial and viral adhesion and invasion, and the ability to sense microbial-associated molecular patterns through pattern recognition receptors (PRRs) (28).

Primary cultures of small or large IECs have been used to evaluate the effects of microbial virulence factors, toxic compounds, and antimicrobial factors in cattle. Moreover, those primary cultures have been also used for the study of innate immune responses through PRRs signaling (28–33). Soft mechanical agitation combined with enzymatic digestion using dispase and collagenase has been proved to successfully release viable intact bovine colonic cells. However, these cells suspensions contained contaminating non-epithelial cells (mostly fibroblasts) and therefore, a series of purification steps was required to obtain relatively pure bovine colonic cells. The development of primary bovine cell lines from rectum, colon and ileum was reported by Dibb-Fuller et al. (29). Those bovine primary cell cultures were successfully used to evaluate the interaction of several intestinal pathogenic bacteria with bovine IECs and to determine mechanisms of adherence and invasion. More recently, Zhan et al. (34) successfully cultured primary bovine IECs and established a novel clone cell method. Authors demonstrated the expression of E-cadherin and cytokeratin 18, as well as characteristics of epithelial-like morphology in this new cell line. However, the immunological characteristics of cells or viral infections have not been evaluated in those systems.

As mentioned earlier, viral infections in livestock animals could cause a fatal disease that implicate serious economic loses. Therefore, the effective and non-costly control of this type of infections is a key factor for improving animal production. It is believed that the clear and detailed understanding of viral pathogenicity as well as host immune response in the bovine host is necessary to develop strategies capable of reducing infectious disease caused by these viruses. In this sense, a deeper understanding of the molecular interactions of virus with bovine IECs is necessary for the development of better prevention strategies to improve protection in animals.

Bovine virus pathology and immune response have been studied mainly in heterologous systems including mouse models (35) and human cell lines (36). Taking into consideration the differences in viral strains, the specific receptors for virus uptake, the factors required for viral replication and pathogenesis as well as the specific species variations in innate immune responses; the information generated in those heterologous models may not be fully applicable to cattle. Therefore, scientists have tried to establish bovine systems for the study of viral infections. One of the earliest works able to confirm that cultured bovine IECs were susceptible to BRV infection was reported by Kaushik et al. (37). Epithelial cultures obtained from jejunal and ileal tissues were incubated with BRV and both cell types were similarly infected with the viral pathogen. Long incubation times of BRV with the epithelial cultures coming from jejunal and ileal tissues resulted in extensive cellular damage and reduced cell viability, which is in line with the knowledge that BRV is a lytic virus. Furthermore, BRV particles were recovered from the culture supernatants confirming that viral replication occurred in bovine IECs (37). However, the immune response was not studied. In addition, those epithelial cell cultures contained fibroblasts, and therefore if the immune response is evaluated in this system it cannot be discriminated whether the response (cytokine production, for example) is mediated by one or both cells, especially considering that the authors also demonstrated that BRV infected and replicated in fibroblasts (37).

Bovine primary IEC cultures have been of value to study the molecular mechanisms involved in diseases caused by pathogens. However, the cellular and molecular interactions of beneficial or commensal microorganisms with bovine IECs cells have been less examined. In addition, the intestinal cell lines established from adult cattle may have limitations in the study of infections with BRV, BVDV, or BCV, since these viruses infect IECs in the gut of young calves (4, 5).

In order to understand: (i) the pathogenesis of bovine viral infections and the subsequent gastrointestinal diseases, (ii) the role of bovine IECs in the generation of mucosal immune responses, and (iii) the effect of beneficial microbes that may be used to advantageously modulate the antiviral immune response in bovine IECs; we have developed an immortalized bovine IEC line from young calves: bovine intestinal epithelial (BIE) cells (38).

Bovine intestinal epithelial cells have an epithelial-like morphology and they grow forming a monolayer with cells that establish close contact between them (38). Scanning electron microscopy analysis revealed that 3-day-old BIE cells have microvilli-like structures on their surface that are irregular and slender. These cellular structures increase in complexity as the cells grow as observed in 10-day-old BIE cells (38). The evaluations of the expression of cytokeratin and specific villin protein, which are known as markers of epithelial cells, have demonstrated that BIE cells are strongly positive for both proteins. In contrast, vimentin and desmin that are markers for mesenchymal cells and muscle cells, respectively, were not found in BIE cells.

Bovine intestinal epithelial cells also expressed the cell-to-cell adhesion molecules ZO-1 and beta-catenin (39). Both proteins were strongly positive in the cell-to-cell contact region when cells reached confluence. Moreover, the functional integrity of BIE cells gradually increased with time as indicated by studies of TEER and paracellular permeability (38). These results provide clear evidence of the intestinal epithelial nature of BIE cells.

IMMUNOBIOLOGY OF BOVINE EPITHELIAL CELLS

Significant progress has been made in the understanding of both the beneficial and detrimental roles of TLR3 in innate antiviral immune responses in mucosal tissues (6, 40). Therefore, to decipher the exact role of TLR3 in antiviral defenses in IECs is

of value to understand the mechanisms that activate and regulate the intestinal immune system of the host. Few studies have been conducted on cattle. Those studies are of importance since the determination of the mechanisms involved in the activation and regulation of TLR3 in bovine IECs could give the scientific basis for the development of efficient preventive or therapeutic strategies for reducing severity and mortality of viral diseases, including oral vaccines and functional feeds. Then, the expression of mRNAs of TLRs was evaluated in BIE cells and it was reported that all the genes for these receptors were expressed in this cell line (41). TLR1, TLR3, TLR4, and TLR6 were strongly expressed while TLR5, TLR9, TLR2, and TLR7 were expressed modestly. We were especially interested in expression of TLR3 as the most important receptor detecting double-stranded genomic RNA (dsRNA) from viruses.

Therefore, to confirm these findings, we further examined the expression of TLR3 protein in BIE cells by immunohistochemical analysis and demonstrated that this PRRs is strongly expressed in the cytoplasm of BIE cells (42). Of note, no TLR3expression was detected at the BIE cell surface. Therefore, BIE cells, in addition to displaying characteristics of epithelial cells like those mentioned earlier such as microvilli-like structures, and strong expression of cell-to-cell junctional proteins (38), they also express TLR3 and thus are similar to the IECs of other species.

The innate immune response induced by TLR3 activation in BIE cells was also studied. BIE cells were treated with the TLR3 agonist poly(I:C) and an upregulation of type I interferon (IFN), and proinflammatory cytokines expression was detected. The changes in the expression of inflammatory factors induced by poly(I:C) in BIE cells correlate with the changes reported in various intestinal viral infections of cattle and other hosts. For instance, enhanced gene expression of CCL5 (RANTES), CXCL10 (IP-10), CXCL8 (IL-8), and CCL2 (MCP-1) were observed in rotavirus-infected HT-29 cells (43, 44). In addition, *in vitro* studies showed that the challenge of bovine intestinal tissues with BRV or BCV activated TLR3, upregulated nuclear factor κB (NF-κB) and increase IL-6 production (4). These findings indicate that BIE cells are valuable tools for the *in vitro* study of immune responses mediated by TLR3 in bovine IECs.

Bovine rotavirus is able to induce a potent inflammatory response mediated by IFN and IFN-induced genes as well as inflammatory cytokines. In this regard, studies performed in HT29 cells infected with BRV (A5-13 strain) demonstrated that viral infection significantly upregulated most of the IFN-inducible genes including IL-18, IFN-α-inducible protein 6, IFN-induced transmembrane protein 3, TAP1, DDX58 [retinoic acid inducible gene-I (RIG-I)], and 2′-5′-oligoadenylate synthetase (OAS) 1 as well as several cytokines such as IL-8, CCL5, CXCL10, and CXCL11 (36). Few studies evaluated the BRV infection and its immune responses by using *in vivo* or *in vitro* bovine systems. By performing an intestinal loop surgical technique, Aich et al. (4) investigated the innate immune responses against bovine BRV in newborn calves. BRV (field isolate BRV85) challenge was able to induce accumulation of fluid and visible histological alterations in the gut of infected animals. Moreover, transcriptional profile of gene expression analysis and qPCR revealed that BRV enhanced TLR3, NF-κB p65, and IL-6. In addition, IRF1, a transcriptional regulator involved in the activation of IFN responses, was activated after rotaviral challenge (4).

We demonstrated that 10-days old BIE cells have developed microvilli-like structures on their surface (45). These characteristics of BIE cells together with their capacity to respond to TLR3 activation allowed us to hypothesize that this cell line could be a valuable *in vitro* tool for studying the interactions between BRV and bovine IECs. Therefore, we compared the infection capacity of four rotavirus strains in BIE cells including human (Wa), murine (EW), porcine (OSU), and bovine (UK), and we found that BIE cells can be effectively infected with the four rotavirus strains (45). Our results showed that 3-day-old BIE cells were more resistant to rotavirus infection than 10-day cultured cells, which probably related to the differences in the length and number of microvilli present on their surfaces. As we mentioned previously, the presence of these cellular structures is important for rotaviral infection since it was suggested that differentiated non-dividing mature enterocytes express the factors that are essential for the efficient rotavirus infection and replication (46, 47). In addition, we found significant differences regarding the viral titers when rotaviruses of different origins were compared. BIE cells were highly infected by bovine and porcine strains, whereas human and murine rotavirus showed a lower capacity to infect this bovine cell line (45). We also observed that viral titers were higher in BIE cells infected with UK than OSU strains, confirming that BRV strain isolated from cattle has a higher capacity to infect these cells. This is in line with previous studies that reported that the infection of porcine small intestinal epithelial (IPEC-J2) cells with OSU rotavirus induced a higher cytopathic effect and significantly reduced cell survival when compared with Wa strain (48). Moreover, our results in BIE cells are also in agreement with our results in porcine IECs (PIE cells) that showed a higher capacity of OSU rotavirus to infect those cells when compared to UK, Wa, or EW strains (49).

The innate immune response triggered by BRV infection in BIE cells was also characterized (**Figure 1**). We observed that BRV challenge activated antiviral PRRs in BIE cells and induced immune responses characterized by IFN regulatory factor-3 (IRF3) and NF-κB activation, with the subsequent upregulation of IFN-β and inflammatory chemokines and cytokines. Those results are in agreement with the innate immune mechanisms described for BRV infection in several experimental models as mentioned previously (4, 35, 36). Of interest, we also observed that UK rotavirus was able to induce a stronger innate immune response in BIE cells than OSU strain as demonstrated by the higher levels of expression of inflammatory factors IL-6, IL-8, MCP-1, and IFN-β.

The, BIE cells have several characteristics that make them extremely interesting for the study of BRV pathogenesis, and the molecular mechanisms involved in the generation of innate immune responses. In addition, this cell line could be of value for the evaluation of treatments aimed to beneficially modulate antiviral defenses and reduce inflammatory-mediated damage.

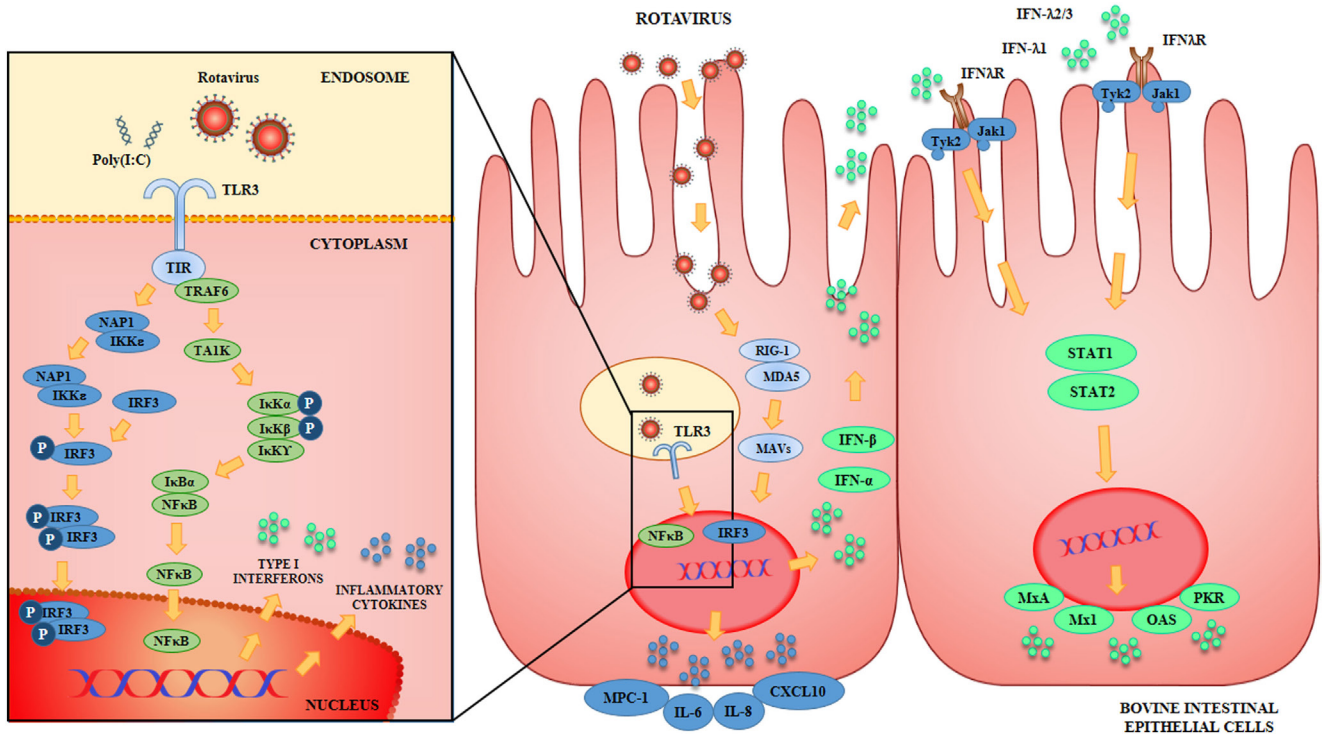


FIGURE 1 | Antiviral Innate immune response against rotavirus in bovine intestinal epithelial (BIE) cells. Rotavirus double-stranded genomic RNA activates toll-like receptor 3 (TLR3), retinoic acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in BIE cells. Cellular signaling cascades are activated and converge at the level of interferon (IFN) regulatory factor-3 (IRF3) that upregulate the expression of type I (IFN-α, IFN-β) and type III (IFNλ1, IFNλ2/3) IFN, which in turn induces the synthesis of IFN-stimulated genes with antiviral activities including: myxovirus resistance 1 IFN-inducible protein (Mx1), MxA, ribonuclease L (RNaseL), 2′-5′-oligoadenylate synthetase (OAS), and protein kinase R (PKR). Antiviral PRRs also activate nuclear factor κB (NF-κB) pathway and induce the secretion of proinflammatory cytokines and chemokines including: interleukin 6 (IL-6), IL-8, monocyte chemotactic protein 1 (MCP-1/CCL2), and IFN gamma-induced protein 10 (IP-10/CXCL10).

BOVINE IECs AS A MODEL TO SELECT AND CHARACTERIZE IMMUNOBIOTICS WITH ANTIVIRAL ACTIVITY

The capacity of beneficial microbes to differentially modulate the response of BIE cells to TLR3 stimulation was evaluated by using several lactobacilli and bifidobacteria strains (42, 45) (**Figure 2**). Some strains such as *L. rhamnosus* LA-2, *S. thermophilus* TMC1543 (42), *B. infantis* MCC12, and *B. breve* MCC1274 (45) were able to enhance IFN-β levels after poly(I:C) challenge. The improved production of IFN-β by BIE cells after TLR3 activation induced by those probiotic strains may have significant *in vivo* effects in the protection against enteric viruses. It is well known that IFN-α and IFN-β are important factors of the innate immune response against viral infections. Type I IFNs, after their interaction with the IFN-α/β receptor (IFNAR), upregulate the expression of hundred of antiviral proteins capable to reduce or inhibit viral replication and promote viral clearance. In this regard, transcriptomic analyses of bovine intestinal tissues after the challenge with BRV or BCV have shown that the expression of several IFN-regulated genes is reduced, supporting the conclusion that both viruses have developed mechanism(s) to inhibit immune responses mediated by IFNs (4). Moreover, it has been reported that BVDV is able to impair the induction of type I IFN, which not only affect innate immunity, but in addition interferes with the appropriate development of adaptive immune defenses (5, 50). Based on these findings, immunobiotics that enhance IFN-β production in BIE cells could have a prominent role in the reinforcement of innate and adaptive immune responses against bovine intestinal virus.

As the duration and intensity of proinflammatory factors secretion after TLR3 activation by viral dsRNA can become harmful to the host (51), we also evaluated the levels of key inflammatory cytokines and chemokines in BIE cells including IL-6, IL-8, and MCP-1. Our results indicated that BIE cells pretreated with the probiotic strain *L. casei* TMC0409 (42) produced lower levels of MCP-1, IL-6, and IL-8 when compared with control cells after stimulation with poly(I:C). It has been well established that the unregulated activation of TLR3 is capable to mediate detrimental inflammatory responses in the intestine, thus contributing to the tissue damage induced by viral infections (6, 52). Therefore, the diminished production of proinflammatory factors after the exposure to immunobiotics may allow a better control of the inflammatory responses and reduce the tissue injury mediated by this mechanism. In this way, beneficial bacteria like the TMC0409 strain may offer a different protection mechanism against bovine viral infection.

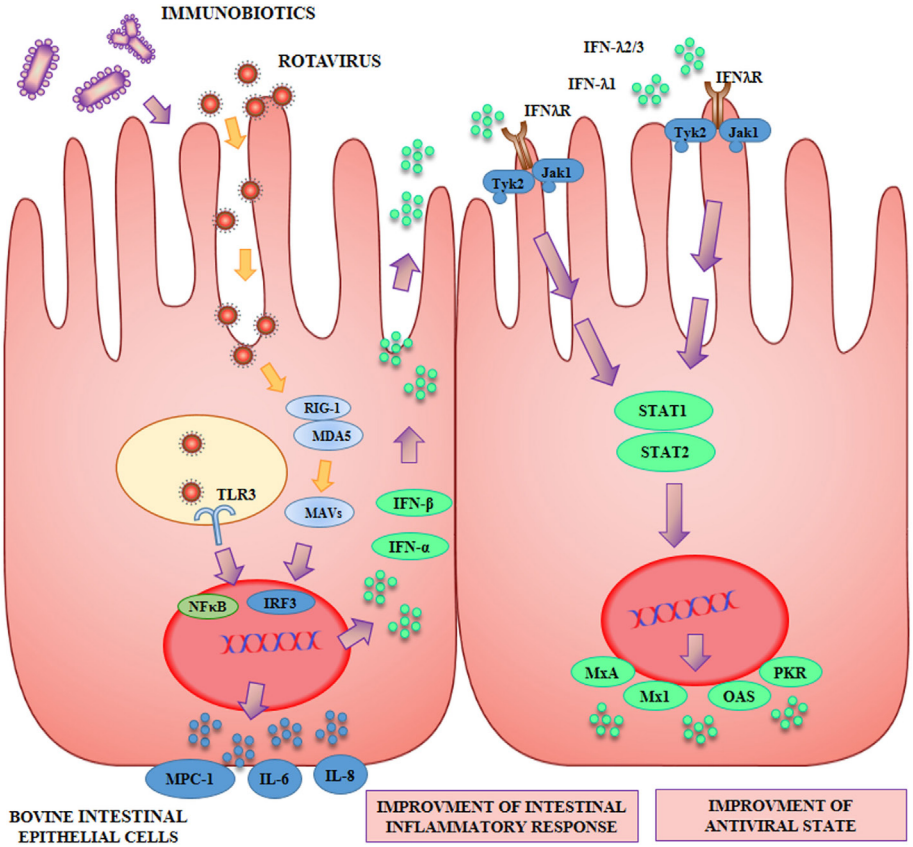


FIGURE 2 | Beneficial effects of immunobiotics on the antiviral innate immune response against rotavirus in bovine intestinal epithelial (BIE) cells. Rotavirus doublestranded genomic RNA activates toll-like receptor 3 (TLR3), retinoic acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in IECs. Cellular signaling cascades mediated by interferon (IFN) regulatory factor-3 (IRF3) upregulate the expression of type I (IFN-α, IFN-β), and type III (IFNλ1, IFNλ2/3) IFN, which in turn induces the synthesis of IFN-stimulated genes with antiviral activities including: myxovirus resistance 1 IFN-inducible protein (Mx1), MxA, ribonuclease L (RNaseL), 2′-5′-oligoadenylate synthetase (OAS), and protein kinase R (PKR). Antiviral PRRs also activate nuclear factor κB (NF-κB) pathway and induce the secretion of proinflammatory cytokines and chemokines including: interleukin 6 (IL-6), IL-8, monocyte chemotactic protein 1 (MCP-1/CCL2), and IFN gamma-induced protein 10 (IP-10/CXCL10). Preventive treatment of BIE cells with immunobiotics increase the activation of IRF3, improve the production of the antiviral factors and differentially regulate the expression of inflammatory mediators.

In conclusion, the BIE cells *in vitro* system was of value for the efficient screening of two types of immunomodulatory probiotic strains capable to improve protection against viral intestinal diseases in the bovine host: (i) strains with the ability to increase antiviral defenses like *L. rhamnosus* LA-2, *B. infantis* MCC12 and *B. breve* MCC1274 and (ii) strains with anti-inflammatory capacities like *L. casei* TMC0409.

Considering the mentioned results and that some recent research indicated that the presence of bifidobacteria in the gut of young calf is associated with a good health status (53), we next aimed to evaluate the capacity of the selected immunobiotic bifidobacteria strains to improve resistance of BIE cells to viral challenge.

Studies with the porcine IPEC-J2 cell line, demonstrated that *L. rhamnosus* GG reduced the mucin and IL-6 secretion response triggered by porcine rotavirus, diminishing the inflammatory damage (48). Our own studies in PIE cells also demonstrated that selected immunobiotic strains were capable to upregulate IFN-β expression in response to poly(I:C) stimulation (54, 55). Moreover, we recently demonstrated that *B. infantis* MCC12 and *B. breve* MCC1274 were capable to significantly improve the resistance of PIE cells to porcine rotavirus infection (49). Both immunobiotic bifidobacteria strains significantly enhanced the expression of IFN-β, MxA and ribonuclease L (RNaseL) in infected PIE cells, reducing viral replication. Therefore, we aimed to determine whether the studies performed in our laboratory using porcine IECs could be reproduced in BIE cells. Then, we evaluated whether immunobiotic *B. infantis* MCC12 and *B. breve* MCC1274 were able to protect BIE cells against BRV infection.

We showed that BIE cells treated with bifidobacteria were more resistant to BRVs infection, and that MCC12 and MCC1274 treatments significantly increased IFN-β in BRV-infected BIE cells. This is in line with the observation that BRV replication is restricted in susceptible cells by preventive treatment with recombinant IFN-β (56). Likewise, administration of recombinant IFN-β to newborn calves prior to BRV challenge suppresses virus replication and diminishes disease severity (57). A recent study reported the antiviral activity of bacterial strains in mice and Caco-2 cells, in

which the probiotic *B. longum* SPM1206 and *L. ruminis* SPM0211 induced the expression of IFN-β in response to human rotavirus (58). Moreover, these probiotic strains had the capacity to inhibit RVs infection through the increase IFN signaling component (STAT1), and IFN-inducible antiviral effectors MXA, protein kinase R (PKR), and OAS in mice. In line with these findings, our recent immunotranscriptomic analysis showed that both *B. infantis* MCC12 and *B. breve* MCC1274 are able to improve the expression of several antiviral factors through their capacity to improve IFN-β production. Both bifidobacteria improved the expression of IFN-α and IFN-β as well as the antiviral factors RNASEL, MX1, and MX2 when compared to controls. In addition, bifidobacteria increased the expression of NLRP3 [Albarracin et al. (59), *in preparation*]. In agreement with the central role of IFN-β in the protection of BIE cells against BRV, we also observed that *B. breve* MCC1274 induced an earlier and higher activation of TRAF3, higher levels of IFN-β and significantly lower titers of BRV in infected BIE cells when compared with *B. infantis* MCC12. The mechanism(s) (probiotic molecules and host receptors and signaling pathways) by which these immunobiotic bifidobacteria induce higher expression of IFN-β in BRV-infected BIE cells is an interesting topic for future research.

CONCLUSION

Prophylactic administration of low doses of antibiotics has been historically used to promote the growth and avoid infectious diseases in livestock animals. However, due to the emergence of antibiotic resistant microbes, several governments in countries around the world have prohibited the use of antibiotics as growth promoters for animals.

One of the most important challenges of agricultural immunology therefore is to find alternatives for developing drug-independent safe food production systems by modulating the immune system of animals. The work reviewed here encourages the research of probiotics to beneficially modulate the immune system of the bovine host. This review provides comprehensive information on the innate antiviral immune response of bovine IECs against virus, which can be further studied for the development of strategies aimed to improve antiviral defenses. The analyzed data also suggest that beneficial microbes have a great potential to be used as antiviral alternatives able to reduce severity of infections in the bovine host.

The development of specific *in vitro* study systems for cattle such as BIE cells as well as the selection and characterization of microbes that exert beneficial functions specifically and efficiently in the bovine host are key points for the successful development of immunomodulatory feeds aimed to protect against infections and reduce or avoid the use of antibiotics.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to the design, writing, and editing of the review article.

FUNDING

This study was supported by a Grant-in-Aid for Scientific Research (B)(2) (24380146, 16H05019), Challenging Exploratory Research (23658216, 26660216, 16K15028), and Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects from the Japan Society for the Promotion of Science (JSPS) to HK and by an ANPCyT–FONCyT Grant PICT-2013 (No. 3219) to JV. This work was also supported by JSPS Core-to-Core Program A (Advanced Research Networks) entitled: "Establishment of international agricultural immunology research-core for a quantum improvement in food safety." This study was also supported by grants for "Scientific Research on Innovative Areas" from the Ministry of Education, Culture, Science, Sports and Technology (MEXT) of Japan (grant numbers: 16H06429, 16K21723, and 16H06435).

REFERENCES

1. Cappellini OR. *Dairy Development in Argentina*. Rome: FAO (2011).
2. Greiser-Wilke I, Grummer B, Moennig V. Bovine viral diarrhoea eradication and control programmes in Europe. *Biologicals* (2003) 31(2):113–8. doi:10.1016/S1045-1056(03)00025-3
3. Richter V, Lebl K, Baumgartner W, Obritzhauser W, Käsbohrer A, Pinior B. A systematic worldwide review of the direct monetary losses in cattle due to bovine viral diarrhoea virus infection. *Vet J* (2017) 220:80–7. doi:10.1016/j.tvjl.2017.01.005
4. Aich P, Wilson HL, Kaushik RS, Potter AA, Babiuk LA, Griebel P. Comparative analysis of innate immune responses following infection of newborn calves with bovine rotavirus and bovine coronavirus. *J Gen Virol* (2007) 88:2749–61. doi:10.1099/vir.0.82861-0
5. Lee SR, Pharr GT, Boyd BL, Pinchuk LM. Bovine viral diarrhea viruses modulate toll-like receptors, cytokines and co-stimulatory molecules genes expression in bovine peripheral blood monocytes. *Comp Immunol Microbiol Infect Dis* (2008) 31:403–18. doi:10.1016/j.cimid.2007.06.006
6. Villena J, Vizoso-Pinto MG, Kitazawa H. Intestinal innate antiviral immunity and immunobiotics: beneficial effects against rotavirus infection. *Front Immunol* (2016) 7:563. doi:10.3389/fimmu.2016.00563
7. Kandasamy S, Vlasova AN, Fischer DD, Chattha KS, Shao L, Kumar A, et al. Unraveling the differences between Gram-positive and Gram-negative probiotics in modulating protective immunity to enteric infections. *Front Immunol* (2017) 8:334. doi:10.3389/fimmu.2017.00334
8. Nader-Macías ME, Otero MC, Espeche MC, Maldonado NC. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. *J Ind Microbiol Biotechnol* (2008) 35:1387–95. doi:10.1007/s10295-008-0438-2
9. Maldonado NC, de Ruiz CS, Otero MC, Sesma F, Nader-Macías ME. Lactic acid bacteria isolated from young calves-characterization and potential as probiotics. *Res Vet Sci* (2012) 92:342–9. doi:10.1016/j.rvsc.2011.03.017
10. Uyeno Y, Shigemori S, Shimosato T. Effect of probiotics/prebiotics on cattle health and productivity. *Microbes Environ* (2015) 30(2):126–32. doi:10.1264/jsme2.ME14176
11. Abe F, Ishibashi N, Shimamura S. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J Dairy Sci* (1995) 78(12):2838–46. doi:10.3168/jds.S0022-0302(95)76914-4
12. Işik M, Ekimler F, Ozen N, Firat MZ. Effects of using probiotics on the growth performance and health of dairy calves. *Turk J Vet Anim Sci* (2004) 28(1):63–9.
13. Ewaschuk JB, Naylor JM, Chirino-Trejo M, Zello GA. *Lactobacillus rhamnosus* strain GG is a potential probiotic for calves. *Can J Vet Res* (2004) 68(4):249.
14. Ewaschuk JB, Zello GA, Naylor JM. *Lactobacillus* GG does not affect D-lactic acidosis in diarrheic calves, in a clinical setting. *J Vet Intern Med* (2006) 20(3):614–9. doi:10.1892/0891-6640(2006)20[614:LGDNAD]2.0.CO;2
15. Galvão KN, Santos JE, Coscioni A, Villaseñor M, Sischo WM, Berge ACB. Effect of feeding live yeast products to calves with failure of passive transfer

on performance and patterns of antibiotic resistance in fecal *Escherichia coli*. *Reprod Nutr Dev* (2005) 45(4):427–40. doi:10.1051/rnd:2005040
16. Ramaswami N, Chaudhary LC, Agarwal N, Kamra DN. Effect of lactic acid producing bacteria on the performance of male crossbred calves fed roughage based diet. *Asian-australas J Anim Sci* (2005) 18(8):1110. doi:10.5713/ajas.2005.1110
17. Timmerman HM, Mulder L, Everts H, Van Espen DC, Van Der Wal E, Klaassen G, et al. Health and growth of veal calves fed milk replacers with or without probiotics. *J Dairy Sci* (2005) 88(6):2154–65. doi:10.3168/jds.S0022-0302(05)72891-5
18. Yasuda K, Hashikawa S, Sakamoto H, Tomita Y, Shibata S, Fukata T. A new synbiotic consisting of *Lactobacillus casei* subsp. casei and dextran improves milk production in Holstein dairy cows. *J Vet Med Sci* (2007) 69(2):205–8. doi:10.1292/jvms.69.205
19. Batista CG, Coelho SG, Rabelo E, Lana AMQ, Carvalho AU, Reis RB, et al. Performance and health of calves fed milk without antimicrobials residue or milk from mastitis treated cows with or without probiotic. *Arq Bras Med Vet Zootech* (2008) 60(1):185–91. doi:10.1590/S0102-09352008000100026
20. Sun P, Wang JQ, Zhang HT. Effects of *Bacillus* subtilis natto on performance and immune function of preweaning calves. *J Dairy Sci* (2010) 93(12):5851–5. doi:10.3168/jds.2010-3263
21. Qadis AQ, Goya S, Yatsu M, Yoshida YU, Ichijo T, Sato S. Effects of a bacteria-based probiotic on subpopulations of peripheral leukocytes and their cytokine mRNA expression in calves. *J Vet Med Sci* (2014) 76(2):189–95. doi:10.1292/jvms.13-0534
22. Foditsch C, Pereira RVV, Ganda EK, Gomez MS, Marques EC, Santin T, et al. Oral administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers. *PLoS One* (2015) 10(12):e0145485. doi:10.1371/journal.pone.0145485
23. Zhang R, Zhou M, Tu Y, Zhang NF, Deng KD, Ma T, et al. Effect of oral administration of probiotics on growth performance, apparent nutrient digestibility and stress-related indicators in Holstein calves. *J Anim Physiol Anim Nutr* (2016) 100(1):33–8. doi:10.1111/jpn.12338
24. Fouladgar S, Shahraki AD, Ghalamkari GR, Khani M, Ahmadi F, Erickson PS. Performance of Holstein calves fed whole milk with or without kefir. *J Dairy Sci* (2016) 99(10):8081–9. doi:10.3168/jds.2016-10921
25. Marcondes MI, Pereira TR, Chagas JC, Filgueiras EA, Castro MM, Costa GP, et al. Performance and health of Holstein calves fed different levels of milk fortified with symbiotic complex containing pre- and probiotics. *Trop Anim Health Prod* (2016) 48(8):1555–60. doi:10.1007/s11250-016-1127-1
26. Drackley JK. Calf nutrition from birth to breeding. *Vet Clin North Am Food Anim Pract* (2008) 24(1):55–86. doi:10.1016/j.cvfa.2008.01.001
27. Oikonomou G, Teixeira AG, Foditsch C, Bicalho ML, Machado VS, Bicalho RC. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. *PLoS One* (2013) 8:e63157. doi:10.1371/journal.pone.0063157
28. Bridger PS, Mohr M, Stamm I, Fröhlich J, Föllmann W, Birkner S, et al. Primary bovine colonic cells: a model to study strain-specific responses to *Escherichia coli*. *Vet Immunol Immunopathol* (2010) 137(1–2):54–63. doi:10.1016/j.vetimm.2010.04.010
29. Dibb-Fuller MP, Best A, Stagg DA, Cooley WA, Woodward MJ. An in-vitro model for studying the interaction of *Escherichia coli* O157:H7 and other enteropathogens with bovine primary cell cultures. *J Med Microbiol* (2001) 50(9):759–69. doi:10.1099/0022-1317-50-9-759
30. Follmann W, Weber S, Birkner S. Primary cell cultures of bovine colon epithelium: isolation and cell culture of colonocytes. *Toxicol In Vitro* (2000) 14:435–45. doi:10.1016/S0887-2333(00)00033-3
31. Hoey DEE, Sharp L, Currie C, Lingwood CA, Gally DL, Smith DGE. Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. *Cell Microbiol* (2003) 5(2):85–97. doi:10.1046/j.1462-5822.2003.00254.x
32. Rusu D, Loret S, Peulen O, Mainil J, Dandrifosse G. Immunochemical, biomolecular and biochemical characterization of bovine epithelial intestinal primocultures. *BMC Cell Biol* (2005) 6:42. doi:10.1186/1471-2121-6-42
33. Baskaran S, Venkitanarayanan A. Plant-derived antimicrobials reduce *E. coli* O157:H7 virulence factors critical for colonization in cattle gastrointestinal tract in vitro. *Biomed Res Int* (2014) 2014:212395. doi:10.1155/2014/212395
34. Zhan K, Lin M, Liu MM, Sui YN, Zhao GQ. Establishment of primary bovine intestinal epithelial cell culture and clone method. *In Vitro Cell Dev Biol Anim* (2017) 53(1):54–7. doi:10.1007/s11626-016-0082-5
35. Rathi R, Kadian SK, Khurana B, Grover YP, Gulati BR. Evaluation of immune response to bovine rotavirus following oral and intraperitoneal inoculation in mice. *Indian J Exp Biol* (2007) 45:212–6.
36. Bagchi P, Nandi S, Chattopadhyay S, Bhowmick R, Halder UC, Nayak MK, et al. Identification of common human host genes involved in pathogenesis of different rotavirus strains: an attempt to recognize probable antiviral targets. *Virus Res* (2012) 169(1):144–53. doi:10.1016/j.virusres.2012.07.021
37. Kaushik RS, Begg AA, Wilson HL, Aich P, Abrahamsen MS, Potter A, et al. Establishment of fetal bovine intestinal epithelial cell cultures susceptible to bovine rotavirus infection. *J Virol Methods* (2008) 148(1–2):182–96. doi:10.1016/j.jviromet.2007.11.006
38. Miyazawa K, Hondo T, Kanaya T, Tanaka S, Takakura I, Itani W, et al. Characterization of newly established bovine intestinal epithelial cell line. *Histochem Cell Biol* (2010) 133(1):125–34. doi:10.1007/s00418-009-0648-3
39. Hollande F, Lee DJ, Choquet A, Roche S, Baldwin GS. Adherens junctions and tight junctions are regulated via different pathways by progastrin in epithelial cells. *J Cell Sci* (2003) 116:1187–97. doi:10.1242/jcs.00321
40. Kitazawa H, Villena J. Modulation of respiratory TLR3-anti-viral response by probiotic microorganisms: lessons learned from *Lactobacillus rhamnosus* CRL1505. *Front Immunol* (2014) 5:201. doi:10.3389/fimmu.2014.00201
41. Takanashi N, Tomosada Y, Villena J, Murata K, Takahashi T, Chiba E, et al. Advanced application of bovine intestinal epithelial cell line for evaluating regulatory effect of lactobacilli against heat-killed enterotoxigenic *Escherichia coli*-mediated inflammation. *BMC Microbiol* (2013) 13:54. doi:10.1186/1471-2180-13-54
42. Chiba E, Villena J, Hosoya S, Takanashi N, Simazu T, Aso H, et al. A newly established bovine intestinal epithelial cell line is effective for in vitro screening of potential antiviral immunobiotic microorganisms for cattle. *Res Vet Sci* (2012) 93:688–94. doi:10.1016/j.rvsc.2011.10.002
43. Rollo EE, Kumar KP, Reich NC, Cohen J, Angel J, Greenberg HB, et al. The epithelial cell response to rotavirus infection. *J Immunol* (1999) 163(8):4442–52.
44. Xu J, Yang Y, Wang C, Jiang B. Rotavirus and coxsackievirus infection activated different profiles of toll-like receptors and chemokines in intestinal epithelial cells. *Inflamm Res* (2009) 58(9):585–92. doi:10.1007/s00011-009-0022-x
45. Kobayashi H, Kanmani P, Ishizuka T, Miyazaki A, Soma J, Albarracin L, et al. Development of an in vitro immunobiotic evaluation system against rotavirus infection in bovine intestinal epitheliocytes. *Benef Microbes* (2017) 8(2):309–21. doi:10.3920/BM2016.0155
46. Lin CL, Chen SC, Liu SY, Chen KT. Disease caused by rotavirus infection. *Open Virol J* (2014) 8:14–9. doi:10.2174/1874357901408010014
47. Chang K, Kim Y, Saif LJ. Rotavirus and reovirus. 10th ed. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. *Diseases of Swine*. West Sussex, UK: Wiley-Blackwell (2012). p. 621–34.
48. Liu F, Li G, Wen K, Bui T, Cao D, Zhang Y, et al. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. *Viral Immunol* (2010) 23:135–49. doi:10.1089/vim.2009.0088
49. Ishizuka T, Kanmani P, Kobayashi K, Miyazaki A, Soma J, Suda Y, et al. Immunobiotic bifidobacteria strains modulate rotavirus immune response in porcine intestinal epitheliocytes via pattern recognition receptor signaling. *PLoS One* (2016) 11:e0152416. doi:10.1371/journal.pone.0152416
50. Peterhans E, Jungi TW, Schweizer M. BVDV and innate immunity. *Biologicals* (2003) 31(2):107–12. doi:10.1016/S1045-1056(03)00024-1
51. Vercammen E, Staal J, Beyaert R. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. *Clin Microbiol Rev* (2008) 21(1):13–25. doi:10.1128/CMR.00022-07
52. Zhou R, Wei H, Sun R, Zhang J, Tian Z. NKG2D recognition mediates toll-like receptor 3 signaling-induced breakdown of epithelial homeostasis in the small intestines of mice. *Proc Natl Acad Sci U S A* (2007) 104(18):7512–5. doi:10.1073/pnas.0700822104
53. Bunešová V, Domig KJ, Killer J, Vlková E, Kopečný J, Mrázek J, et al. Characterization of bifidobacteria suitable for probiotic use in calves. *Anaerobe* (2012) 18(1):166–8. doi:10.1016/j.anaerobe.2011.09.008
54. Hosoya S, Villena J, Shimazu T, Tohno M, Fujie H, Chiba E, et al. Immunobiotic lactic acid bacteria beneficially regulate immune response

triggered by poly(I:C) in porcine intestinal epithelial cells. *Vet Res* (2011) 42:111. doi:10.1186/1297-9716-42-111
55. Villena J, Kitazawa H. Modulation of intestinal TLR4-inflammatory signalling pathways by probiotic microorganisms: lessons learned from *Lactobacillus jensenii* TL2937. *Front Immunol* (2014) 4:512. doi:10.3389/fimmu.2013.00512
56. Papp H, Laszlo B, Jakab F, Ganesh B, De Grazia S, Matthijnssens J, et al. Review of group A rotavirus strains reported in swine and cattle. *Vet Microbiol* (2013) 165:190–9. doi:10.1016/j.vetmic.2013.03.020
57. Arnold MM, Sen A, Greenberg HB, Patton JH. The battle between rotavirus and its host for control of the interferon signaling pathway. *PLoS Pathog* (2013) 9:e1003064. doi:10.1371/journal.ppat.1003064
58. Kang JY, Lee DK, Ha NJ, Shin HS. Antiviral effects of *Lactobacillus ruminis* SPM0211 and *Bifidobacterium longum* SPM1205 and SPM1206 on rotavirus-infected Caco-2 cells and a neonatal mouse model. *J Microbiol* (2015) 53:796–803. doi:10.1007/s12275-015-5302-2
59. Albarracin L, Kobayashi H, Iida H, Sato N, Nochi T, Aso H, et al. Transcriptomic analysis of the innate antiviral immune response in porcine intestinal epithelial cells: influence of immunobiotic lactobacilli. *Front Immunol* (2017) 8:57. doi:10.3389/fimmu.2017.00057

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 22 June 2018
doi: 10.3389/fnut.2018.00052

Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection

*Laurien H. Ulfman[1], Jeanette H. W. Leusen[2], Huub F. J. Savelkoul[3,4], John O. Warner[5] and R. J. Joost van Neerven[1,3]**

[1] FrieslandCampina, Amersfoort, Netherlands, [2] Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, Netherlands, [3] Wageningen University & Research, Cell Biology and Immunology, Wageningen, Netherlands, [4] Allergy Consortium Wageningen, Wageningen, Netherlands, [5] National Institute of Health Research, Collaboration for Leadership in Applied Health Research and Care for NW London, Imperial College, London, United Kingdom

This review aims to provide an in depth overview of the current knowledge of the effects of bovine immunoglobulins on the human immune system. The stability and functional effects of orally ingested bovine immunoglobulins in milk products are described and potential mechanisms of action are discussed. Orally ingested bovine IgG (bovine IgG) can be recovered from feces, ranging from very low levels up to 50% of the ingested IgG that has passed through the gastrointestinal tract. In infants the recovered levels are higher than in adults most likely due to differences in stomach and intestinal conditions such as pH. This indicates that bovine IgG can be functionally active throughout the gastrointestinal tract. Indeed, a large number of studies in infants and adults have shown that bovine IgG (or colostrum as a rich source thereof) can prevent gastrointestinal tract infections, upper respiratory tract infections, and LPS-induced inflammation. These studies vary considerably in target group, design, source of bovine IgG, dosage, and endpoints measured making it hard to draw general conclusions on effectiveness of bovine immunoglobulin rich preparations. Typical sources of bovine IgG used in human studies are serum-derived IgG, colostrum, colostrum-derived IgG, or milk-derived immunoglobulins. In addition, many studies have used IgG from vaccinated cows, but studies using IgG from nonimmunized animals have also been reported to be effective. Mechanistically, bovine IgG binds to many human pathogens and allergens, can neutralize experimental infection of human cells, and limits gastrointestinal inflammation. Furthermore, bovine IgG binds to human Fc receptors which, enhances phagocytosis, killing of bacteria and antigen presentation and bovine IgG supports gastrointestinal barrier function in *in vitro* models. These mechanisms are becoming more and more established and explain why bovine IgG can have immunological effects *in vivo*. The inclusion of oral bovine immunoglobulins in specialized dairy products and infant nutrition may therefore be a promising approach to support immune function in vulnerable groups such as infants, children, elderly and immunocompromised patients.

Keywords: bovine immunoglobulins, milk, colostrum, immune, infection, allergy

OPEN ACCESS

Edited by:
Willem Van Eden, Utrecht University, Netherlands

Reviewed by:
Brandt D. Pence, University of Memphis, United States
Claudio Nicoletti, Università degli Studi di Firenze, Italy

***Correspondence:**
R. J. Joost van Neerven
joost.vanneerven@wur.nl

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *23 April 2018*
Accepted: *30 May 2018*
Published: *22 June 2018*

Citation:
Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO and van Neerven RJJ (2018) Effects of Bovine Immunoglobulins on Immune Function, Allergy, and Infection. Front. Nutr. 5:52. doi: 10.3389/fnut.2018.00052

HUMAN IMMUNOGLOBULINS IN MILK

The first year of life is essential for infants to develop their immune system. At the same time the child is exposed to many new and potentially immunogenic stimuli such as dietary components (food) allergens and the microbiota that colonize the gastrointestinal tract. Therefore, during this year, it is crucial to have tightly regulated immune responses to prevent inflammatory responses to these (stimuli) food antigens, allergens, and commensal bacteria.

It is known that human breast milk and bovine milk contain many factors that are involved in immune development. Milk contains immunoglobulins, prebiotic oligosaccharides, extracellular vesicles (e.g., exosomes), antimicrobial proteins such as lysozyme, lactoperoxidase, and lactoferrin, also immunomodulatory cytokines such as IL-10 and TGF-β. In addition, breastfeeding has been shown to provide protection against gastrointestinal and respiratory tract infections (1, 2). It prevents excessive immune responses during immune development in early life (3) which may be attributed to the orchestrated activity of the above mentioned milk components. Most of these components—with the exception of human milk oligosaccharides—are relatively well-conserved between bovine and human milk. Bovine milk and its components have clear functional effects on the human immune system (4). However, as many other immunomodulatory molecules are present in milk, the individual contribution of each of these to disease prevention is difficult to determine (5).

As early as 1892, Paul Ehrlich demonstrated that mouse maternal immunoglobulins confer passive immunity to their murine offspring (6, 7). Immunoglobulins (or antibodies) are glycoproteins produced by plasma/B cells that specifically recognize and bind antigens, that are present on e.g. bacteria and viruses. As the immunoglobulins have a high degree of specificity they assist in the destruction of specific pathogens. In addition to this specific recognition, various human immunoglobulin isotypes (IgM, IgG, IgA), and subclasses (IgG1-4, IgA1-2) differ in their biological features, structure, target specificity and distribution. Whilst IgG is the most predominant antibody in the serum, IgA is the main isotype of the immunoglobulins found in human milk and colostrum, followed by IgG and IgM.

In mature human breast milk, IgA is present at levels around 1 mg/ml, whilst in colostrum levels can reach up to 5–12 mg/ml (8, 9). The specificity of maternal IgA reflects the past exposure of the mother to bacteria and viruses present on mucosal surfaces in the gastrointestinal and respiratory tract and thus there is a transfer of immunological memory of the mother to the infant. Breastmilk IgA and IgG can promote neutralization and killing of pathogens and can also modulate immune function in infants, thereby protecting them against inflammatory responses (10–14). Therefore, the concept of vaccinating mothers against pathogens to optimize the protection of their nursing children has increasing interest (15, 16). Indeed in the UK both influenza and pertussis immunization are recommended during pregnancy and others such as for Group B streptococcal infection are now being tested *in vivo* (17).

The levels of serum IgA produced by the newborn infant itself are very low (18). At the age of 1 year, secretory (s)IgA is only 20% of the level observed in adults and circulating levels only reach maturity by the age of 4 years (18). Aberrant IgA responses in the gut have been described in several gastrointestinal diseases (19). Furthermore, delayed increases in IgA in the infant and low maternal IgA levels in breast milk have been associated with a higher risk of infection, allergy, and autoimmune diseases (20).

sIgA also has an important role in the development of oral tolerance toward the gut microbiota. Kohler showed that fecal sIgA concentrations peak at 1 month of age in exclusively breastfed infants compared to exclusively formula fed infants and decline to levels observed in formula fed infants around 5 months. From this timepoint onwards, no difference was observed between fecal sIgA concentration between breastfed and formula fed infants (21). In line with this, other studies showed that fecal sIgA concentration in exclusively formula-fed infants were only 33% of breastfed infants' levels at 1 month and only reached comparable levels to breastfed infants at 9 months of age [(21), and reviewed in (22)]. Low levels of fecal IgA in infants have been associated with a higher risk of allergy (23). Thus, the supply of sIgA in breast milk has a potentially pivotal role in enhancing intestinal immunity in early life and help in establishing a healthy outcome (24). However, interpreting the results of human observational studies of birth cohorts in relation to health outcomes is difficult as considerable variability exists in the breast milk content of immune active molecules both between mother/infant pairs and within such pairs at the different sampling points. Numerous genetic and environmental influences affect the levels of immune factors in milk and as yet no study has been able to account for the variability (22). Furthermore, as there are many other immunomodulatory molecules in the milk, the individual contribution of each to disease prevention is unknown (5).

Just as the multiple advantages of breast milk described, cow's milk consumption has also been associated with specific health outcomes. Several epidemiological studies have shown a positive association between the consumption of unprocessed cow's milk and a reduced risk for developing allergy, most prominently allergic asthma (25–27). This association was not found for the consumption of heated farm milk and commercial high heat treated milk (e.g., UHT) (28). The consumption of these different processed cow milk types is consumed on top of regular breastmilk and formula milk consumption and can thus be considered as weaning food. The levels of intact milk protein in these cow's milks correlated with the protective effect on asthma (28), suggesting that intact milk protein confer this effect but not denatured protein which occurs upon heating (29, 30). Several factors that are present in breast milk as well as in cow's milk can be linked to this effect on allergy (4). In addition, raw milk consumption in the first year of life is also associated with decreased risk of upper respiratory tract infections and otitis media (31).

This well-established protective function of immunoglobulins in breast milk, and in neonatal health in farm animals, has meant that bovine immunoglobulins have been studied extensively for their putative effect on human health.

BOVINE IMMUNOGLOBULINS FROM MILK, COLOSTRUM, AND SERUM

Bovine milk contains many components that have immunomodulatory and antimicrobial properties. Bovine immunoglobulins (bIg), in particular bovine IgG, have been studied since the 1970's for their potential effects on immunity and infection in humans.

Bovine IgG can not only bind to a wide range of pathogenic bacteria and viruses (32–40), but also to many allergens (41). In addition, the specificity of the immunoglobulins in the milk or colostrum can be increased by vaccinating the cows before collecting their milk or colostrum. After vaccination hyperimmune colostrum is strongly enriched for IgG1 that recognizes the pathogen included in the vaccine. Indeed, the first studies on the use of oral bovine immunoglobulins to prevent and treat gastrointestinal infections have focused on the use of hyperimmune colostrum containing rotavirus-specific immunoglobulins (40, 42). These were used for the treatment and prophylaxis of rotavirus infection in infants and children and showed good efficacy in several studies (described in section Treatment of Git Infection in Infants and Children and Prevention of Gastrointestinal Tract infection). Since these early studies, many studies have been performed utilizing different *in vitro* systems, animal models and human studies in order to determine mechanisms of action and their applicability for human health.

Sources and Origin of Bovine IgG

In contrast to humans, in cow's milk IgG is the main isotype present, especially in colostrum, followed by IgA and IgM (43). There are 2 subclasses of IgG known in cows: IgG1 and IgG2. IgG1 is the main isotype of immunoglobulin found in bovine milk and colostrum. In mature milk IgG1 is the dominant isotype, whereas sIgA and IgM are present at ∼5–10 fold lower levels. The concentration of IgG1 in mature milk is around 200–500 μg/ml although levels reported in literature differ depending on type of measurement technique used (44, 45).

In colostrum the IgG levels are much higher, reaching up to 50–100 mg/ml in the first days after birth [reviewed in (46)]. These high concentrations are necessary for the calf because cows cannot transfer IgG across the placenta. Calves are thus dependent on the transfer of IgG from colostrum into the blood, and calves that do not receive colostrum after birth are immunocompromised and prone to infection. High levels of colostrum-derived IgG in blood of calves are associated with reduced risks of pneumonia (47). Likewise, colostrum intake by calves is crucial to protect against gastrointestinal tract infections. This uptake of IgG occurs partly via passive transport across the epithelium when it is not yet fully closed, but also actively via FcRn, the neonatal IgG receptor that is expressed on intestinal epithelium in neonatal calves (48, 49).

This high level of IgG in colostrum has meant that many studies have used colostrum as an IgG rich source for studies, rather than purified IgG (1). However, in colostrum—as in mature milk—many other factors are present that can have immunological effects, but compared to IgG their concentrations in colostrum are relatively low. For this reason, colostrum is often regarded as an IgG1 preparation even though additional factors are present. More recently, bovine immunoglobulins isolated from serum—that is also high in IgG and low in IgM and IgA—have been used to study their effects on chronic and severe gastrointestinal disturbances in humans (50).

Maternal serum IgG levels in cows decrease before delivery (51), shown by following labeled IgG1 and IgG2 transfer from blood into colostrum (52). The majority of all maternal IgG in colostrum thus seems to be transported from the serum into colostrum and milk (52, 53). The immunoglobulins in milk and colostrum can therefore be considered as a good reflection of the immunoglobulins in maternal serum.

Digestion of Bovine IgG After Oral Administration

In order to be used as an active, prophylactic ingredient in human food products, a significant amount of bovine immunoglobulins must be able to survive passage through the stomach into the small intestine, or even throughout the entire gastrointestinal (GI) tract. This has been the subject of several studies, reviewed in detail in (54).

An early study by Hilpert et al. showed that Enteropathogenic *E. coli* (EPEC)-specific IgG or active fragments thereof are present in the stools of infants that received immunoglobulins from cows immunized against EPEC (55). In line with this, in the stools from children with rotavirus that received oral colostral immunoglobulins anti-rotaviral immunoglobulin activity could be detected (56). This data indicate that IgG can survive passage through the GI tract and retain functionality.

In a study of ileostomy patients, a total of 50% of all orally administered IgG1 from an bovine immunoglobulin concentrate could be recovered in ileal fluid (57). Interestingly, no enhanced survival was seen by combining the preparation with antacid or omeprazole (a proton pump inhibitor) to counter the effect of low gastric pH, suggesting that pH regulation in the stomach does not enhance the survival of the IgGs.

Two other studies have shown that up to 10–20% of the immunoglobulins may survive GI passage in infants (58) and adults (59). However another study, showed only 3% of orally ingested bovine IgG could survive the passage through the GI tract in healthy adults (60). Encapsulation of the immunoglobulins in entero-protective capsules that dissolve at pH 6 and higher, increased GI tract survival of bovine IgG from 3 to 30% (60). Similarly, in mice consuming bovine colostrum, 5% of the IgG could be recovered in the colon, which was increased to >20% for encapsulated colostrum (61).

In contrast, two other studies could not recover significant amounts of bovine IgG in stools of healthy adults (36, 62). Bogstedt (62) showed <0.01% of orally ingested bovine IgG recovery in stool in healthy adults. Furthermore, Lissner (36) showed low recovery and only in 3 out of 8 healthy volunteers after ingestion of 15 g of colostrum powder. It was noted that the longer residence time in the large intestine is determining the observed low survival of IgG, especially when compared to

other studies in which people with diarrhea were investigated (62). Furthermore, a recent study showed that even though orally ingested bovine IgG can be detected in fecal matter, bovine IgG is not taken up into circulation in adult human volunteers (63). An explanation of the different recoveries of bovine IgG in fecal matter after oral administration by adults could rely on differences in time of fecal matter sampling, the analytical methods used or set up of the study. Kelly et al. (60) who showed the 3% recovery level tracked fecal passage by oral ingestion of carmin red combined witha 3 days stool collection. As the duration of fecel transfer differs per person, and if no tracker is used (36, 62), it is difficult to predict the peak of bovine IgG in feces. The risk is that the peak may be missed. Furthermore, differences in protease inhibitors have been reported therefore a cocktail (60) might be more effective compared to a single trypsin inhibitor (64).

Even though these findings consistently show survival of bovine IgG throughout the GI tract, the amounts recovered in feces vary from trace amounts (0.01%) up to 50% between different studies most likely due to differences in study methodology.

Collectively, the data indicates that a significant amount of orally ingested bovine IgG immunoglobulins are present throughout the GI tract, especially so in infants as they have a higher gastric pH and lower levels of proteolysis in the GI tract. The recent insights in the role of maternal breast milk immunoglobulins and its role in microbiota development in the infant stress the importance of keeping these structures intact. This is further discussed in section Immunological effects of Oral immunoglobulins of other species.

Specificity of Bovine IgG in Normal Milk and Colostrum

The presence of bovine IgG1 against rotavirus in normal cow's milk was first demonstrated in the 1970's by Ellens et al. (37). This was followed by a series of studies on binding of bovine IgG to many human bacterial pathogens including *Yersinia enterocolitica, Campylobacter jejuni*, *Escherichia coli*, *Klebsiella pneumoniae, Serratia marescens, Salmonella typhimurium, Staphylococcus, Streptococcus, Cryptosporidium, Helicobacter, E.coli EHEC O157:H7, Pseudomonas*, and Rotavirus (32–36). Bovine colostrum also contains IgG1 and IgA antibodies directed against Necrotizing enterocolitis (NEC)-associated pathogens such as *Klebsiella, Citrobacter, Enterobacter* and *Serratia* (65).

In addition to recognizing gastrointestinal tract associated pathogens, bovine Igs can also bind to respiratory pathogens such as human Respiratory Syncitial Virus (RSV), influenza virus and *Streptococcus pneumoniae* (38). This may in part explain the finding by Loss et al. that raw milk consumption in the first year of life is also associated with fewer upper respiratory tract infections and otitis media (31).

In addition to immunoglobulins specific for infectious microorganisms, Collins et al. have shown that cow's milk and colostrum can also contain immunoglobulins that are specific for aeroallergens like rye-grass pollen, house dust mite, aspergillus, and wheat allergens (41).

Although bovine milk IgG has been investigated to a larger extent than IgA, it is assumed that the specificities are similar between the two. Furthermore, a recent report compared the specificity between bovine sIgA and human sIgA isolated from milk to pathogenic and commensal bacteria and concluded that the binding characteristics of both human and bovine sIgA from milk were similar (66).

In summary, bovine immunoglobulins can bind to a wide range of human intestinal and respiratory bacterial—as well as viral pathogens, and can also bind to inhalation and some food allergens. **Figure 1** shows the proposed effects of bovine IgG along the gastrointestinal tract.

IN VIVO STUDIES ON EFFECTS OF BOVINE IGG AND COLOSTRUM ON GASTROINTESTINAL TRACT INFECTIONS

Studies on the effect of bovine immunoglobulins on human immune function and susceptibility to infection have been performed with a wide range of Immunoglobulin rich products which can be categorized in three groups: IgG-isolates from colostrum or milk, IgG-rich colostrum, and serum-derived IgG. These products have either been tested for prophylactic or therapeutic effects in field settings or in controlled challenge models.

Treatment of Git Infection in Infants and Children

A number of studies have been performed in which (hyperimmune) colostrum or immunoglobulins derived thereof were used for treatment in infants and young children and are summarized in **Table 1**. Four independent studies describe rotavirus-diarrhea treatment with hyperimmune colostrum products. Two studies were double blind, placebo controlled studies (67, 68) performed in Bangladesh and another two were controlled studies in Europe (58, 69). Three of the studies showed significant clinical effects including a reduction in duration of diarrhea (67) and stool frequency (67, 68) the duration of rotavirus excretion (58) and need for oral rehydration solution (ORS) (68). In the fourth study, a trend was noted toward shorter duration and decreased stool frequencies in the actively treated group, but were not significant compared to the control group (69). All these studies on treatment were performed in infants of a similar age (∼4–30 months old) but with bovine immunoglobulin doses ranging from 3 g (100 ml colostrum) to 20 g (based on a 10 kg child receiving 2 g/kg body weight).

An early treatment study providing a specific milk immunoglobulin concentrate (1 g/kg/day for 10 days) against enteropathogenic *E. coli* (EPEC) to infants (10 days−18 months old) suffering from enteropathogenic *E. coli* (EPEC) induced diarrhea showed stool cultures being negative for EPEC in 84% of the cases (70). The control group suffered from diarrhea induced by other *E. coli* strains that did not receive the milk immunoglobulin concentrate and resulted in negative stool cultures in only 11% (1/9) of the cases. In another, double blind, placebo controlled study, performed in

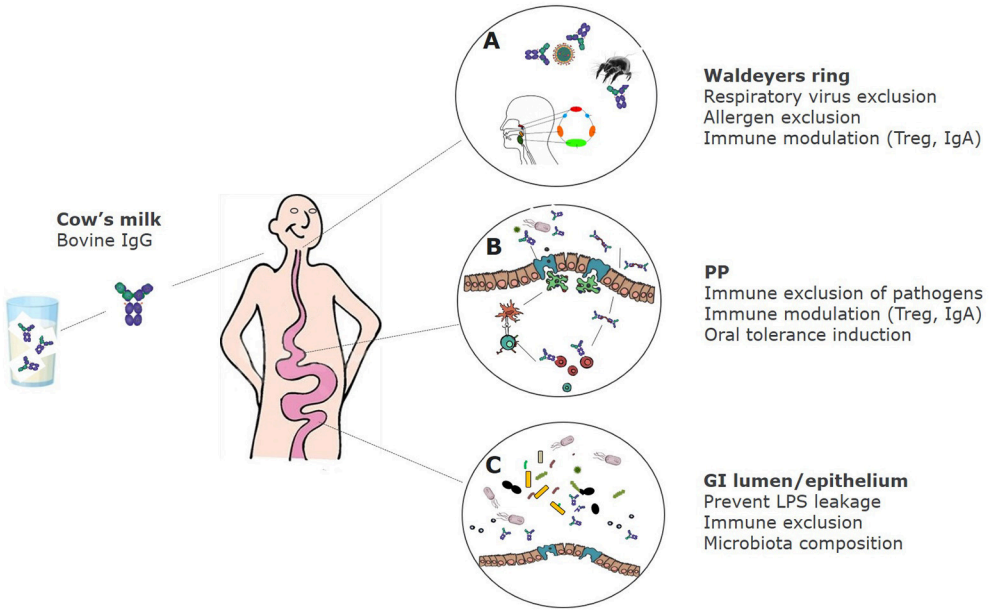


FIGURE 1 | Proposed effects of bovine IgG at various locations in the GI tract. **(A)** After ingestion bovine IgG can encounter swallowed respiratory pathogens and inhaled allergens. This can lead to partial immune exclusion (especially when milk is regurgitated and enters the nasopharynx) and immune modulation in the tonsils that comprise Waldeyer's ring. **(B)** In the small intestine IgG can also exclude pathogens by preventing adhesion to epithelial surfaces, but may also promote the uptake of immune complexes of IgG with pathogens via Fc receptors, resulting in regulatory immune responses and induction of IgA. **(C)** In the colon, IgG prevents leakage of LPS, can modify microbiota composition and short-chain fatty acids (SCFA) production, and can prevent adhesion of pathogens.

Bangladesh Casswall et al. could not demonstrate a significant effect of an oral bovine immunoglobulin milk concentrate from cows hyperimmunized with enterotoxicogenic *E. coli* (ETEC) and EPEC strains on the duration, ORS intake, stool frequency or duration of *E.coli*-induced acute diarrhea (71). Within this study, children (4–24 months of age) that already had *E. coli* induced diarrhea were included and given 20 g of immunoglobulin concentrate for 4 days. This is a 20-fold higher dose than described in the study by Mietens et al. (70). Since no effects were seen in the Bangladesh study, it was hypothesized that the strains used for vaccination of the cows and the strains that infected the children differed too much.

For other pathogens such as *Helicobacter pylori* and *Shigella*, the use of hyperimmune colostrum in treatment has been less successful compared to rotavirus and *E. coli* induced diarrhea. In a study performed in infants in rural Bangladesh *Helicobacter pylori*-infected children (4–29 months of age) treatment with purified immunoglobulins from hyperimmune colostrum failed to eradicate *H. pylori* infection (72). In this double blind, placebo controlled study, children were treated for 1 month with 1 g hyperimmune colostrum containing 0.7 g of immunoglobulins per day. Although hyperimmune colostrum against *Helicobacter pylori* prevented adhesion of helicobacter *in vitro*, and treated an infection *in vivo* in mice (75), an effect *in vivo* in humans was not observed. Specificity of the strains used for the vaccine or age of the target group are two factors that might be responsible for the differing outcomes. Hyperimmune colostrum of cows immunized with *Shighella* did not have an effect on *Shighella* dysenteria-infected children (1–12 years of age) in Bangladesh in a double blind, placebo controlled trial (73). No significant differences were seen in duration of diarrhea, fever, stool frequency and visible blood in the stool between the actively treated (100 ml of hyperimmune colostrum 3 times per day) and the control group. Interestingly, a single placebo controlled study reported that children (0–18 years, mean age of 2) suffering from Shiga toxin-producing *E. coli* induced diarrhea and treated with a normal (non-hyperimmune) colostrum resulted in significantly reduced stool frequency (74). Yet, no effects were shown on pathogen numbers or complications of infection. One of the factors which might explain the difference in the observed effect was the timing of the start of treatment. In the study that did show a beneficial effect of treatment was started 24–48 h after diarrhea and in the other study only after 48–72 h. Other reasons such as study power cannot be excluded. Intervention studies have been described in literature testing immunoglobulin preparations for treatment and/or prevention of parasites and fungi reviewed by Hammarstrom et al. (76).

Although not all studies discussed above show effects of bovine IgG in the treatment of on-going gastrointestinal tract infections in children and infants, the majority of the studies indicate that bovine IgG, especially after prior bovine vaccination with the specific organism, can help in shortening the duration of gastrointestinal infections.

TABLE 1 | Treatment of GIT infections in infants and children.

Subject characterisitics	Product, dosage, and duration	Type of study	Reported outcome	References
3 months−6 year Japanese children with rotavirus diarrhea (active $n = 18$/placebo $n = 26$)	Hyper immune colostrum (titer 1:1280 – 1:5120) 20–50 mL/day, 3 days	Randomized	No significant differences between Rota colostrum recipients and controls.	(40)
6–24 months old Bangladeshian children with rotavirus diarrhea (active $n = 35$/placebo $n = 33$)	Hyperimmune- vs. normal colostrum rota strains WA, RV5, RV3 and ST3. 100-fold titer difference 100 mL/day, 3 days	Randomized, double blind	Reduction of duration and severity of diarrhea in treatment group	(67)
4–24 months old Bangladeshian children with rotavirus diarrhea (active $n = 40$ placebo $n = 40$)	Hyper immune colostrum 4 × 2.5 g/day, 4 days	Double blind	Decreased stool output and -frequency; increased, and faster clearance of rotavirus from the stool	(68)
<2 year old German children with rotavirus diarrhea [acive $n = 46$; placebo $n = 45$ (low titer)] and [active $n = 43$; placebo $n = 30$ (high titer)]	Bovine antibody concentrate. Low and high rotatiters (low: f1.100 high: =1:6.000) 2 g/kg bw/day, 5 days	Open controlled	Low titers, no effect. High titers; trend for reduced duration diarrhea, sign. reduction viral shedding.	(58)
6–30 months old Finnish children with rotavirus diarrhea ($n = 42$; $n = 42$; $n = 41$)	Hyperimmune- vs. standard colostrum vs. milk SA rotavirus titers: 1:597 vs. 1:128 vs. 1:16 4 × 100 mL/day, 4 days	Randomized, double blind	Trend but no significant differences for weight gain, reduced duration and frequency of diarrhea in hyperimmune group compared to other 2 groups	(69)
10 day−18 months German children with ETEC diarrhea ($n = 60$)	Hyperimmune milk (0–7 days) 0.32 g/kg/day 1 g/kg, 10 days	Open	Negative stool culture in 84% of the patients	(70)
4–24 months Bangladeshian children with a history of acute watery diarrhea for ‘48 h (active $n = 32$; placebo $n = 31$)	Hyperimmune- vs. non-immune milk Ig against EPEC and ETEC, 45, 40% respectively. 20 g/day, 4 days	Placebo controlled, randomized, double blind	No effect of hyperimmune milk on any of the outcomes.	(71)
4–29 months old *H. pylori* infected Bangladeshian children (active $n = 12$;placebo $n = 12$)	Hyperimmune Ig preparation vs. non-immune colostrum, 1 g/day, 30 days	Placebo controlled, double blind pilot	No effect was observed by hyperimmune product on H pylori infection as determined by UBT	(72)
1–12 year old Bangladeshian children with bloody mucoid diarrhea < 5 days, Shigellosis (active $n = 34$; placebo $n = 35$)	Hyperimmune vs. non-immune colostrumtiter 3,200–6,400 anti-shigella 3 × 100 mL, 3 days	Placebo controlled, randomized	No difference between hyper and non-immune colostrum group in any of the outcomes	(73)
1 months−18 year old German children with *E. coli* associated diarrhea (active $n = 13$;placebo $n = 14$)	Immunoglobulin concentrate (Lactobin) vs. placebo 80% protein, > 65% Immunoglobulin 3× 7 g, 14 days	Placebo controlled, double blind	Stool frequency was reduced in Ig Concentrate treated group. No effects on carriage pathogens and complications of infections.	(74)

Treatment of Gastrointestinal Infections in Adults With Hyperimmune Colostrum

In a single, human trial with adult patients suffering from *Clostridium difficile* associated diarrhea, Van Dissel et al. (77) showed that a whey protein concentrate (WPC40) from *Clostridium* immunized cows containing a high concentration of specific sIgA might prevent relapsing clostridium-induced diarrhea after antibiotic treatment. The trial was uncontrolled, and should therefore be considered as preliminary. In addition, the study also showed that the hyperimmune WPC40 could prevent lethality in a hamster model of *Clostridium* infection.

Similarly, in a prospective randomized double blind study to compare treatment of *Clostridium difficile* associated diarrhea patients were treated with either colostral immunoglobulins or metronidazole. The study showed that colostral immunoglobulins were as effective in the prevention of *clostridium difficile*-associated diarrhea as treatment with metronidazole (78).

A recent trial treating *Helicobacter pylori*-infected adults with milk of immunized cows (randomized double-blind trial) showed an increased clearance of intragastric *Helicobacter pylori* (79). A third gastrointestinal pathogen that has been studied for its' potential to be treated with bovine hyperimmune colostrum is *cholera*-induced diarrhea. In this paper the results of two randomized, controlled clinical trials were reported but no effect on reducing stool volumes was observed (80).

Prevention of Gastrointestinal Tract Infection Upon a Pathogen Challenge of Healthy Volunteers

To assess the biological activity of new nutritional concepts, studies have been set up that utilize adult human volunteers that were given immunoglobulin preparations derived from hyperimmune colostrum orally before a challenge with live pathogens. These studies are mainly performed with products containing immunoglobulins specific for *E.coli* to prevent travellers' diarrhea.

In the first study, Tacket et al. (81) showed a clear prophylactic effect of hyperimmune colostrum from cows immunized with a range of *E.coli* serotypes. In this study

volunteers received an oral challenge with 10^9 enterotoxicogenic *E.coli*. The volunteers received either hyperimmune colostrum (3 times/day) or a control immunoglobulin concentrate without *E. coli*-specific immunoglobulins. None of the volunteers in the hyperimmune colostrum, while 9/10 in the control group developed diarrhea. Similar preventive effects of *E.coli* specific immunoglobulins were reported by Freedman et al. (82). However, in a follow-up study no effect could be seen using an enteric coated *E.coli* specific immunoglobulin product (83). It could be that the immunoglobulins in enteric capsules were not released until they reached the colon, whilst the infection is initiated in the small intestine (60).

In a placebo controlled challenge study with ETEC H10407, oral hyperimmune bovine IgG isolated from hyperimmune colostrum protected against ETEC-induced diarrhea (84). In a challenge model with an 078 ETEC strain, colostrum protein formulated in tablet form was shown to confer protection against diarrhea in two separate randomized, double blind placebo controlled studies (85).

In a similar challenge study, healthy volunteers were infected with *Cryptosporidium parvum* and prophylactically treated with a hyperimmune colostrum (86). In this study only a non-significant trend toward less diarrhea was noted in the treated group.

The treatment of GI-tract infections in adults, the prophylactic approach in challenge studies with healthy volunteers indicates that hyperimmune immunoglobulins can alleviate or shorten GI tract infections in a well-controlled challenge setting.

Prevention of Gastrointestinal Tract Infection

It was first shown in the 1960s that bovine colostral immunoglobulins protected calves against gastrointestinal bacterial and viral infections (87, 88). This has prompted researchers to investigate the efficacy of bovine immunoglobulins in humans. Alarge number of studies have now shown efficacy of bovine immunoglobulins in preventing natural infection in humans as well as in animal infection models (76). Studies on prevention of gastrointestinal tract infections in infants are summarized in **Table 2**. This concept has been tested extensively for anti-rotavirus antibodies. One of the first studies to demonstrate functional effects of bovine immunoglobulins in humans was a study by Ebina et al. (40) showing that bovine colostrum from naturally rotavirus infected cows protected young children against rotavirus diarrhea during a rotavirus epidemic. A similar protective effect of rotavirus-specific bovine colostral immunoglobulins was described by Davidson in a controlled study in hospitalized Australian children aged 3–15 months (42). In a later study, Davidson confirmed this in Hong Kong and India (89). However, an infant formula with a bovine milk immunoglobulin concentrate from cows immunized with *E. coli* EPEC and Rotavirus did not protect against the development of diarrhea in a controlled study carried out in Chile (91). The reason of the lack of efficacy is not known but the authors speculated that the dose (0.12 g/kg/day) might have been too low. Specificity of the antibodies was in line with the observed pathogens in the study and therefore an unlikely reason for the observed lack of effectivity. Yet, a preventive effect of bovine colostrum immunoglobulins of cows immunized with rotavirus was shown in a study in 3–7 months of old infants in America (90). This was also shown for hyperimmune colostrum against E.coli in a study in 3–6 month old infants in Iraq (92). Infants in the active group had significantly lower incidence in diarrhea and episodes of diarrhea were shorter in duration during a 6 months follow up period. In this study, no effect was seen in children receiving colostrum immunoglobulins from non-immunized cows implying that specific antibodies were needed for the observed benefit. Indeed, rotavirus-specific IgG1 immunoglobulins from bovine milk were shown to protect mice from rotavirus-induced diarrhea (39). Similar findings were reported by others in rotavirus infection models in mice (95, 96), cows (97, 98), and piglets (99). The bovine immunoglobulins were also shown to functionally inhibit the replication of human rotaviruses in *in vitro* tissue culture experiments. This protective effect of hyperimmune bovine IgG has not only been demonstrated for rotavirus in *in vivo* animal models but also for other pathogens such as enteropathogenic *E. coli* bacteria (100) and *Helicobacter pylori* (72, 75).

Preventive studies do not always focus on a specific pathogen to induce diarrhea. In fact, scoring disease episodes within a specific time is a commonly used approach to test whether a specific product lowers the incidence of the disease. Two studies from India and Egypt used this approach and have shown decreased diarrhea in children that received (non-hyperimmune) colostrum products (93, 94). Both studies included children with recurrent infections prior to the start of the study, but unfortunately as both were uncontrolled the colostrum products were not compared to a placebo. Therefore, these results should be interpreted with caution.

The strongest evidence on protective effects of bovine immunoglobulins from non-immunized cows comes from studies in HIV patients with recurrent diarrhea (32, 101–106) and are summarized in **Table 3**. As HIV patients are strongly immunosuppressed as a result of the depletion of CD4+ T cells, they have diminished capacity to resist infections and are highly susceptible to diarrhea, especially induced by *Cryptosporidium, Amoeba* and *Campylobacter*. The interest in colostrum for treating HIV infected people came from an initial report that described a positive effect of an immunoglobulin preparation derived from colostrum of non-immunized cows (32, 101). This was confirmed in another study using a colostrum-based porridge (103). Stool frequencies in these studies decreased from ∼7 times/24 h period to 1–3 times/24 h. In addition to reduced stool frequency, decreased fatigue scores and increased weight and CD4+ T cell counts were also noted (104–106).

These studies clearly demonstrate that the administration of passive immunity in the form of bovine immunoglobulins can be protective against a range of pathogens and is especially effective

TABLE 2 | Prevention of Gastrointestinal tract infections in infants.

Age of infants /children	Product, dosage, and duration	Type of study	Reported outcome	References
3 months–6 year old Japanese infants (active $n = 18$; control $n = 26$) and 1 month–3 year old Japanese orphaned infants (active $n = 6$; control $n = 7$)	Hyperimmune colostrum 0.6 g IgG + 0.02–0.05 g IgA/day 20 mL	Randomized	Prevention of rota infection but no effect on duration of diarrhea, bowel movements or virus shedding in stool.	(40)
3–15 months old Australian infants (active $n = 55$; control $n = 56$)	Hyperimmune colostrum 50 mL/ 1 day, 10days	Randomized, controlled	Effective; 9/ 65 control children and 0/55 treated children acquired rotavirus infection	(42)
1–36 months old infants from HongKong and India (actve $n = 50$; placebo $n = 102$)	Hyperimmune colostrum 2 g/day. 3x a day hospital stay + 3days after	Randomized double blind	0/23, 0/27 in treated and 5/50 and 8/52 in control group showed rota infection in India and HK resp.	(89)
3–7 months old American infants (actve $n = 31$; control $n = 33$)	Hyperimmune colostrum (0.2 mg/ml in formula) >360 mL formula/day, max 6 months	Randomized, controlled	No. of days with diarrhea and rota associated diarrhea sign lower in treatment group. Incidence not sign lower.	(90)
3–6 months old Chilean children (active $n = 124$; control $n = 108$)	Milk Ig concentrate, immunized cows 1 g/day 0.5% wt/wt rota + 0.5% wt/wt EPEC immune conc. 6 months	Double blind	No effect on incidence nor duration of diarrhea	(91)
3–6 months old Iraqi infants ($n = 125$)	Hyperimmune colostrum 0.5 g/kg bw	Randomized double blind	Lower incidence of diarrhea	(92)
1–6 year old Egyption children with recurrent URTI and/or diarrhea (GITI) ($n = 160$)	Bovine colostrum 3 g/day for < 2 year 6gr/day for >2 years, 4 weeks	Open, non-comparative	Lower number of episodes and hospitalizations for GITI (and URTI)	(93)
1–8 year old Indian children with recurrent URTI and/or diarrhea ($n = 605$)	Bovine colostrum 3gr/day, 12 weeks	Open, non-comparative	Lower number of episodes and hospitalizations for GITI (and URTI)	(94)

in immune compromised individuals. Interestingly, overall it seems that treatment and prevention of rotavirus infection with bovine IgG from vaccinated cows is more effective than the same approach for pathogenic bacteria. However, in bacterial challenge models, bovine IgG of vaccinated cows is more effective against bacterial pathogens than in prevention and treatment of normal infections. In these challenge models the pathogen with which the volunteers are challenged is the very same bacterial strain that is used for vaccination of the cows. As a result it is expected that the "coverage" of bacterial epitopes by the IgG raised in the cows is complete, and therefore effective in subsequent challenge models. In a field trial this coverage might not be always complete with the result that the bacteria still finds a way to resist the limited action of the antibodies. Indeed, bacteria have evolved to escape the immune system (107, 108).

In relation to treatment and prevention of natural infections by rotavirus, we speculate that the virus has fewer surface proteins and (oligo)saccharides involved in adhesion and infection than more complex bacterial pathogens. As the prevention and treatment is done with IgG raised against different strains of rotavirus and bacteria than the ones that may be actually the cause of infection, the chance of cross protection of bovine IgG to other strains of similar pathogens is better for rotavirus than for bacteria. This could be because of the more limited numbers of relevant cell surface antigens that are needed to initiate infection with rotavirus.

IN VIVO STUDIES OF BREASTFEEDING AND BOVINE IGG AND COLOSTRUM ON RESPIRATORY TRACT INFECTIONS AND THE RELATION WITH ALLERGY

Breastfeeding And Prevention of Respiratory Tract Infections: A Link to Asthma?

Respiratory tract infections can be divided into upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI). Especially URTI are very common in childhood. Likewise, otitis media, which is infection of the middle ear is highly prevalent, and has partial overlap in symptoms with URTI. Multiple cohort studies have shown that breastfed infants have a lower risk to develop respiratory infections and otitis media compared to formula fed infants (1–3, 109, 110). Duijts (111) et al. showed that exclusive breastfeeding up to 6 months tended to be more protective for upper and lower respiratory tract infections compared to 4 months exclusive breastfeeding. Yet for recurrent upper respiratory infections defined as >3 episodes of influenza/cold Chantry (2) et al. did not observe a difference between not breastfed, < 1 months, 1–3 months, 4–5 months or >6 months breastfed infants. Interestingly, all groups that were breastfed for < 6 months had a 2-fold higher chance to develop recurrent otitis media compared to infants that were breastfed for >6 months. In line with this Ip et al.

TABLE 3 | prevention of GIT in HIV patients.

Number of subjects	Country of study	Product, dosage, and duration	Type of study	Outcome	References
$n = 37$	Germany	Igs from bovine colostrum (Lactobin) 10 g/day, 10 days	Non-controlled pilot study	Mean daily stool frequency decreased from 7.4 to 2.2 at the end of the treatment	(32)
$n = 1$	UK	Igs from bovine colostrum (Lactobin) 50 g/day, 14 days	Case report	Clinical improvement of diarrhea and elimination of parasite.	(101)
$n = 25$	Germany	Igs from bovine colostrum 10 g/day, 10 days, 20 g/day 10 days in non-responders	Prospective, open, uncontrolled	Complete (40%) or partial (24%) remission of diarrhea in 64% of the patients	(102)
$n = 30$	Nigeria	Bovine colostrum product (ColoPlus) 2 × 50 g/day, colostrum 32% containing 3–4 g IgG /50 g	Open label observational study	Reduced nr defecations/day. Increase haemaglobulin, albumin. Alleviated fatique, increased CD4+ cells.	(103)
$n = 8$	America	Serum derived bovine Ig 2.5 g/day, 8 weeks	Open label	Improvement in symptoms with reduced bowel movements/day ($P = 0.008$) and improvements in stool consistency ($P = 0.008$)	(104)
$n = 850$	Uganda	Colostrum based food	Field trial	Improved nutritional and immune status (increased body weight, decreased fatigue, transient rise in CD4 Tells)	(105)
Active $n = 45$; control $n = 42$	Uganda	Colostrum based supplement 2 × 50 g/day, 4 weeks	Randomized single-blind controlled trial	Daily stool frequency decraeased by 79% during study period in colostrum group compared to 58% in control group($p < 0.001$)	(106)

showed in a meta-analysis of 5 studies that breastfeeding was associated with a reduced risk of otitis media (1). In summary, it is not clear what the optimal duration and amount of breastfeeding is for protection against these infections. The effects found differ between studies and between types of outcome (pneumonia, upper vs. lower respiratory infections, and otitis media). Much of the variation could be attributed to the impact of gene/environment interactions having diverse effects on levels of immune-modulatory molecules in breast milk between study subjects (112, 113).

There is a complex relationship between early respiratory tract infection and the subsequent evolution of allergy [Summarized in (114)]. The previously labeled hygiene hypothesis suggested that early infection was associated with less subsequent allergy and allergic disease, most notably asthma. There is a credible immunological mechanism to explain this mutually exclusive phenomenon in that interferon gamma (IFN-γ) generated during infection down-regulates T-helper lymphocyte-2 (Th2) activity associated with the production of interleukin-4 (IL-4) which promotes IgE production. In addition infection also up-regulates T-regulatory cell (T-reg) activity, which has additional controlling effects on Th2 and Th1 responses. This latter effect would explain why both allergic (Th2) diseases and auto-immune (Th1) diseases have both increased in relatively affluent communities and co-exist more frequently than by chance. However, while in some communities, active infection such as measles, and immunization with live organisms such as BCG, has been associated with less allergy, it is more likely that the commensal human microbiome has the most important influence in promoting normal immune regulation. This has resulted in the more accurate descriptor; "the microbial exposure hypothesis." It is likely that changes in the commensal normal human microbiome in communities adopting a Western life-style have increased susceptibility to all non-communicable inflammatory diseases (115).

If we consider this microbial exposure hypothesis true, there is a problem with the interpretation of epidemiological cohort studies showing an increased frequency of respiratory infections in children who subsequently develop allergic sensitization and disease. The underlying defects in immune response increasing the risks of infection may be the same that increase the likelihood of allergy. This may thus imply that the infection/allergy association is not cause and effect.

However, there are certain predominantly infant viral infections that have been specifically associated with the later development of asthma. Rhino-virus (RV), the common cold virus (particularly with the RV-C) induced wheezing in infancy predicts a very high probability of later asthma (115). To a lesser extent this process has also been attributed to infant bronchiolitis due to RSV infections, although recent outcomes of the MAKI study in which infants in the first year of life were treated with palivizumab against RSV or not treated suggest that although palivizumab treatment resulted in a significant reduction in wheezing days during the first year of life, the incidence of asthma at 6 years of age did not differ between the groups (116, 117). In addition, it has long been known that acute gastro-enteritis in infancy increases the risk of allergic sensitization to food proteins, most notably from cow's milk, if the exposure to allergenic

proteins occurs while there is still intense gut inflammation. The latter provides the co-stimulatory signals to trigger a sensitizing response (115). Thus breast-feeding with its known protective effects against GI and RSV infection will reduce such events. In addition the human milk oligosaccharides that are present in breast milk facilitate the development of a beneficial microbiome which through generation of short-chain fatty acids enhances T-cell regulation amongst a number of other beneficial effects (118).

Bovine IgG and Colostrum: Effects on Respiratory Tract Infections in Preclinical Models

Several *in vitro* and animal studies have shown effects of bovine IgG and colostrum in viral respiratory tract infections. Bovine immunoglobulins can bind to—and *in vitro* even neutralize—RSV, a common childhood pathogen resulting in upper respiratory tract infections in infants especially in the first year of life (38). Furthermore, bovine immunoglobulins from non-immunized cows have been shown to be able to bind to human RSV as well as to influenza virus, and are able to prevent the infection of Hep2 cells by human RSV *in vitro* (38). In addition, dietary bovine colostrum could reduce the severity of infection and viral titers in a murine model of RSV infection (119). Specificity of the antibodies against respiratory viruses might further enhance efficiency in prevention and/or treatment of disease as was discussed above for gastrointestinal pathogens. Indeed, bovine IgG isolated from hyperimmune colostrum, as well as F(ab')2 fragments thereof could prevent infection with influenza PR8 virus after intranasal application in mice (120). This indicates that Fc receptor-independent mechanisms such as neutralization or immune complex formation are the mechanism behind this finding. In a follow up study (non-immunized) oral bovine colostrum reduced the severity of influenza infection, by reducing viral load and preventing loss of body-weight (121). In addition, splenic NK activity, as well as the production of IgA producing B cells in the small intestine and lungs, was noted in the colostrum group. For RSV, however, this non-specific response might be less relevant since the F protein of bovine RSV has great similarities to human RSV (122) which explains the presence of specific antibodies against human RSV in bovine milk of non-immunized cows.

Prevention of Upper Respiratory Tract Infections (Urtis) And Otitis Media (OM) in Children and Infants With Bovine IgG and Colostrum

Epidemiological data previously showed that infants that receive unprocessed (raw) bovine milk as a weaning food in the first year of life had a lower chance to get respiratory infections and otitis media compared to infants that received ultra heat-treated (UHT) milk (28). A hypothesis is that the lack of intact proteins in UHT milk vs. the presence of these proteins in unpasteurized cow's milk explains the observed effect. Indeed, bovine IgG and colostrum have been reported to prevent upper respiratory tract infections in children, adults, elderly people and athletes (93, 94, 123–130) and these studies are summarized in **Table 4**.

Five studies have shown a reduced incidence or severity of upper respiratory tract infections in children that received colostrum from non-immunized cows. Of these studies, two were double blind and the other 3 studies were prospective, open or uncontrolled. The first double blind placebo controlled study was performed in IgA-deficient children with viral upper respiratory tract infections that received a sucking tablet containing 14 mg of colostrum three times per day which lowered the infection severity score after 1 week (123). In another study, 195 children of 3–9 years old received either three colostrum or control milk powder chewing tablets per day for 2 months corresponding to 0.5 g colostrum or control milk powder per day (127). The children receiving colostrum tablets, especially in the 3–6 years age group, had a reduced upper respiratory tract infection frequency, compared to placebo, as well as a reduced number of reported sick days. A prospective open, non-controlled, study in children suffering from recurrent episodes of URTI or diarrhea were given powdered colostrum and showed a reduction of these infections compared to baseline (93). In another large, uncontrolled open study, significant decreases in diarrhea and upper respiratory tract infection (URTI) episodes were reported in Indian children receiving an oral colostrum for 12 weeks (94). The same was noted in a retrospective study comparing two groups of children, one that received a product containing colostrum plus a probiotic for 4 months, and the other that received a bacterial extract for 3 months (124). Children that received the colostrum/probiotic combination had a reduction in respiratory tract infections requiring antibiotic therapy compared to the children receiving bacterial extracts.

These studies suggest that bovine colostrum can prevent upper respiratory tract infections although the open, non-controlled prospective studies should be interpreted with care. In line with studies in children, the use of colostrum to prevent respiratory tract infections has also been studied in adults and elderly people. In adults suffering from frequent upper respiratory tract infections, supplementation with a bovine IgG and lactoferrin containing whey protein fraction (600 mg/day for 90 days) showed a reduced incidence of the common cold and cold-associated symptoms compared to the placebo group in a double blind randomized, placebo controlled study (125). Finally, decreased numbers of self-reported upper respiratory tract infections have also been noted after colostrum supplementation in athletes (126, 128, 130, 131), and colostrum prevented influenza infection in elderly volunteers comparable to influenza vaccination (129). In the latter study, colostrum was consumed for 2 months and flu episodes were scored for 3 months. Also, the effect of colostrum supplementation alone, colostrum combined with vaccination or vaccination alone was studied in a high-risk cardiovascular subject group on flu-associated complications in the hospital. Colostrum groups showed significant lower flu-associated complications compared to the vaccination only group.

Intervention studies aiming to reduce otitis media using bovine milk proteins such as immunoglobulins are scarce. Recently, an infant formula containing milk fat globule

TABLE 4 | Prevention of URTI with bovine IgG and colostrum.

Subject characterisitics	Type of study	Product, dosage, and duration	Reported outcome	References
INFANTS/CHILDREN				
1–6 year old Egyptian children with recurrent URTI and/or diarrhea ($n = 160$)	open, non-comparative	Colostrum, 3 g/day for < 2 year 6 g/day for >2 years, 4 weeks	Lower number of episodes and hospitalizations for URTI (and GITI)	(93)
1–8 y old Indian children with recurrent URTI/diarrhea ($n = 605$)	open, non-comparative	Colostrum 3 g/day, 12 weeks	Lower number of episodes and hospitalizations for URTI (and GITI)	(94)
5–17 year old Turkish IgA deficient infants with recurrent URTI (active $n = 16$; placebo $n = 15$)	Placebo controlled, randomized, double blind	Colostrum 3 × suckling tablet (incl 14 mg colostrum+2.2 mg lysozyme) /d, 3 days	Lower infection severity score in treatment group, no effect on salivary IgA	(123)
3–9 year old healthy japanese children (active $n = 103$; placebo $n = 104$)	Placebo controlled, randomized, double blind	Late colostrum (10% Igs)- vs. semi skimmed milk tablets 0.5 g/day. 9 weeks	Frequency and duration of URTI was lower in the treatment group vs. the control group, especially in the 3–6 year old children	(127)
3–7 year old Italian children with recurrent URTI (active $n = 67$; placebo $n = 100$)	Retrospective observational study	Sinerga (incl. colostrum, incl probiotics) vs. bacterial extracts 1 sachet (a 3 g)/day, 10 days month 1, 20 days months 2,3,4	Greater reduction in the frequency of respiratory infections that needed antibiotic therapy in the group of children supplemented with Sinerga than in the group treated with bacterial extracts.	(124)
ADULTS				
>18 years old australian adults with 3 or more URTI in the last 6 months (active $n = 53$; placebo $n = 52$)	Placebo controlled, randomized, double blind	Bovine lactoferrin /whey protein Ig-rich fraction (Lf/IgF) vs. placebo 2 × 300 mg/day, 90 days	# Cold events over 90 days significant lower in treatment group compared to control group. Duration and severity of cold was not different between groups.	(125)
14–27 year old Trained swimmers and age matched controls (4 groups) from New Zealand (active $n = 25$; placebo $n = 28$)	Placebo controlled, randomized, double blind	Low protein colostrum powder (3% Ig w/w) vs. isocaloric placebo 2 × 25 g/day, 10 weeks	A non-significant trend for lower upper respiratory symptoms in athletes consuming colostrum vs. placebo was reported. No effects in control group. No differences between groups wrt saliva and serum Igs.	(126)
18–50 year old adults in the UK (active $n = 25$; placebo $n = 28$)	Placebo controlled, randomized, double blind	Colostrum vs. iso-energetic/-macronutrient placebo 2 × 25 g/day, 10 weeks	Significantly lower proportion of days with URI during the 12 weeks in the COL group (5%) compared to the PLA group (9%). No difference on *in vitro* immune functionality, except for lower bacterial load in saliva of colostrum subjects.	(17)
25–30 year old trained Australian cyclists	Placebo controlled, randomized, double blind	Colostrum protein concentrate (CPC, 20% Ig) vs. whey protein 10 g/day, 8 weeks	Trend toward reduced incidence of upper respiratory illness symptoms in the bovine CPC group ($P = 0.055$). Diverse immune parameters changed in CPC group.	(131)
18–35 year old Australian healthy volunteers (active $n = 93$; placebo $n = 81$)	Placebo controlled, randomized, double blind	Colostrum protein concentrate (CPC) vs. whey protein 3 × 20 g/day, 8 weeks	Incidence but not duration of URTI was significantly lower in CPC group compared to whey group.	(128)
50–60 year old Italian healthy volunteers ($n = 41$ vs. $n = 36$ vs. $n = 39$ vs. $n = 23$)	Randomized study	Vaccination ± colostrum product vs. colostrum only vs. no prophylaxis 1 tablet a 400 mg (25-40% Ig)/day, 8 weeks	Number of days with flu was 3 times higher in the non-colostrum compare to the colostrum treated group (colostrum+vacc 14 vs. vacc only 57 vs. colostrum only 13 episodes vs. non-treated 41)	(129)
ELDERLY				
60–70 year old Italian elderly people with high risk for influenza (heart/lung problems) ($n = 21$ vs. $n = 20$ vs. $n = 19$)	Randomized study	vaccination ± colostrum product vs. colostrum only vs. no prophylaxis 1 tablet a 400 mg (25–40% Ig)/day, 8 weeks	The incidence of complications and hospital admission was higher in the group that received only a vaccination compared with the colostrum groups.	(129)

membrane components (proteins and phospholipids) reduced the incidence of otitis media in infants 2–6 months of age in a double blind placebo controlled study (132). Interestingly, a component of the proteins in this milk fraction are immunoglobulins (133).

Taken together, there is an increasing number of studies that suggest a role for bovine IgG or colostrum in preventing or ameliorating viral respiratory tract infections. To what extent this protective effect against infection will also affect allergy prevalence remains to be established. However, the reduced prevalence of allergic conditions amongst farming families in many countries (134) has been directly associated with the use of unpasteurized milk by pregnant and lactating mothers, and their infants after weaning (27, 135). Maternal consumption of farm dairy products during pregnancy resulted in reduced levels of pro-inflammatory cytokine production from stimulated cord blood mononuclear cells from the very large multinational European study (PASTURE) (136).

BOVINE IMMUNOGLOBULINS: PUTATIVE MECHANISM OF ACTION

Bovine immunoglobulins can exert their effects on several levels: via direct effects on potential pathogens, via enhancing clearance of pathogens, via influencing intestinal barrier function, and via modulating immune function. This section will discuss the mechanistic evidence available to date.

Direct Effects of Immunoglobulins Toward Pathogens

The primary role of immunoglobulins on mucosal surfaces is to bind to pathogens to prevent their entry into the body. This process is termed immune exclusion. During immune exclusion the pathogens as well as the immunoglobulins remain confined to the intestinal lumen and the immunoglobulins prevent adhesion to intestinal epithelium. Hyperimmune bovine immunoglobulins can prevent the adhesion of pathogens to intestinal epithelial cells (75) and even immunoglobulins from non-immunized cows can prevent adhesion of some pathogens (65, 137). As an example, the adhesion of *Clostridium difficile* to human intestinal epithelium cells (Caco-2 cells) was inhibited dose-dependently by normal bovine colostral whey (137) and spray dried colostrum from normal cows was shown to inhibit the adhesion of several necrotizing enterocolitis-associated pathogens to HT-29 colonic epithelial cells (65).

Effects on Barrier Function and Gastrointestinal Inflammation

At the next level, there are indications that bovine immunoglobulins and colostrum can also support intestinal barrier function. When intestinal barrier function is compromised, bacterial products such as LPS, food allergens, as well as pathogens can passively cross the epithelial layer and cause inflammation and infection in the mucosa. Bovine colostrum can inhibit the NF-κB signaling pathway and induction of pro-inflammatory cytokines in HT29 cells, suggesting colostrum has direct anti-inflammatory effects on intestinal epithelium (138). Furthermore, bovine IgG can also have anti-inflammatory effects by preventing translocation of bacterial components across the epithelial layer. This was investigated in a co-culture model of intestinal epithelial C2BBe1 cells and THP-1 cells. In this model bovine serum derived IgG could prevent the translocation of bacterial components over the epithelium, thus preventing inflammatory responses to bacterial ligands in the underlying THP1 cells (139).

To further investigate the potentially mitigating role of bovine IgG in inflammatory responses, effects of immunoglobulins and colostrum have been addressed in human and animal models for necrotizing enterocolitis (NEC), irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), non-steroid anti-inflammatory drug (NSAID)-induced gut damage and perioperative endotoxemia. IgG purified from hyperimmune colostrum could protect mice against TNBS-induced colitis (140). Mice receiving hyperimmune colostral IgG before colitis induction had reduced weight loss, improved histological score and increased serum IL-10 as well as increased numbers of Tregs compared to controls. Similar findings were reported for bovine serum IgG from non-immunized cows in a bacterial-induced colitis model (141), a chemotherapy-induced mucositis model (142) and a pathogen infection model (143). Likewise, serum-derived bovine IgG reduced mucosal expression of pro-inflammatory cytokines and prevented reduction of barrier function through preventing reduction of tight junction proteins ZO-1 in a mouse model for IBD (144). Bovine IgG has also been studied in IBS, where it improved symptom scores in IBS patients with recurrent diarrhea (145). In addition, oral bovine immunoglobulin induced some improvement in a subgroup of IBD patients (146).

Animal models indicate that colostrum may prevent NEC. Preterm piglets fed normal bovine colostrum had better growth and lower NEC incidence, NEC severity and intestinal cytokine production compared to piglets fed formula feeding (147, 148). The use of bovine colostrum is under investigation in preterm infants. In the safety phase of a pilot study bovine colostrum was well-tolerated in the pre-term infants, and further studies are ongoing (149).

Two studies have addressed the effect of immunoglobulin-enriched non-immune colostrum on peri-operative endotoxaemia as a measure for peri-operative infections. In the first study by Bölke et al. a reduced peri-operative endotoxaemia was noted after abdominal surgery, suggesting a stabilization of gut barrier function and neutralization of endotoxins by colostrum (150). A second study, however, could not confirm this effect after coronary bypass surgery, possibly because the dose used was lower (151). Finally, non-steroidal anti-inflammatory drugs (NSAID)-induced intestinal damage has been shown to be reduced after colostrum treatment in animal experiments (152) and NSAID-induced increases in intestinal permeability were reduced in human volunteers receiving colostrum (153). However, these effects are probably not mediated by bovine IgG but by TGF-β that is also present in colostrum (152).

These findings indicate that bovine IgG (probably in concert with additional colostrum components) prevents bacterial transfer and leakage of LPS over intestinal epithelium, modulates the expression of epithelial tight junction proteins and inhibits intestinal inflammation. This is comparable to findings in studies on the role of IgA in breastfeeding in animal models (154–157), suggesting that oral immunoglobulins from cows may have a similar mode of action as breastmilk-derived IgA.

Immune Effects

Immunoglobulins consist of an antigen binding domain (in the variable region of the molecule) and a constant region. This constant region is essential for inducing effector functions of immunoglobulins such as phagocytosis and clearance of pathogens, complement fixation, antigen presentation and immune regulation. These downstream effector functions of immunoglobulins are dependent on binding of the constant region to immunoglobulin Fc receptors (Fc receptors). These receptors are primarily expressed on the cell surface of immune cells. Secretory IgA (sIgA) does not have these effector functions because it is a dimer containing a secretory component, and therefore the constant part is not available for binding to receptors.

Interestingly, bovine IgG is able to bind to Fcγ receptors on human cells. bovine IgG was shown to bind to human monocytes, neutrophils and macrophages, as well as to B cells (158, 159). Coating (opsonisation) of *Streptococcus mutans* with bovine IgG induced phagocytosis and killing of the bacteria by human leucocytes (160). Similar findings were reported for *S. epidermidis* and RSV (38).

Bovine IgG inhibited pokeweed mitogen-induced secretion of immunoglobulins in PBMC cultures, probably mediated by FcγR as F(ab')2 fragments had no effect (161). This was later confirmed by another group, who suggested that bovine IgG binds to FcγRII, the only FcR expressed on B cells (159). Indeed, also binding of bovine IgG1 to FcγRII on phagocytes (monocytes and PMN) was shown by Jungi in 1989 and to be dependent on immune complexes (158). Now it is known that two types of FcγRII exist: The activating FcγRIIa, expressed on monocytes, dendritic cells and neutrophils, and the inhibitory FcγRIIb, mainly expressed on B cells and mast cells (162–164). Whereas, activating FcR contain an ITAM (immunoreceptor tyrosin activation motif), the inhibitory FcγRIIb contains an ITIM (immunoreceptor tyrosin based inhibitory motif) in its intracellular tail. When FcγRIIb is crosslinked, the downstream signaling of the ITIM leads to a inhibition of activating signals, resulting in dampening of the immune response, i.e. antibody production. On the other hand, binding of immune complexes or opsonized pathogens to activating FcR on phagocytes will lead to activation: phagocytosis, antigen presentation and cytokine production. The homology of the extracellular part of FcγRIIa and FcγRIIb is very high: 92%. However, human IgG2 binds to FcgRIIa but not to FcgRIIb (165).

The fact that bovine IgG can bind to human FcγRII was confirmed more recently (38, 166). These two research groups demonstrated directly that bovine IgG is able to form immune complexes with bacterial and viral pathogens, mediates FcγR-mediated uptake and antigen presentation, as well as phagocytosis and killing in phagocytes.

Less is known on the interaction of bovine IgG with other receptors. Bovine IgG1 can also bind to a non-classical receptor for IgG, the human neonatal Fc receptor FcRn, albeit with lower affinity compared to human IgG (167). FcRn is expressed on intestinal epithelium in humans throughout life (168, 169). However, evidence suggests that bovine IgG is not taken up into circulation of adult human volunteers (36, 63) and unpublished observations). It is therefore not clear at present if and what the consequence of binding of bovine IgG to FcRn is.

In contrast to short-lived passive immunity, adaptive immune responses occur during the lifetime of an individual as an adaptive response to specific infecting pathogens on subsequent exposures and in many cases, confer lifelong protective immunity to reinfection with the same pathogens. An adaptive immune response involves activation, selection, and clonal proliferation of T and B cells. After encountering an antigen, T cells proliferate and differentiate into antigen-specific effector cells, while B cells proliferate and differentiate into antibody-secreting cells.

Adaptive immune responses are initiated by activation of CD4+ T helper cells after antigen presentation by antigen presenting cells (APC). APC take up exogenous (protein) antigens from their environment via pinocytosis or receptor-mediated endocytosis, degrade them into peptides, and present these peptides in the context of MHC class II molecules to the T-cell receptor (CD3) on antigen-specific T cells (170).

Facilitated antigen presentation has been described for cell surface expressed Fc receptors on professional antigen presenting cells. Fc receptors can internalize bacteria and viruses as well as protein antigens that are bound by circulating serum immunoglobulins, resulting in killing of pathogenic microorganisms, and antigen presentation to initiate immune responses.

Low- and intermediate affinity Fc receptors have a low affinity for monomeric immunoglobulins, but have a high affinity for immune complexes. As a result, these receptors will only bind immunoglobulins when they are in complexes together with the antigens they recognize. Indeed, for Fcγ receptors (171, 172), Fcα receptors (173) and Fcε receptors (174, 175) it has been shown that immune complexes are taken up more efficiently by antigen presenting cells than the antigens alone. These studies demonstrate that antigen-specific responses of CD4+ T helper cells are facilitated strongly by specific antigen uptake via Fc receptors on antigen-presenting cells, resulting in more efficient immune responses. This is especially relevant when low antigen doses are present.

In line with this a recent study shows increased CD8+ T cell activation in mice infected with RSV when they received bovine colostrum orally (119). Therefore, in addition to immune exclusion by direct binding of bovine IgG to pathogens, FcγRII mediated pathogen clearance and killing by macrophages and neutrophils, bovine IgG may also play a role in enhancing adaptive immune responses by increasing T and B cell responses to pathogens.

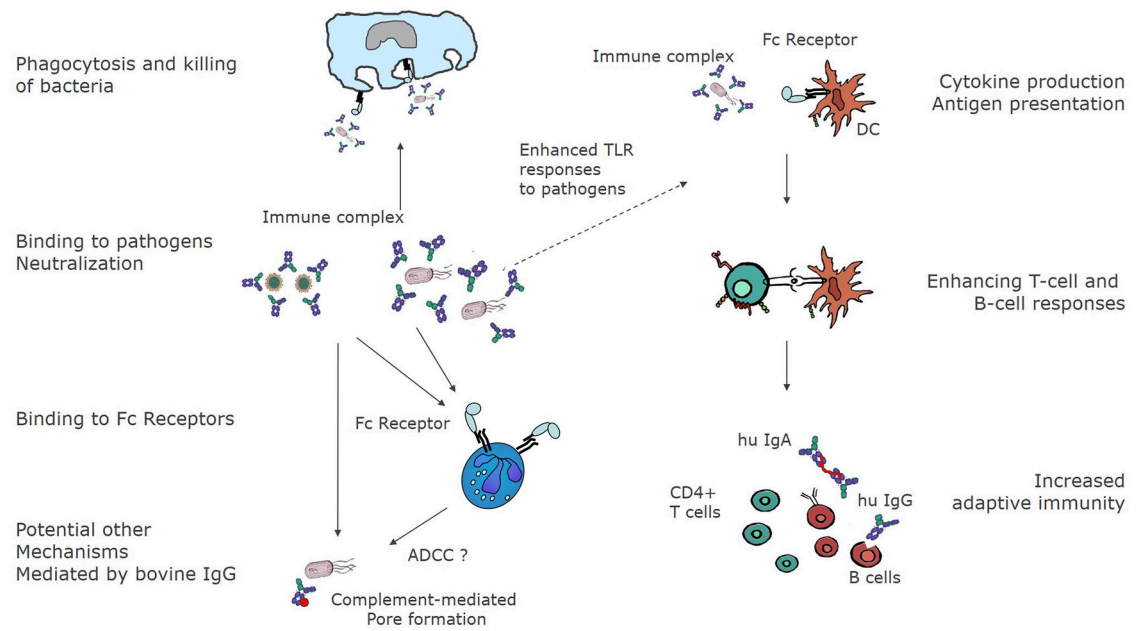


FIGURE 2 | Immunological mechanisms of bovine immunoglobulins. Bovine immunoglobulins can modify innate as well as adaptive immunity. By binding directly to pathogens, bovine immunoglobulins can bind to FcγR bearing innate immune cells, leading to phagocytosis and killing. In some cases, as for RSV, bovine IgG may also fully neutralize human pathogens as demonstrated for RSV *in vitro* (38). Other mechanisms of pathogen elimination may be complement mediated pore formation and killing, and ADCC. On the other hand, as bovine IgG-pathogen immune complexes bind to FcγR, receptor mediated uptake and antigen processing is enhanced, resulting in increased T, and ultimately B-cell responses to the pathogens.

Experiments in murine animal models have also found increased NKT in mice that ingested colostrum containing LPS-specific bovine IgGs (176). Similarly, increased NK activity was reported in spleen and Peyer's patch of mice that ingested bovine hyperimmune colostrum (121, 143, 177). It is not clear at present if these NK cells are induced by IgG or by other factors present in colostrum.In summary, **Figure 2** shows the mechanism of action of bovine IgG on the immune system.

Immunological Effects of Oral Immunoglobulins of Other Species

In addition to oral bovine immunoglobulins, several studies with murine, porcine and human oral immunoglobulins have elucidated mechanisms that may also occur after ingestion of bovine immunoglobulins.

A series of recent studies in mice have shown yet another potential mechanism for the presence of immunoglobulins in the intestinal lumen. These studies have shown that locally produced intestinal IgA can bind to intestinal bacteria and modify the microbiota composition as well as immune responses toward bacteria in the gut (178–184). These studies suggest that oral immunoglobulins may also have an effect on microbiota development and composition.

Indeed, maternal sIgA in breast milk was shown to significantly modify microbiota composition at weaning and later in life, limit translocation of pathogens in suckling pups and ameliorated DSS induced colitis (154). In addition, IgG2 and IgG3 as well as sIgA in breast milk were shown to bind to commensal bacteria, dampen mucosal T cell activation, and prevent bacterial translocation to the mesenteric lymph nodes (155). Similar findings were reported previously, showing that both sIgA and IgG in breast milk can play a role in immune exclusion as well as in dampening T cell responses to intestinal bacteria (156, 157).

Similar effects of IgG in breast milk of mice have been obtained in a study in which immune complexes of murine breast milk IgG and allergens prevented the development of experimentally induced asthma (185). IgG-allergen immune complexes were transferred from breast milk into the suckling pups via FcRn, and induced FoxP3+ regulatory T cells that were needed for the protective effect on asthma development. This was recently confirmed in another study (186). Interestingly, in humans this process also occurs, but in humans it occurs when IgG-allergen complexes cross the placenta. This results in much lower rates of infant sensitization to that allergen (187).

As described above, bovine milk contains IgG directed against inhalation allergens (41). The functional relevance and possible applications of such allergen-specific bovine immunoglobulins are not clear at this time, but they may play a role in the protective association of raw milk consumption with reduced prevalence of allergies (4).

Immune complexes of maternal breast milk immunoglobulins with maternal GI microbiota components have been shown to be crucial for delivering tryptophan-derived AhR ligands that promote barrier function in the suckling pups in an IL-22 and Innate Lymphocytic Cell type 3 (ILC3)-dependent manner (188).

Oral ingestion of porcine immunoglobulin also contributes to prevention of inflammatory tissue damage in LPS-induced acute lung inflammation in mice. Oral IgG enhanced the expression of IL-10 producing Treg, decreasing Th1 and Th2 cytokines and decreasing chemokine production in the lung in response to LPS (189).

These studies indicate that oral immunoglobulins may help to regulate immune responses to the microbiota as well as allergens—on the one hand promoting immune exclusion of pathogens, and on the other hand preventing excessive immune responses to commensal bacteria. Studies on effects of bovine Ig and colostrum ingestion on fecal microbiota composition in infants during colonization of the GI tract have not been performed to date, but based on these findings it may be expected that oral intake of bovine IgG may similarly affect microbiota composition, allergy and immune development in humans.

CONCLUDING REMARKS

The protective role of IgA in breast milk is well-documented. IgA protects infants against infection, shapes the microbiota and creates a non-inflammatory response against the microbiota, thus preventing intestinal inflammation.

As described above, bovine IgG has a number of similar effects: binding to human-relevant pathogens, effects on phagocytosis mediated through Fc receptors for human IgG and prevention of infection in human studies. So functionally, bovine IgG does have effects on the human immune system beyond just binding to potential pathogens. In addition, especially in young infants, IgG does seem to pass through the gastrointestinal tract without being fully degraded by digestion, thus leaving its functional aspects intact. As the fine specificity as well as the effector functions of bovine IgG are not identical to breast milk IgA (and IgG), one question that remains to be answered in clinical studies is if inclusion of functionally active bovine immunoglobulins in infant nutrition can fully restore the lack of maternal IgA in bottle fed children.

Immune support by bovine immunoglobulins as an alternative for breast milk-derived IgA is especially relevant in the period just after birth in case the mother cannot breastfeed her child and is dependent on bottle feeding, but also in the weaning period at around 4–6 months when respiratory and gastrointestinal infections are known to increase as the passively acquired maternal IgG in serum, as well as the maternal breast milk-derived IgA in the gastrointestinal tract of the infant, have decreased to very low levels.

It should be stated though, that the increased prevalence of infections after weaning is not completely related to deprivation of IgA but is also linked to the introduction of new foods, an increased exposure to the outside world and in part by the absence of additional protective factors in breast milk.

Currently, formula fed children receive no or very low levels of functionally active bovine immunoglobulins. This is because current foods processing technologies as well as legislation strongly depend on heating as the technique of choice to achieve microbiological safety. Overcoming these challenges by applying novel food processing technologies may enable the application of bovine immunoglobulins in infant nutrition.

Future research will be needed to confirm if this will indeed result in a reduction in gastrointestinal and respiratory infections, and if this will also be associated with a decreased prevalence of asthma that is linked to early respiratory tract infections in infants.

AUTHOR CONTRIBUTIONS

LU, JL, HS, JW, and RvN all contributed to the writing of the manuscript.

FUNDING

JW is funded by NIHR-CLAHRC for NW London. The views expressed in this publication are those of the author and not those of the NHS, the NIHR or the Department of Health.

ACKNOWLEDGMENTS

The authors would like to thank Prescilla Jeurink for critically reading the manuscript. The authors would like to thank STW and the project members of STW MIRACLE project nr 13017 for their support.

REFERENCES

1. Ip S, Chung M, Raman G, Trikalinos TA, Lau J. A summary of the Agency for Healthcare Research and Quality's evidence report on breastfeeding in developed countries. *Breastfeed Med.* (2009) 4(Suppl 1):S17–30. doi: 10.1089/bfm.2009.0050
2. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and associated decrease in respiratory tract infection in US Children. *Pediatrics* (2006) 117:425–32. doi: 10.1542/peds.2004-2283
3. Section on Breastfeeding. Breastfeeding and the use of human milk. *Pediatrics* (2012) 129:e827–41. doi: 10.1542/peds.2011-3552
4. van Neerven RJJ, Knol EF, Heck JML, Savelkoul HFJ. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol.* (2012) 130:853–8. doi: 10.1016/j.jaci.2012.06.050
5. Munblit D, Boyle RJ, Warner JO. Factors affecting breast milk composition and potential consequences for development of the allergic phenotype. *Clin Exp Allergy* (2015) 45:583–601. doi: 10.1111/cea.12381
6. Ehrlich P. *The Collected Papers of Paul Ehrlich Vol. II Immunology and Cancer Research*. London:Himmelweit Pergamon (1957).
7. Kaufmann SHE. Immunology's foundation : the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat Immunol.* (2008) 9:705–712. doi: 10.1038/ni0708-705

8. Goldman AS, Garza C, Nichols BL, Goldblum RM. Immunologic factors in human milk during the first year of lactation. *J Pediatr.* (1982) 100:563–7.
9. Hanson L. Comparative immunological studies of the immune globulins of human milk and of blood serum. *Int Arch Allergy Appl Immunol.* (1961) 18:241–67. doi: 10.1159/000229177
10. Fink K, Zellweger R, Weber J, Manjarrez-Orduno N, Holdener M, Senn BM, et al. Long-term maternal imprinting of the specific B cell repertoire by maternal antibodies. *Eur J Immunol.* (2008) 38:90–101. doi: 10.1002/eji.200737872
11. Zinkernagel R. Maternal antibodies, childhood infections, and autoimmune diseases. *N Engl J Med.* (2001) 345:1331–5. doi: 10.1056/NEJMra012493
12. Zinkernagel RM. On natural and artificial vaccinations. *Annu Rev Immunol.* (2003) 21:515–46. doi: 10.1146/annurev.immunol.21.120601.141045
13. Navarini AA, Krzyzowska M, Lang KS, Horvath E, Hengartner H, Niemialtowski MG, et al. Long-lasting immunity by early infection of maternal-ntibody-protected infants. *Eur J Immunol.* (2010) 40:113–6. doi: 10.1002/eji.200939371
14. Perre P Van De. Transfer of antibody via mother' s milk. *Vaccine* (2003) 21:3374–6. doi: 10.1016/S0264-410X(03)00336-0
15. Maertens K, De Schutter S, Braeckman T, Baerts L, Van Damme P, De Meester I, et al. Breastfeeding after maternal immunisation during pregnancy: providing immunological protection to the newborn: a review. *Vaccine* (2014) 32:1786–92. doi: 10.1016/j.vaccine.2014.01.083
16. Marchant A, Sadarangani M, Garand M, Dauby N, Verhasselt V, Pereira L, et al. Maternal immunisation: collaborating with mother nature. *Lancet Infect Dis.* (2017) 17:e197–208. doi: 10.1016/S1473-3099(17)30229-3
17. Jones C, Heath P. Antenatal immunization. *Hum Vaccin Immunother* (2014) 10:2118–22. doi: 10.4161/hv.29610
18. Wedgwood JF, Palmer R, Weinberger B. Development of the ability to make IgG and IgA in newborns and infants. *Mt Sinai J Med.* (1994) 61:409–15.
19. Brandtzaeg P. Update on mucosal immunoglobulin A in gastrointestinal disease. *Curr Opin Gastroenterol.* (2010) 26:554–63. doi: 10.1097/MOG.0b013e32833dccf8
20. Yel L. Selective IgA deficiency. *J Clin Immunol.* (2010) 30:10–6. doi: 10.1007/s10875-009-9357-x
21. Köhler H, Donarski S, Stocks B, Parret A, Edwards C, Schroten H. Antibacterial characteristics in the feces of breast-fed and formula-fed infants during the first year of life. *J Pediatr Gastroenterol Nutr.* (2002) 34:188–93.
22. Munblit D, Peroni DG, Boix-Amorós A, Hsu PS, Van't Land B, Gay MCL, et al. Human milk and allergic diseases: an unsolved puzzle. *Nutrients* (2017) 9:1–41. doi: 10.3390/nu9080894
23. Kukkonen K, Kuitunen M, Haahtela T, Korpela R, Poussa T, Savilahti E. High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. *Pediatr Allergy Immunol.* (2010) 21:67–73. doi: 10.1111/j.1399-3038.2009.00907.x
24. Brandtzaeg P. The mucosal immune system and its integration with the mammary glands. *J Pediatr.* (2010) 156:S8–15. doi: 10.1016/j.jpeds.2009.11.014
25. Waser M, Michels KB, Bieli C, Fl Ãistrup H, Pershagen G, Von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37:661–670. doi: 10.1111/j.1365-2222.2006.02640.x
26. Braun-Fahrländer C, Von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41:29–35. doi: 10.1111/j.1365-2222.2010.03665.x
27. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358:1129–33. doi: 10.1016/S0140-6736(01)06252-3
28. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol.* (2011) 128:766–773.e4. doi: 10.1016/j.jaci.2011.07.048
29. Mainer G, Sánchez L, Ena JM, Calvo M. Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. *J Food Sci.* (1997) 62:1034–38.
30. Van Neerven, RJJ. The effects of milk and colostrum on allergy and infection: mechanisms and implications. *Front. Anim.* (2014) 4:16–22 doi: 10.2527/af.2014-0010
31. Loss G, Depner M, Ulfman LH, van Neerven RJJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. *J Allergy Clin Immunol.* (2015) 135:56–62. doi: 10.1016/j.jaci.2014.08.044
32. Rump JA, Arndt R, Arnold A, Bendick C, Dichtelmuller H, Franke M, et al. Treatment of diarrhoea in human immunodeficiency virus-infected patients with immunoglobulins from bovine colostrum. *Clin Investig.* (1992) 70:588–94.
33. Stephan W, Dichtelmuller H, Lissner R. Antibodies from colostrum in oral immunotherapy. *J Clin Chem Clin Biochem.* (1990) 28:19–23.
34. Kelly GS. Bovine colostrums: a review of clinical uses. *Altern Med Rev.* (2003) 8:378–94.
35. Lissner R, Schmidit H, Karch H. A standard immunoglobulin preparation produced from bovine colostra shows antibody reactivity and neutralization activity against Shiga-like toxins and EHEC-hemolysin of *Escherichia coli* O157:H7. *Infection* (1996) 24:378–83. doi: 10.1007/BF01716084
36. Lissner R, Thurmann PA, Merz G, Karch H. Antibody reactivity and fecal recovery of bovine immunoglobulins following oral administration of a colostrum concentrate from cows (Lactobin) to healthy volunteers. *Int J Clin Pharmacol Ther.* (1998) 36:239–45.
37. Ellens D, de Leeuw P, Straver P. The detection of rotavirus specific antibody in colostrum and milk by ELISA. *Ann Rech Vet.* (1978) 9:337–42.
38. den Hartog G, Jacobino S, Bont L, Cox L, Ulfman LH, Leusen JHW, et al. Specificity and effector functions of human RSV-Specific IgG from bovine milk. *PLoS ONE* (2014) 9:e112047. doi: 10.1371/journal.pone.0112047
39. Yolken RH, Losonsky GA, Vonderfecht S, Leister F, Wee SB. Antibody to human rotavirus in cow's milk. *N Engl J Med.* (1985) 312:605–10.
40. Ebina T, Sato A, Umezu K, Ishida N, Ohyama S, Ohizumi A, et al. Prevention of rotavirus infection by cow colostrum antibody against human rotaviruses. *Lancet* (1983) 2:1029–30.
41. Collins AM, Roberton DM, Hosking CS, Flannery GR. Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. *Int Arch Allergy Appl Immunol.* (1991) 96:362–7. doi: 10.1159/000235523
42. Davidson GP, Daniels E, Nunan H, Moore AG, Whyte PBD, Franklin K, et al. Passive immunisation of children with bovine colostrum containing antibodies to human rotavirus. *Lancet* (1989) 2:709–712. doi: 10.1016/S0140-6736(89)90771-X
43. Stelwagen K, Carpenter E, Haigh B, Hodgkinson A, Wheeler TT. Immune components of bovine colostrum and milk. *J Anim Sci* (2009) 87:3–9. doi: 10.2527/jas.2008-1377
44. Heck JML, van valenberg HJF, Dijkstra J, van Hooijdonk ACM. Seasonal variation in the Dutch bovine raw milk composition. *J Dairy Sci.* (2009) 92:4745–55. doi: 10.3168/jds.2009-2146
45. Jensen RG. *Handbook of Milk Composition*. San Diego, CA: Academic Press (1995).
46. Korhonen H, Marnila P, Gill HS. Bovine milk antibodies for health. *Br J Nutr.* (2000) 84 (Suppl. 1):S135–46. doi: 10.1017/S0007114500002361
47. Virtala AMK, Gröhn YT, Mechor GD, Erb HN. The effect of maternally derived immunoglobulin G on the risk of respiratory disease in heifers during the first 3 months of life. *Prev Vet Med.* (1999) 39:25–37.
48. Bush L, Aguilera M, Adans G, Jones E. Absorption of colostral immunoglobulins by newborn dairy calves. *J Dairy Sci.* (1971) 54:1547–9. doi: 10.3168/jds.S0022-0302(71)86063-0
49. Cervenak J, Kacskovics I. The neonatal Fc receptor plays a crucial role in the metabolism of IgG in livestock animals. *Vet Immunol Immunopathol.* (2009) 128:171–7. doi: 10.1016/j.vetimm.2008.10.300
50. Van Arsdall M, Haque I, Liu Y, Rhoads JM. Is there a role for the enteral administration of serum-derived immunoglobulins in human gastrointestinal disease and pediatric critical care nutrition? *Adv Nutr An Int Rev J.* (2016) 7:535–543. doi: 10.3945/an.115.011924
51. Larson B. Transfer of specific blood serum proteins to lacteal secretions near parturition. *J Dairy Sci.* (1958) 41:1033–44. doi: 10.3168/jds.S0022-0302(58)91047-6

52. Sasaki M, Davis CL, Larson BL. Production and Turnover of IgG1 and IgG2 Immunoglobulins in the Bovine around Parturition. *J Dairy Sci.* (1976) 59:2046–55. doi: 10.3168/jds.S0022-0302(76)84486-4
53. Baumrucker CR, Bruckmaier RM. Colostrogenesis: IgG1 transcytosis mechanisms. *J Mammary Gland Biol Neoplasia* (2014) 19:103–17. doi: 10.1007/s10911-013-9313-5
54. Jasion VS, Burnett BP. Survival and digestibility of orally-administered immunoglobulin preparations containing IgG through the gastrointestinal tract in humans. *Nutr J.* (2015) 14:22. doi: 10.1186/s12937-015-0010-7
55. Hilpert H, Gerber H, de Peyer E, Nussl D. Gastrointestinal passage of bovine anti-*E. coli* milk immunoglobulins (Ig) in infants. *Nestle Res News* (1974) 75:134–8.
56. Pacyna J, Siwek K, Terry SJ, Roberton ES, Johnson RB, Davidson GP. Survival of rotavirus antibody activity derived from bovine colostrum after passage through the human gastrointestinal tract. *J Pediatr Gastroenterol Nutr.* (2001) 32:162–7. doi: 10.1097/00005176-200102000-00013
57. Warny M, Fatimi A, Bostwick EF, Laine DC, Lebel F, Lamont JT, et al. Bovine immunoglobulin concentrate-clostridium difficile retains C difficile toxin neutralising activity after passage through the human stomach and small intestine. *Gut* (1999) 44:212–7. doi: 10.1136/gut.44.2.212
58. Hilpert H, Brüssow H, Mietens C, Sidoti J, Lerner L, Werchau H. Use of bovine milk concentrate containing antibody to rotavirus to treat rotavirus gastroenteritis in infants. *J Infect Dis.* (1987) 156:158–66.
59. Roos N, Mahe S, Benamouzig R, Sick H, Rautureau J, Tome D. 15N-labeled immunoglobulins from bovine colostrum are partially resistant to digestion in human intestine. *J Nutr.* (1995) 125:1238–44.
60. Kelly CP, Chetham S, Keates S, Bostwick EF, Roush AM, Castagliuolo I, et al. Survival of anti-Clostridium difficile bovine immunoglobulin concentrate in the human gastrointestinal tract. *Antimicrob Agents Chemother* (1997) 41:236–41.
61. Lee J, Kang HE, Woo HJ. Stability of orally administered immunoglobulin in the gastrointestinal tract. *J ImmunolMethods* (2012) 384:143–7. doi: 10.1016/j.jim.2012.06.001
62. Bogstedt AK, Hammarstrom L, Robertson AK. Letter to the editor survival of immunoglobulins from different species through the gastrointestinal tract in healthy adult volunteers : implications for. *Antimicrob Agents Chemother* (1997) 41:4804.
63. Shaw AL, Mathews DW, Hinkle JE, Petschow BW, Weaver EM, Detzel CJ, et al. Absorption and safety of serum-derived bovine immunoglobulin/protein isolate in healthy adults. *Clin Exp Gastroenterol.* (2016) 9:365–75. doi: 10.2147/CEG.S120118
64. Li A, Kärnell A, Pham Thi Huan, Phung DC, Nguyen BM, Luong NT, et al. Safety and immunogenicity of the live oral auxotrophic Shigella flexneri SFL124 in adult Vietnamese volunteers. *Vaccine* (1993) 11:180–9.
65. Brooks HJL, McConnell MA, Corbett J, Buchan GS, Fitzpatrick CE, Broadbent RS. Potential prophylactic value of bovine colostrum in necrotizing enterocolitis in neonates: an *in vitro* study on bacterial attachment, antibody levels and cytokine production. *FEMS Immunol Med Microbiol.* (2006) 48:347–54. doi: 10.1111/j.1574-695X.2006.00151.x
66. Hodgkinson AJ, Cakebread J, Callaghan M, Harris P, Brunt R, Anderson RC, et al. Comparative innate immune interactions of human and bovine secretory IgA with pathogenic and non-pathogenic bacteria. *Dev Comp Immunol.* (2017) 68:21–5. doi: 10.1016/j.dci.2016.11.012
67. Mitra AK, Mahalanabis D, Ashraf H, Unicomb L, Eeckels R, Tzipori S, et al. Hyperimmune cow colostrum reduces diarrhoea due to rotavirus: a double-blind, controlled clinical trial. *Acta Paediatr.* (1995) 84:996–1001. doi: 10.1111/j.1651-2227.1995.tb13814.x
68. Sarker S, Casswall T, Mahalanabis D, Alam N, Albert M, Brussow H, et al. Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunized bovine colostrum. *Pediatr Infect Dis J.* (1998) 17:1149–54. doi: 10.1097/00006454-199812000-00010
69. Ylitalo S, Uhari M, Rasi S, Pudas J, Leppaluoto J. Rotaviral antibodies in the treatment of acute rotaviral gastroenteritis. *Acta Paediatr* (1998) 87:264–7.
70. Mietens C, Keinhorst H, Hilpert H, Gerber H, Amster H, Pahud JJ. Treatment of infantile *E. coli* gastroenteritis with specific bovine anti-*E. coli* milk immunoglobulins. *Eur J Pediatr.* (1979) 132:239–252. doi: 10.1007/BF00496847
71. Casswall TH, Sarker SA, Faruque SM, Weintraub A, Albert MJ, Fuchs GJ, et al. Treatment of enterotoxigenic and enteropathogenic *Escherichia coli*-induced diarrhoea in children with bovine immunoglobulin milk concentrate from hyperimmunized cows: a double-blind, placebo-controlled, clinical trial. *Scand J Gastroenterol.* (2000) 35:711–8.
72. Casswall TH, Sarker SA, Albert MJ, Fuchs GJ, Bergström M, Björck L, et al. Treatment of Helicobacter pylori infection in infants in rural Bangladesh with oral immunoglobulins from hyperimmune bovine colostrum. *Aliment Pharmacol Ther.* (1998) 12:563–8.
73. Ashraf H, Mahalanabis D, Mitra K, Tzipori S, Fuchs GJ. Hyperimmune bovine colostrum in the treatment of shigellosis in children: a double-blind, randomized, controlled trial. *Acta Paediatr.* (2001) 90:1373–8.
74. Huppertz HI, Rutkowski S, Busch DH, Eisebit R, Lissner R, Karch H. Bovine colostrum ameliorates diarrhea in infection with diarrheagenic *Escherichia coli*, Shiga toxin-producing *E. coli*, and *E. coli* expressing intimin and hemolysin. (1999) 29:452–6.
75. Casswall T, Nilsson H, Bjorck L, Sjostedt S, Xu L, Nord C, et al. Bovine anti-Helicobacter pylori antibodies for oral immunotherapy. *Scand J Gastroenterol.* (2002) 37:1380–85. doi: 10.1080/003655202762671242
76. Hammarstrom L, Weiner C. Targeted antibodies in dairy-based products. *Adv Exp Med Biol.* (2008) 606:321–43. doi: 10.1007/978-0-387-74087-4_13
77. Dissel JT Van, Groot N De, Hensgens CMH, Numan S, Kuijper EJ, Veldkamp P, et al. Bovine antibody-enriched whey to aid in the prevention of a relapse of Clostridium difficile- associated diarrhoea: preclinical and preliminary clinical data. *J MedMicrobiol.* (2005) 54:197–205. doi: 10.1099/jmm.0.45773-0
78. Mattila E, Anttila VJ, Broas M, Marttila H, Poukka P, Kuusisto K, et al. A randomized, double-blind study comparing Clostridium difficile immune whey and metronidazole for recurrent Clostridium difficile-associated diarrhoea: efficacy and safety data of a prematurely interrupted trial. *ScandJ Infect Dis.* (2008) 40:702–8. doi: 10.1080/00365540801964960
79. Hu D, Zhang F, Zhou J, Xu B, Zhang H, Qiang H, et al. The clearance effect of bovine anti-Helicobacter pylori antibody-containing milk in O blood group Helicobacter pylori-infected patients: a randomized double-blind clinical trial. *J Transl Med.* (2015) 13:205. doi: 10.1186/s12967-015-0558-1
80. McClead Jr. RE, Butler T, Rabbani GH. Orally administered bovine colostral anti-cholera toxin antibodies: results of two clinical trials. *Am J Med.* (1988) 85:811–6. doi: 10.1016/S0002-9343(88)80026-3
81. Tacket CO, Losonsky G, Link H, Hoang Y, Guesry P, Hilpert H, et al. Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic *Escherichia coli*. *N Engl J Med.* (1988) 318:1240–3. doi: 10.1056/NEJM198805123181904
82. Freedman DJ, Tacket CO, Delehanty A, Maneval DR, Nataro J, Crabb JH. Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic *Escherichia coli*. *J Infect Dis.* (1998) 177:662–7.
83. Tacket CO, Losonsky G, Livio S, Edelman R, Crabb J, Freedman D. Lack of prophylactic efficacy of an enteric-coated bovine hyperimmune milk product against enterotoxigenic *Escherichia coli* challenge administered during a standard meal. *J Infect Dis.* (1999) 180:2056–9. doi: 10.1086/315157
84. Savarino SJ, Tribble DR, Porter CK, O'Dowd A, Cantrell JA, Sincock SA, et al. Prophylactic efficacy of hyperimmune bovine colostral antiadhesin antibodies against enterotoxigenic *Escherichia coli* diarrhea: a randomized, double-blind, placebo-controlled, phase 1 trial. *J Infect Dis.* (2017) 216:7–13. doi: 10.1093/infdis/jix144
85. Otto W, Najnigier B, Stelmasiak T, Robins-Browne RM. Randomized control trials using a tablet formulation of hyperimmune bovine colostrum to prevent diarrhea caused by enterotoxigenic *Escherichia coli* in volunteers. *ScandJ Gastroenterol.* (2011) 46:862–8. doi: 10.3109/00365521.2011.574726
86. Okhuysen PC, Chappell CL, Crabb J, Valdez LM, Douglass ET, DuPont HL. Prophylactic effect of bovine anti-Cryptosporidium hyperimmune colostrum immunoglobulin in healthy volunteers challenged with Cryptosporidium parvum. *Clin Infect Dis.* (1998) 26:1324–9.
87. Griffiths O V. Colostral immunity against salmonella infection in calves. *N Z Vet J* (1969) 17:50. doi: 10.1080/00480169.1969.33779

88. Royal WA, Robinson RA, Duganzich DM. Colostral immunity against salmonella infection in calves. *N Z Vet J.* (1968) 16:141–5. doi: 10.1080/00480169.1968.33761
89. Davidson G, Tam J, Kirubakaran C. Passive protection against hospital acquired symptompatic rota virus gasteroenteritis in India and Hong Kong. *J Pediatr Gastroenterol Nutr.* (1994) 19:351. doi: 10.1097/00005176-199410000-00102
90. Turner RB, Kelsey DK. Passive immunization for prevention of rotavirus illness in healthy infants. *Pediatr Infect Dis J.* (1993) 12:718–22.
91. Brunser O, Espinoza J, Figueroa G, Araya M, Spencer E, Hilpert H, et al. Field trial of an infant formula containing anti-rotavirus and anti- *Escherichia coli* milk antibodies from hyperimmunized cows. *J Pediatr Gastroenterol Nutr.* (1992) 15:63–72. doi: 10.1097/00005176-199207000-00010
92. Tawfeek HI, Najim NH, Al-Mashikhi S. Efficacy of an infant formula containing anti-*Escherichia coli* colostral antibodies from hyperimmunized cows in preventing diarrhea in infants and children: a field trial. *Int J Infect Dis.* (2003) 7:120–8. doi: 10.1016/S1201-9712(03)90007-5
93. Saad K, Abo-Elela MGM, El-Baseer KAA, Ahmed AE, Ahmad F-A, Tawfeek MSK, et al. Effects of bovine colostrum on recurrent respiratory tract infections and diarrhea in children. *Medicine (Baltimore)* (2016) 95:e4560. doi: 10.1097/MD.0000000000004560
94. Patel K, Rana R. Pedimune in recurrent respiratory infection and diarrhoea–the Indian experience–the pride study. *Indian J Pediatr.* (2006) 73:585–91. doi: 10.1007/BF02759923
95. Inagaki M, Yamamoto M, Cairangzhuoma, Xijier, Yabe T, Uchida K, et al. Multiple-dose therapy with bovine colostrum confers significant protection against diarrhea in a mouse model of human rotavirus-induced gastrointestinal disease. *J Dairy Sci.* (2013) 96:806–14. doi: 10.3168/jds.2012-5847
96. Inagaki M, Yamamoto M, Xijier C, Uchida K, Yamaguchi H, Kawasaki M, et al. *In vitro* and *in vivo* evaluation of the efficacy of bovine colostrum against human rotavirus infection. *Biosci Biotechnol Biochem.* (2010) 74:680–2. doi: 10.1271/bbb.90862
97. Parreño V, Marcoppido G, Vega C, Garaicoechea L, Rodriguez D, Saif L, et al. Milk supplemented with immune colostrum: protection against rotavirus diarrhea and modulatory effect on the systemic and mucosal antibody responses in calves experimentally challenged with bovine rotavirus. *Vet Immunol Immunopathol.* (2010) 136:12–27. doi: 10.1016/j.vetimm.2010.01.003
98. Saif L, Smith KL. Enteric Viral Infections of Calves and Passive Immunity. *J Dairy Sci.* (1985) 68:206–28. doi: 10.3168/jds.S0022-0302(85)80813-4
99. Schaller JP, Saif LJ, Cordle CT, Candler J, Winship TR, Smith KL. Prevention of human rotavirus-induced diarrhea in gnotobiotic piglets using bovine antibody. *J Infect Dis.* (1992) 165:623–30. doi: 10.1093/infdis/165.4.623
100. Funatogawa K, Ide T, Kirikae F, Saruta K, Nakano M, Kirikae T. Use of immunoglobulin enriched bovine colostrum against oral challenge with enterohaemorrhagic *Escherichia coli* O157:H7 in mice. *Microbiol Immunol.* (2002) 46:761–6. doi: 10.1111/j.1348-0421.2002.tb02761.x
101. Shield J, Melville C, Novelli V, Anderson G, Scheimberg I, Gibb D, et al. Bovine colostrum immunoglobulin concentrate for cryptosporidiosis in AIDS. *Arch Dis Child* (1993) 69:451–3. doi: 10.1136/adc.69.4.451
102. Plettenberg A, Stoehr A, Stellbrink HJ, Albrecht H, Meigel W. A preparation from bovine colostrum in the treatment of HIV-positive patients with chronic diarrhea. *Clin Investig.* (1993) 71:42–5. doi: 10.1007/BF00210962
103. Florén C-H, Chinenye S, Elfstrand L, Hagman C, Ihse I. ColoPlus, a new product based on bovine colostrum, alleviates HIV-associated diarrhoea. *Scand J Gastroenterol.* (2006) 41:682–6. doi: 10.1080/00365520500380817
104. Asmuth DM, Ma Z-M, Albanese A, Sandler NG, Devaraj S, Knight TH, et al. Oral serum-derived bovine immunoglobulin improves duodenal immune reconstitution and absorption function in patients with HIV enteropathy. *Aids* (2013) 27:2207–17. doi: 10.1097/QAD.0b013e328362e54c
105. Odong PO, Angwech PJ, Obol J, Kuule J, Florén C. Management of HIV in Children Using a Bovine Colostrum-Based Food Product — An Observational Field Study. *AIDS* (2015) 5:100–4. doi: 10.4236/wja.2015.52012
106. Kaducu FO, Okia SA, Upenytho G, Elfstrand L, Floron CH. Effect of bovine colostrum-based food supplement in the treatment of HIV-associated diarrhea in Northern Uganda: a randomized controlled trial. *Indian J Gastroenterol.* (2011) 30:270–6. doi: 10.1007/s12664-011-0146-0
107. de Jong MF, Alto NM. Cooperative Immune Suppression by *Escherichia coli* and *Shigella* Effector Proteins. *Infect Immun.* (2018) 86:e00560–17. doi: 10.1128/IAI.00560-17
108. Koymans KJ, Vrieling M, Gorham RD, van Strijp JAG. Staphylococcal immune evasion proteins: structure, function, and host adaptation. *Curr Top Microbiol Immunol.* (2017) 409:441–489. doi: 10.1007/82_2015_5017
109. Ip S, Chung M, Raman G, Chew P, Magula N, DeVine D, et al. *Breastfeeding and Maternal and Infant Health Outcomes in Developed Countries Evidence Report/Technology Assessment* . Rockville, MD (2007). p. 1–186.
110. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom millennium cohort study. *Pediatrics* (2007) 119:e837–42. doi: 10.1542/peds.2006-2256
111. Duijts L, Jaddoe VW V, Hofman A, Moll HA. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. *Pediatrics* (2010) 126:e18–25. doi: 10.1542/peds.2008-3256
112. Haschke F, Haiden N, Thakkar SK. Nutritive and bioactive proteins in breastmilk. *Ann Nutr Metab.* (2016) 69:17–26. doi: 10.1159/000452820
113. Urwin HJ, Zhang J, Gao Y, Wang C, Li L, Song P, et al. Immune factors and fatty acid composition in human milk from river/lake, coastal and inland regions of China. *Br J Nutr.* (2013) 109:1949–61. doi: 10.1017/S0007114512004084
114. Warner J, Turner P. Allergy. In *The Science of Paediatrics*, Carroll W, editor London: Elsevier. (2017) p. 297–316. Available online at: https://www.elsevier.com/books/the-science-of-paediatrics-mrcpch-mastercourse/lissauer/978-0-7020-6313-8
115. Brown EM, Arrieta M-C, Finlay BB. A fresh look at the hygiene hypothesis: how intestinal microbial exposure drives immune effector responses in atopic disease. *Semin Immunol.* (2013) 25:378–87. doi: 10.1016/j.smim.2013.09.003
116. Scheltema NM, Nibbelke EE, Pouw J, Blanken MO, Rovers MM, Naaktgeboren CA, et al. Respiratory syncytial virus prevention and asthma in healthy preterm infants: a randomised controlled trial. *Lancet Respir Med.* (2018) 6:257–64. doi: 10.1016/S2213-2600(18)30055-9
117. Blanken MO, Rovers MM, Molenaar JM, Winkler-Seinstra PL, Meijer A, Kimpen JLL, et al. Respiratory syncytial virus and recurrent wheeze in healthy preterm infants. *N Engl J Med.* (2013) 368:1791–9. doi: 10.1056/NEJMoa1211917
118. Wopereis H, Sim K, Shaw A, Warner JO, Knol J, Kroll JS. Intestinal microbiota in infants at high risk for allergy: effects of prebiotics and role in eczema development. *J Allergy Clin Immunol.* (2017) 141:1334–42. doi: 10.1016/j.jaci.2017.05.054
119. Xu ML, Kim HJ, Wi GR, Kim H-J. The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse. *J Microbiol.* (2015) 53:661. doi: 10.1007/s12275-015-5353-4
120. Ng WC, Wong V, Muller B, Rawlin G, Brown LE. Prevention and treatment of influenza with hyperimmune bovine colostrum antibody. *PLoS ONE* (2010) 5:e13622. doi: 10.1371/journal.pone.0013622
121. Wong EB, Mallet J-F, Duarte J, Matar C, Ritz BW. Bovine colostrum enhances natural killer cell activity and immune response in a mouse model of influenza infection and mediates intestinal immunity through toll-like receptors 2 and 4. *Nutr Res.* (2014) 34:318–25. doi: 10.1016/j.nutres.2014.02.007
122. Meyer G, Deplanche M, Schelcher F. Human and bovine respiratory syncytial virus vaccine research and development. *Comp Immunol Microbiol Infect Dis.* (2008) 31:191–225. doi: 10.1016/j.cimid.2007.07.008
123. Patiroglu T, Kondolot M. The effect of bovine colostrum on viral upper respiratory tract infections in children with immunoglobulin A deficiency. *Clin RespirJ.* (2013) 7:21–26. doi: 10.1111/j.1752-699X.2011.00268.x
124. Nigro A, Nicastro A, Trodella R. Retrospective observational study to investigate Sinerga, a multifactorial nutritional product, and bacterial extracts in the prevention of recurrent respiratory infections in children. *Int J Immunopathol Pharmacol.* (2014) 27:455–60. doi: 10.1177/039463201402700318

125. Vitetta L, Coulson S, Beck SL, Gramotnev H, Du S, Lewis S. The clinical efficacy of a bovine lactoferrin/whey protein Ig-rich fraction (Lf/IgF) for the common cold: a double blind randomized study. *Compl Ther Med.* (2013) 21:164–71. doi: 10.1016/j.ctim.2012.12.006
126. Crooks C, Cross M, Wall C, Ali A. Effect of bovine colostrum supplementation on respiratory tract mucosal defenses in swimmers. *Int J Sport Nutr Exerc Metab.* (2010) 20:224–35. doi: 10.1123/ijsnem.20.3.224
127. Uchida K, Yamagucki H, Kawasaki M, Yamashita K, Kaji N. Bovine late colostrum (colostrum 6 or 7 days after parturition) supplement reduces symptoms of Upper Respiratory Tract Infection in Infant. *Jap J Clin Nutr.* (2010) 31:122–7.
128. Brinkworth G, Buckley J. Concentrated bovine colostrum protein supplementation reduces the incidence of self-reported symptoms of upper respiratory tract infection in adult males. *Eur J Nutr.* (2003) 42:228–32. doi: 10.1007/s00394-003-0410-x
129. Cesarone MR, Belcaro G, Di Renzo A, Dugall M, Cacchio M, Ruffini I, et al. Prevention of influenza episodes with colostrum compared with vaccination in healthy and high-risk cardiovascular subjects: the epidemiologic study in San Valentino. *Clin Appl Thromb Hemost* (2007) 13:130–6. doi: 10.1177/1076029606295957
130. Jones AW, March DS, Curtis F, Bridle C. Bovine colostrum supplementation and upper respiratory symptoms during exercise training: a systematic review and meta-analysis of randomised controlled trials. *BMC Sports Sci Med Rehabil.* (2016) 8:21. doi: 10.1186/s13102-016-0047-8
131. Shing CM, Peake J, Suzuki K, Okutsu M, Pereira R, Stevenson L, et al. Effects of bovine colostrum supplementation on immune variables in highly trained cyclists. *J Appl Physiol.* (2007) 102:1113–22. doi: 10.1152/japplphysiol.00553.2006
132. Timby N, Hernell O, Vaarala O, Melin M, Lönnerdal B, Domellöf M. Infections in infants fed formula supplemented with bovine milk fat globule membranes. *J Pediatr Gastroenterol Nutr.* (2015) 60:384–9. doi: 10.1097/MPG.0000000000000624
133. Zavaleta N, Kvistgaard AS, Graverholt G, Respicio G, Guija H, Valencia N, et al. Efficacy of an MFGM-enriched complementary food in diarrhea, anemia, and micronutrient status in infants. *J Pediatr Gastroenterol Nutr.* (2011) 53:561–8. doi: 10.1038/s41538-018-0014-8
134. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol.* (2010) 10:861–8. doi: 10.1038/nri2871
135. Perkin MR, Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol.* (2006) 117:1374–81. doi: 10.1016/j.jaci.2006.03.008
136. Pfefferle PI, Büchele G, Blümer N, Roponen M, Ege MJ, Krauss-Etschmann S, et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE Study. *J Allergy Clin Immunol.* (2010) 125:108–15.e1–3. doi: 10.1016/j.jaci.2009.09.019
137. Naaber P, Lehto E, Salminen S, Mikelsaar M. Inhibition of adhesion of Clostridium difficile to Caco-2 cells. *FEMS ImmunolMed Microbiol.* (1996) 14:205–9. doi: 10.1111/j.1574-695X.1996.tb00288.x
138. An MJ, Cheon JH, Kim SW, Park JJ, Moon CM, Han SY, et al. Bovine colostrum inhibits nuclear factor kappaB-mediated proinflammatory cytokine expression in intestinal epithelial cells. *Nutr Res.* (2009) 29:275–80. doi: 10.1016/j.nutres.2009.03.011
139. Detzel CJ, Horgan A, Henderson AL, Petschow BW, Warner CD, Maas KJ, et al. Bovine immunoglobulin/protein isolate binds pro-inflammatory bacterial compounds and prevents immune activation in an intestinal co-culture model. *PLoS ONE* (2015) 10:e0120278. doi: 10.1371/journal.pone.0120278
140. Ya'acov A Ben, Lichtenstein Y, Zolotarov L, Ilan Y. The gut microbiome as a target for regulatory T cell-based immunotherapy: induction of regulatory lymphocytes by oral administration of anti-LPS enriched colostrum alleviates immune mediated colitis. *BMC Gastroenterol* (2015) 15:154. doi: 10.1186/s12876-015-0388-x
141. Henderson AL, Brand MW, Darling RJ, Maas KJ, Detzel CJ, Hostetter J, et al. Attenuation of colitis by serum-derived bovine immunoglobulin/protein isolate in a defined microbiota mouse model. *Dig Dis Sci.* (2015) 60:3293–303. doi: 10.1007/s10620-015-3726-5
142. Bateman E, Weaver E, Klein G, Wignall A, Wozniak B, Plews E, et al. Serum-derived bovine immunoglobulin/protein isolate in the alleviation of chemotherapy-induced mucositis. *Support Care Cancer* (2016) 24:377–85. doi: 10.1007/s00520-015-2806-6
143. Huang XH, Chen L, Gao W, Zhang W, Chen SJ, Xu LB, et al. Specific IgG activity of bovine immune milk against diarrhea bacteria and its protective effects on pathogen-infected intestinal damages. *Vaccine* (2008) 26:5973–80. doi: 10.1016/j.vaccine.2008.08.040
144. Pérez-Bosque A, Miró L, Maijó M, Polo J, Campbell J, Russell L, et al. Dietary intervention with serum-derived bovine immunoglobulins protects barrier function in a mouse model of colitis. *Am J Physiol Gastrointest Liver Physiol* (2015) 308:G1012. doi: 10.1152/ajpgi.00378.2014
145. Wilson D, Evans M, Weaver E, Shaw AL, Klein GL. Evaluation of serum-derived bovine immunoglobulin protein isolate in subjects with diarrhea-predominant irritable bowel syndrome. *Clin Med Insights Gastroenterol.* (2013) 6:49–60. doi: 10.4137/CGast.S13200
146. Shafran I, Burgunder P, Wei D, Young HE, Klein G, Burnett BP. Management of inflammatory bowel disease with oral serum-derived bovine immunoglobulin. *Therap Adv Gastroenterol.* (2015) 8:331–9. doi: 10.1177/1756283X15593693
147. Jensen ML, Sangild PT, Lykke M, Schmidt M, Boye M, Jensen BB, et al. Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets. *AJP Regul Integr Comp Physiol.* (2013) 305:R4–12. doi: 10.1152/ajpregu.00094.2013
148. Støy ACF, Heegaard PMH, Thymann T, Bjerre M, Skovgaard K, Boye M, et al. Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs. *Clin Nutr.* (2014) 33:322–9. doi: 10.1016/j.clnu.2013.05.013
149. Li Y, Juhl SM, Ye X, Shen RL, Iyore EO, Dai Y, et al. A stepwise, pilot study of bovine colostrum to supplement the first enteral feeding in preterm infants (precolos): study protocol and initial results. *Front Pediatr.* (2017) 5:42. doi: 10.3389/fped.2017.00042
150. Bolke E, Jehle PM, Hausmann F, Daubler A, Wiedeck H, Steinbach G, et al. Preoperative oral application of immunoglobulin-enriched colostrum milk and mediator response during abdominal surgery. *Shock* (2002) 17:9–12. doi: 10.1097/00024382-200201000-00002
151. Bolke E, Orth K, Jehle PM, Schwarz A, Steinbach G, Schleich S, et al. Enteral application of an immunoglobulin-enriched colostrum milk preparation for reducing endotoxin translocation and acute phase response in patients undergoing coronary bypass surgery–a randomized placebo-controlled pilot trial. *WienKlinWochenschr* (2002) 114:923–8.
152. Playford RJ, Floyd DN, Macdonald CE, Calnan DP, Adenekan RO, Johnson W, et al. Bovine colostrum is a health food supplement which prevents NSAID induced gut damage. *Gut* (1999) 44:653–8.
153. Playford RJ, Macdonald CE, Calnan DP, Floyd DN, Podas T, Johnson W, et al. Co-administration of the health food supplement, bovine colostrum, reduces the acute non-steroidal anti-inflammatory drug-induced increase in intestinal permeability. *Clin Sci.* (2001) 100:627–33. doi: 10.1042/cs1000627
154. Rogier EW, Frantz AL, Bruno MEC, Wedlund L, Cohen DA, Stromberg AJ, et al. Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc Natl Acad Sci USA.* (2014) 111:3074–9. doi: 10.1073/pnas.1315792111
155. Koch MA, Reiner GL, Lugo KA, Kreuk LSM, Stanbery AG, Ansaldo E, et al. Maternal IgG and IgA antibodies dampen mucosal t helper cell responses in early life. *Cell* (2016) 165:827–41. doi: 10.1016/j.cell.2016.04.055
156. Harris NL, Spoerri I, Schopfer JF, Nembrini C, Merky P, Massacand J, et al. Mechanisms of neonatal mucosal antibody protection. *J Immunol* (2006) 177:6256–6262. doi: 10.4049/jimmunol.177.9.6256
157. Kramer D, Cebra J. Role of maternal antibody in the induction of virus specific and bystander IgA responses in Peyer's patches of suckling mice. *Int Immunol.* (1995) 7:911–8.
158. Jungi TW, Peterhans E, Pfister H, Fey H. The interaction of ruminant IgG with receptor type II for IgG on human phagocytes. *Immunology* (1989) 66:143–8.
159. Nash GS, MacDermott RP, Schloemann S, Bertovich MJ, O'Neal J, Porter L, et al. Bovine IgG1, but not IgG2, binds to human B cells and inhibits antibody secretion. *Immunology* (1990) 69:361–6.

160. Loimaranta V, Nuutila J, Marnila P, Tenovuo J, Korhonen H, Lilius EM. Colostral proteins from cows immunised with Streptococcus mutans/S. sobrinus support the phagocytosis and killing of mutans streptococci by human leucocytes. *J Med Microbiol.* (1999) 48:917–26.
161. Kulczycki AJ, Nash G, Bertovich M, Burack H, MacDermott R. Bovine milk IgG, but not serum IgG, inhibits pokeweed mitogen-induced antibody secretion by human peripheral blood mononuclear cells. *J Clin Immunol.* (1987) 7:37–45. doi: 10.1007/BF00915423
162. Daeron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J Clin Invest.* (1995) 95:577–85.
163. Nimmerjahn F, Ravetch J V. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol.* (2008) 8:34–47. doi: 10.1038/nri2206
164. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch J V. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature* (1994) 369:340. doi: 10.1038/369340a0
165. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* (2012) 119:5640–49. doi: 10.1182/blood-2012-01-380121
166. Kramski M, Lichtfuss GF, Navis M, Isitman G, Wren L, Rawlin G, et al. Anti-HIV-1 antibody-dependent cellular cytotoxicity mediated by hyperimmune bovine colostrum IgG. *Eur J Immunol.* (2012) 42:2771–81. doi: 10.1002/eji.201242469
167. Ober RJ, Radu CG, Ghetie V, Ward ES. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int Immunol.* (2001) 13:1551–9. doi: 10.1093/intimm/13.12.1551
168. Latvala S, Jacobsen B, Otteneder MB, Herrmann A, Kronenberg S. Distribution of FcRn across species and tissues. *J Histochem Cytochem.* (2017) 65:321–33. doi: 10.1369/0022155417705095
169. Rath T, Baker K, Pyzik M, Blumberg RS. Regulation of immune responses by the neonatal Fc receptor and its therapeutic implications. *Front Immunol.* (2014) 5:22–9. doi: 10.3389/fimmu.2014.00664
170. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol.* (2003) 3:984–93. doi: 10.1038/nri1246
171. Celis E, Zurawski VR, Chang TW. Regulation of T-cell function by antibodies: enhancement of the response of human T-cell clones to hepatitis B surface antigen by antigen-specific monoclonal antibodies. *Proc Natl Acad Sci USA.* (1984) 81:6846–50. doi: 10.1073/pnas.81.21.6846
172. Gosselin EJ, Wardwell K, Gosselin DR, Alter N, Janlanl F, Andpndpm, G. Enhanced antigen presentation using human Fcy receptor (monocyte/macrophage)-specific immunogens. *J Immunol.* (1992) 149:3477–81.
173. Shen L, van Egmond M, Siemasko K, Gao H, Wade T, Lang M, et al. Presentation of ovalbumin internalized via the immunoglobulin-A Fc receptor is enhanced through Fc receptor gamma-chain signaling. *Blood* (2001) 97:205–13. doi: 10.1182/blood.V97.1.205
174. van der Heijden FL, Joost van Neerven RJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic disease. *J Immunol.* (1993) 150:3643–50.
175. Santamaria LF, Bheekha R, Reijsen FC,Van Soler MTP, Suter M, Bruijnzeel-koomen CAFM, et al. Antigen focusing by specific monomeric immunoglobulin e bound to CD23 on. (1993) 30:23–30.
176. Adar T, Ben Ya'acov A, Lalazar G, Lichtenstein Y, Nahman D, Mizrahi M, et al. Oral administration of immunoglobulin G-enhanced colostrum alleviates insulin resistance and liver injury and is associated with alterations in natural killer T cells. *Clin Exp Immunol.* (2012) 167:252–260. doi: 10.1111/j.1365-2249.2011.04511.x
177. Uchida K, Hiruta N, Yamaguchi H, Yamashita K, Fujimura K, Yasui H. Augmentation of cellular immunity and protection against influenza virus infection by bovine late colostrum in mice. *Nutrition* (2012) 28:442–6. doi: 10.1016/j.nut.2011.07.021
178. Kubinak JL, Round JL. Do antibodies select a healthy microbiota? *Nat Rev Immunol.* (2016) 16:767–4. doi: 10.1038/nri.2016.114
179. Dollé L, Tran HQ, Etienne-Mesmin L, Chassaing B. Policing of gut microbiota by the adaptive immune system. *BMC Med* (2016) 14:4–7. doi: 10.1186/s12916-016-0573-y
180. Stephens WZ, Round JL. Previews IgA targets the troublemakers. *Cell Host Microbe* (2014) 16:265–7. doi: 10.1016/j.chom.2014.08.012
181. Jung K, Miyagawa M, Matsuda A, Amagai Y, Oida K, Okamoto Y, et al. Antifungal effects of palmitic acid salt and ultrapure soft water on *Scedosporium apiospermum*. *J ApplMicrobiol.* (2013) 115:711–7. doi: 10.1111/jam.12298
182. Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, et al. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* (2014) 158:1000–10. doi: 10.1016/j.cell.2014.08.006
183. Macpherson AJ, McCoy KD. Independence day for IgA. *Immunity* (2015) 43:416–8. doi: 10.1016/j.immuni.2015.08.024
184. Fransen F, Zagato E, Mazzini E, Fosso B, Manzari C, El Aidy S, et al. BALB/c and C57BL/6 Mice Differ in Polyreactive IgA Abundance, which Impacts the Generation of Antigen-Specific IgA and Microbiota Diversity. *Immunity* (2015) 43:527–540. doi: 10.1016/j.immuni.2015.08.011
185. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, et al. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol.* (2010) 3:461–74. doi: 10.1038/mi.2010.23
186. Ohsaki A, Venturelli N, Buccigrosso TM, Osganian SK, Lee J, Blumberg RS, et al. Maternal IgG immune complexes induce food allergen–specific tolerance in offspring. *J Exp Med.* (2018) 215:91–113. doi: 10.1084/jem.20171163
187. Henderson AJ, Warner JO. Fetal origins of asthma. *Semin Fetal Neonatal Med.* (2012) 17:82–91. doi: 10.1016/j.siny.2012.01.006
188. De Agüero MG, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. *Science* (2016) 351:1296–1302. doi: 10.1126/science.aad2571
189. Maijo M, Miro L, Polo J, Campbell J, Russell L, Crenshaw J, et al. Dietary plasma proteins attenuate the innate immunity response in a mouse model of acute lung injury. *Br J Nutr.* (2012) 107:867–875. doi: 10.1017/S0007114511003655

Conflict of Interest Statement: LU and RvN are employees of FrieslandCampina. JW has been the PI for a trial of a cow milk formula with prebiotics for the prevention of allergy funded by Danone/Nutricia. JL, HS have no conflicts of interest to report.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 12 July 2018
doi: 10.3389/fnut.2018.00060

The Well-Developed Mucosal Immune Systems of Birds and Mammals Allow for Similar Approaches of Mucosal Vaccination in Both Types of Animals

Tomonori Nochi[1,2], *Christine A. Jansen*[3], *Masaaki Toyomizu*[1] *and Willem van Eden*[3]*

[1] International Education and Research Center for Food and Agricultural Immunology, Graduate School of Agricultural Science, Tohoku University, Miyagi, Japan, [2] International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [3] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Pinyi Lu,
DoD, Biotechnology HPC Software Applications Institute (BHSAI), United States

Reviewed by:
Lei Shi,
Georgia State University, United States
Ryan Arsenault,
University of Delaware, United States
Anastasia N. Vlasova,
The Ohio State University, United States

***Correspondence:**
Willem van Eden
w.vaneden@uu.nl

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *31 March 2018*
Accepted: *19 June 2018*
Published: *12 July 2018*

Citation:
Nochi T, Jansen CA, Toyomizu M and Eden W (2018) The Well-Developed Mucosal Immune Systems of Birds and Mammals Allow for Similar Approaches of Mucosal Vaccination in Both Types of Animals. Front. Nutr. 5:60. doi: 10.3389/fnut.2018.00060

The mucosal immune system is a compartmentalized part of the immune system that provides local immunity in the mucosa of the respiratory, gastrointestinal, and digestive tracts. It possesses secondary lymphoid tissues, which contain immune cells, such as T, B, and dendritic cells. Once the cells of the mucosal immune system are stimulated by luminal antigens, including microorganisms, they infiltrate into diffuse areas of mucosal tissues (e.g., respiratory mucosa and lamina propria of intestinal villi) and exhibit immune effector functions. Inducing the antigen-specific immune responses in mucosal tissues by mucosal vaccination would be an ideal strategy for not only humans, but also mammals and birds, to protect against infectious diseases occurring in mucosal tissues (e.g., pneumonia and diarrhea). Infectious diseases cause huge economic losses in agriculture, such as livestock and poultry industries. Since most infectious diseases occur in mucosal tissues, vaccines that are capable of inducing immune responses in mucosal tissues are in high need. In this review, we discuss the current understanding of mucosal immunity in mammals and birds, and recent progress in the development of mucosal vaccines.

Keywords: mucosal immune system, mucosal vaccine, nutritional supplementation, mammals, birds

THE MUCOSAL IMMUNE SYSTEM IN MAMMALS AND BIRDS

The mucosa-associated lymphoid tissues, which are lymphoid structures in the mucosal tissues, form the first line of defense against pathogens that enter the body through the mucosal surfaces lining the respiratory, digestive, and reproduction tracts (**Figure 1**). Fundamentally, the mucosal immune system has evolved to tolerate commensal microbes, while responding quickly and effectively to pathogenic challenges. Although the mucosal immune systems of mammals and birds share many features, which are fundamental to the functioning of mucosa-associated lymphoid tissues, the avian mucosal immune response has unique features. A fundamental difference between mammals and birds is the absence of encapsulated lymph nodes but the presence of diffuse lymphoid tissue in birds.

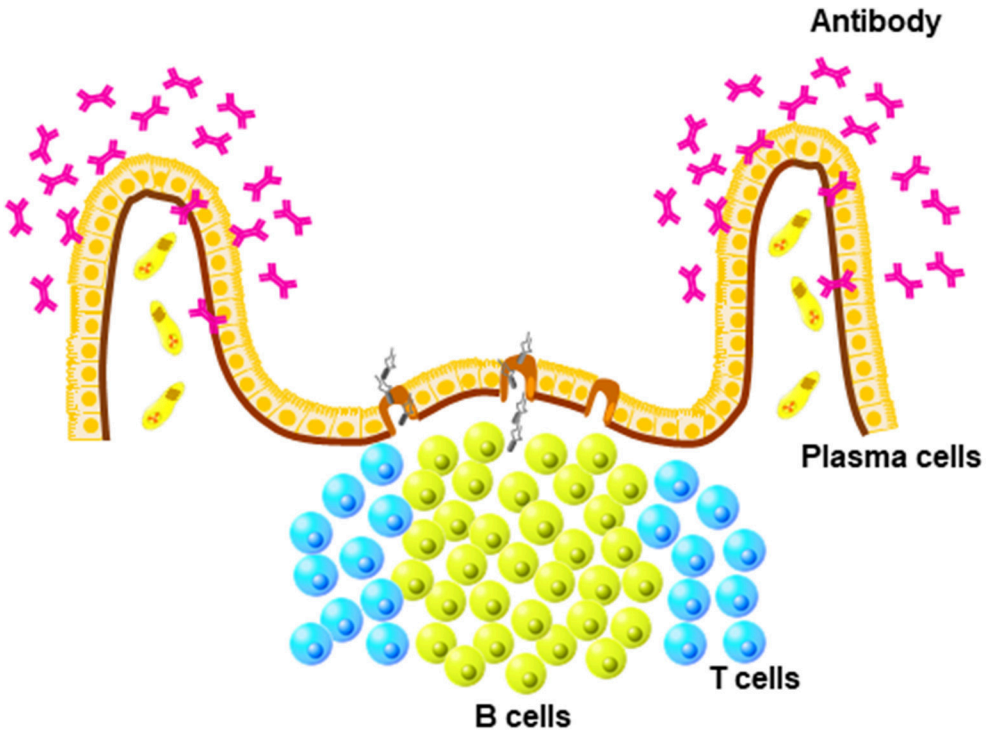


FIGURE 1 | Unique structure of mucosa-associated lymphoid (MALTs). MALTs contain mature B cells that differentiate into antibody-producing plasma cells present in diffuse areas of mucosal tissues, such as lamia propria.

The mucosal tissue of the nose is the first to come into contact with particles and pathogens upon inhalation. In chickens, a major characteristic of the nasal-associated lymphoid tissue (NALT) is the formation of defined areas of B cells with caps composed of $CD4^+$ T cells. Immunoglobulin-producing (Ig^+) B cells are found both within the NALT structures and distributed throughout the epithelium, and are mostly IgY^+ in chickens (1). In mammals, immunoglobulin class-switching from IgM to IgA occurs in the NALT and IgA-producing plasma cells are abundantly present in the nasal cavity. In chickens, closely related to the mucosal tissue of the nose are the head-associated lymphoid tissues, which include the Harderian gland and the conjunctiva-associated lymphoid tissue (CALT). The Harderian gland is located in the orbit of the eye and has the structure of a typical secondary lymphoid organ, with many B cells and plasma cells, germinal centers, and T cell-dependent interfollicular regions with scattered T cells and macrophages (2, 3). It plays an important role in the adaptive mucosal immune response upon ocular exposure to avian pathogens (4, 5). CALT is located on the inner surfaces of the eyelids and is observable in 1-week-old chickens (6). Based on the composition of CALT lymphocytes, the induction of antigen-specific IgA antibody-secreting cells after ocular exposure, the expansion of polymeric immunoglobulin receptors, and the production of IFNγ by lacrimal fluids suggests that CALT plays important roles in the avian mucosal immune response (7). It should be noted that most mammals (e.g., cats, dogs, and humans) also develop CALT. A recent study found that mice possess a similar lymphoid structure in the lacrimal sac, the so-called tear duct-associated lymphoid tissue, which plays an important role in the induction of an immune response by the ocular immunosurveillance system (8).

The chicken respiratory tract is very different from that of mammalians. For example, the avian lung has a unidirectional airflow (9) in contrast to the bidirectional airflow in the human lung. Furthermore, the bird lung is ventilated via air sacs, since birds do not have a diaphragm. A consequence of a unidirectional airflow is that particles are primarily deposited at the caudal regions of the lung (10), which is the part of the lung containing bronchus-associated lymphoid tissue (BALT). These highly organized lymphoid structures together with diffusely distributed cells were described for the first time in 1973 (11). Avian BALT structures are observed around 3–4 weeks post hatching and are fully developed in some birds at the age of 6 weeks (12). Both age and environmental stimuli influence BALT development (13). Furthermore, the number of BALT nodules increases significantly upon infection with pathogenic microorganisms (14). Another difference between the mammalian and chicken lung is the lack of alveolar macrophages at the surface of the air capillaries in chickens (14). Interestingly, a large network of macrophages and dendritic cells (DCs) is present in the mucosa of the larger airways, the linings of the parabronchi (15), and the connective tissue (9). Thus, phagocytic cells are strategically localized at the start of the gas-exchange areas to clear the air of inhaled particles before it reaches the thin

and vulnerable air capillaries. Since chickens lack draining lymph nodes, the location where phagocytic cells present the particles to the immune system remains unclear. Presentation of particles may occur locally in the BALT, in the interstitial follicles between parabronchi, and/or in the spleen.

Gut-associated lymphoid tissues (GALTs) are well developed in birds (16). It consists of lymphoid cells located in the epithelial lining and the lamina propria as well as specialized lymphoid structures such as Peyer's patches and cecal tonsils. Peyer's patches in chicken are clearly visible at 2 weeks of age, and they increase in number with age. Like in mammals, they seem to consist of specialized epithelium with M cells that overlay structured follicles with defined T and B cell areas (17). Cecal tonsils, which are located in the neck region of each ceca, are structurally similar to Peyer's patches (18). Together, the GALT structures play an important role in the induction of immune responses (19).

MUCOSAL VACCINATION IN BIRDS

Vaccination through the mucosa itself is frequently used in the poultry industry, as an economical, efficient, and reliable method to vaccinate large numbers of birds. However, a successful mucosal vaccine must elicit both local and systemic immune responses (20, 21). Poultry vaccines against viral infections consist of either live attenuated viruses or inactivated viruses formulated with a suitable adjuvant. Most live vaccines are applied mucosally via the oculo-nasal route or with a spray, so that the vaccine enters the respiratory tract or is taken up by the head-associated lymphoid tissues where it is recognized and taken up by antigen-presenting cells. The use of a spray is the preferred method for vaccination of birds against respiratory viruses, such as infectious bronchitis virus (22), Newcastle Disease virus, and avian metapneumovirus. However, although deposition patterns after aerosol or spray vaccination are conventionally studied using beads, the deposition pattern of beads is dependent on the bead size, the droplet size of the bead solution, and the age of the chickens. Larger beads ($>3.7\,\mu M$) are mainly deposited in the upper respiratory tract, while smaller beads are distributed throughout the entire respiratory tract (23–25). The highest accumulation of beads occurs at the bifurcations primary to secondary bronchi (24), similar to that observed upon spray vaccination with avian influenza virus (AIV) (10), suggesting that particulate antigens are also taken up in the respiratory tract at these junctions. After entering the respiratory tract, particles are taken up by antigen-presenting cells (26) and are then presented to the immune system.

In addition to spray vaccination, vaccines can be delivered via drinking water. Vaccines dispensed through drinking water end up in the oral cavity with rapid transit to the esophagus and digestive tract. In this case, antigens will be taken up by cells in GALTs and presented to the immune system. Although oral vaccination has been reported to result in protection against *Salmonella* and reduction in necrotic enteritis lesions (27), other reports show less positive results (28). This may be related to the pathogen, type of vaccine, or age of the birds.

Inactivated vaccines are often poorly immunogenic and require additional components (adjuvants) for the induction of a protective immune response (29). These vaccines are formulated with a high antigenic mass of bacterial or viral origin conveyed in a suitable adjuvant, which renders these substances unsuitable for spray vaccination. Therefore, alternative strategies are needed for mucosal application of inactivated vaccines, such as specialized delivery systems or adjuvants with mucoadhesive properties. Several mucosal adjuvants have been employed in chickens and can be divided in two classes based on the mode of action: stimulation of the immune system and/or efficient delivery of vaccine materials. An important group of potential immune stimulators are the toll-like receptor (TLR)-based adjuvants (30). TLRs are pattern recognition receptors, a group of receptors present on immune cells that recognize the conserved molecular structures of pathogens, the so-called microbe-associated molecular patterns. The recognition of pathogens by TLRs results in the immediate activation of the immune system (31). CpG oligodeoxynucleotides (CpG ODNs), the ligand of chicken TLR21, have been reported as potential vaccine adjuvants in chickens. For example, vaccination with NDV and CpG resulted in the induction of specific immune responses and protection (32), and *in vivo* administration of CpG ODNs by itself suppressed the replication of IBV in the chicken embryo (33). Enhanced protection upon CpG ODN administration has also been reported for Marek's disease virus (34), as well as infection with *Salmonella enterica* (35) and *Escherichia coli* (36). Other potential immune stimulators include oligopeptides complexed with an agonistic anti-chicken CD40 monoclonal antibody (37) and the immune potentiator CVCVA5, which induces enhanced immune responses and protection against AIV upon vaccination (38, 39).

Mucoadhesive adjuvants, such as chitosan, have been suggested to increase the mucosal residence time, which results in increased antigen uptake and presentation (40). Rauw and colleagues investigated the effect of chitosan on the mucosal delivery of NDV vaccines in 1-day-old birds and found an enhanced cell mediated immunity in the spleen (41). Also, particulate deliverable systems, such as poly lactic-co-glycolic acid (PLGA) nanoparticles, invoke mechanisms that influence vaccine immunogenicity via enhanced antigen processing (42). Interestingly, vaccinating chickens with PLGA particles encapsulated with inactivated AIV vaccine adjuvanted with CpG ODNs resulted in enhanced antibody responses and a reduction in virus shedding (43). Furthermore, intranasal administration of NDV DNA vaccine-encapsulated nanoparticles in specific-pathogen-free chickens resulted in enhanced humoral and cellular immune responses and protection against challenge with a highly virulent NDV strain (44).

ASPECTS OF ANTIGEN DELIVERY FOR MUCOSAL VACCINES

Approaches of mucosal vaccination, with delivery systems as developed for mammals, may turn out to be similarly effective in birds. In the case of mammals, it is well known that the function

of Peyer's patches in the gut immune system is totally distinct from that of the lamina propria lymphoid tissues of intestinal villi (45). Fundamentally, antigen-specific intestinal immune responses to luminal substances are initiated in Peyer's patches, whereas the actual immune reactions (e.g., IgA production) take place in the intestinal villi (45). Therefore, DCs that prime mature naïve T cells by antigen presentation are frequently found in Peyer's patches; however, DCs are also abundantly distributed in the lamina propria (LP) of the gut intestinal villi, in mammals, despite the absence of lymphoid follicular structures, such as Peyer's patches (46). In birds, the presence of tissue DCs has not been well demonstrated due to the lack of specific antibodies. A first step was made by showing the presence of cells that express the C type lectin receptor DEC205$^+$ in tissues, including bursa and spleen (47). Expression of chicken DEC205 reflects the unique structure and function of the avian immune system (47). In mammals, a subset of the LP DCs, which are monocyte-derived and express CX_3CR1 (a receptor for CX_3CL1), can access the intestinal lumen to directly sample luminal microorganisms by extending their dendrites to regulate immunological tolerance and inflammation (48). A recent study demonstrated that goblet cells, whose primary function is to produce mucus that covers intestinal epithelial surface, have an additional function to deliver luminal antigens to another subset of LP DCs that have differentiated from conventional myeloid DC precursors and express α_E integrin, known as CD103 (49). Among the two DC populations (i.e., CX_3CR1^+ DCs and CD103$^+$ DCs) found in the LP of the gut, CD103$^+$ DCs migrate into the mesenteric lymph nodes that drain the gastrointestinal tract to prime mature T cells for initiation of antigen-specific mucosal immune responses (50). Thus, a strategy that is capable of delivering the vaccine antigen to CD103$^+$ DCs in the LP should be considered as a potential approach to increase the efficacy of mucosal vaccines (50). It should be noted, however, that chickens do not have mesenteric lymph nodes. Therefore, other routes for the delivery of antigens are present in chickens. Interestingly, it was demonstrated that a 12-mer peptide, which was discovered with the use of phage display technology, possesses broad targeting specificity for DCs of humans and mice (51). Moreover, the efficacy of orally administered lactic acid bacteria (LAB) that express the vaccine antigen together with the DC-specific peptide has been confirmed (52). Specifically, oral administration of DC-specific peptide-expressing LAB was shown to effectively induce antigen-specific immune responses in the gastrointestinal tract upon delivery to intestinal DCs (52). However, it is important to note that the mucosal tissues are lined by a tight epithelial barrier and also covered by a thick mucus layer (53). Moreover, CD103$^+$ DCs present in the LP, which is located within intestinal tissues, are still far from the mucosal lumen in which the vaccine antigens are administered (46). Therefore, mucosal vaccines need to cross the physiological barrier (e.g., mucus layer and epithelial layer) to reach CD103$^+$ DCs for initiation of intestinal immune responses. Although markers such as CD103$^+$ are still lacking in birds, it seems reasonable to assume the presence of similar gut antigen-presenting cells in these species.

Another possible vaccine delivery system is with liposomes. More than 50 years ago, the British biophysicist Alec Bangham discovered spherical lipid bilayer structures, so-called liposomes, when testing a new electron microscope introduced in his research institution using dry phospholipid samples that were negatively stained (54). Liposomes are basically formed by phospholipids, which are composed of a hydrophilic head group linked to a hydrophobic tail by a glycerol backbone (55). The size of liposomes varies from small (nanoscale) to large (micro-scale) (55). A well-known biological characteristic of liposomes in vaccine development is the capability of enclosing several different biomaterials, such as protein antigens and nucleic adjuvants, regardless of solubility since liposomes possess amphiphilic features (55). The activity of liposomes can be freely modulated by chemical modification of the surface of the structure. For example, coating of liposomes with polyethylene glycol increases the retention effect in blood, compared with bare liposomes, because the coating allows the liposome to escape from capture by the reticuloendothelial system in the liver and spleen, etc. Another potential modification is to endow liposomes with tropism by conjugating cell-specific antibodies or potential ligand molecules that bind to specific receptors expressed by the target tissues or cells (56). Moreover, recent studies have succeeded in the development of heat-, pH-, enzyme-, and light-dependent liposomes as delivery vehicles that respond to certain stimuli *in vivo* (57). These liposomes have been also used for mucosal vaccine development (55). For example, cationic liposomes that are generated from cationic lipids, such as dimethyldioctadecylammonium bromide (58), 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl], and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (59), can be retained in the mucosal epithelium when administered via the mucosal route. To this end, the vaccine antigens and/or adjuvants enclosed in cationic liposomes are successfully released in the mucosal tissues, which results in immediate processing by DCs and subsequent induction of effective immune responses. Amphiphilic nanometer-sized gels, so-called nanogels, are effective biomaterials that can be used for not only drug delivery but also vaccine development (60). Pullulan, which is a polymer composed of regularly repeating glucose units, described as α(1-4)Glu-α(1-4)Glu-α(1-6)Glu, was first utilized to generate self-assembled nanogels by the addition of multihydrophobic domains consisting of 1.6 cholesteryl groups per 100 glucose units (61). One of the most attractive features of cholesterol-bearing pullulan (CHP) nanogels is the trapping of proteins in the nanoscale matrix, which contains a large amount of water (62, 63). So far, several bioactive proteins, including insulin (64), bovine serum albumin (62), α-chymotrypsin (65), and myoglobin (63), have been successfully encapsulated in CHP nanogels while maintaining activity. Similar to liposomes, the characteristics of CHP nanogels, such as electrical charge, can be freely altered by chemical modification (66). Recent studies of cationic CHP nanogels with encapsulation of several prototypes of vaccine antigens demonstrated that the antigen was effectively sampled by DCs present in the nasal mucosa and subsequent antigen-specific mucosal immune responses were effectively induced in not only mice, but also non-human primates, when administered intranasally (67–69). Because of the high potency of cationic CHP nanogel-based nasal

vaccine, co-administration of a mucosal adjuvant is not required (67–69). Moreover, it should be noted that nasally administered cationic CHP nanogels and encapsulated vaccine antigens do not accumulate in the brain or olfactory bulb (67), suggesting that a strategy for nasal vaccine development using cationic CHP nanogels would be safe without the risk of undesired side effects, such as Bell's palsy (67).

THE POSSIBLE NEGATIVELY INTERFERING EFFECTS OF NUTRIENTS ON MUCOSAL VACCINATION

The mucosal immune system has exquisite qualities for maintaining immunological tolerance and the control of undesirable and counterproductive responses to nutrients. Therefore, successful mucosal vaccination would require overcoming mechanisms of mucosal tolerance. The vaccination effects of LAB illustrate the versatile characteristics of mucosal immune systems. However, intranasal administration of LAB, both live and killed, has been shown to produce an effective vaccination effect leading to protection against infection, oral administration was not effective. To reach any effect, frequent dosing for several weeks or novel delivery or adjuvant strategies was needed. Moreover, tolerization to antigens secreted by orally administered LAB has been reported (70).

Nutrients, such as the dietary antioxidant vitamin A, impact the tolerance of the mucosal immune system to a great extent. $CD103^+$ DCs in the LP can convert vitamin A into retinoic acid, which, in combination with TGFβ, is one of the driving forces in the production of regulatory T cells (Tregs) in the mesenteric lymph nodes (71). Interestingly, vitamin A supplementation during lactation was shown to reduce allergic sensitization in the offspring of mice. Furthermore, through different mechanisms, dietary supplementation with probiotics, prebiotics, and n-3 polyunsaturated fatty acids, is suggested to support oral tolerance and to prevent allergy in early childhood (72). Also, in combination with certain members of the gut microbiota, nutrients are known to promote tolerance. Fermentation of non-digestible dietary carbohydrates (fibers) by the gut microbiota leads to production of short-chain fatty acids, such as acetate, propionate, and butyrate, which also have the capacity to stimulate the expansion and immuno-suppressive capacity of Tregs in the gut (73). L-arginine promotes lymphocyte proliferation, balances pro-inflammatory (IFN-γ and IL-2) and anti-inflammatory (IL-4 and IL-10) cytokines, and increases the secretory IgA (sIgA) level in burn-injured mice (74). In this regard, L-arginine supplementation inhibits *Clostridium perfringens* overgrowth and alleviates intestinal mucosal injury by modulating innate immune responses in chickens by enhancing barrier function and producing NO (75). Another study also suggests that L-arginine supplementation attenuates intestinal mucosal disruption in coccidiosis-challenged chickens probably through suppressing TLR4 and activating mTOR complex 1 pathways (76). Probiotic feeding is also appropriate to manipulate mucosal immunity. After 21 days of treatment with *Lactobacillus acidophilus* as a probiotic on T cells in chicken, the percentages of blood $CD4^+$, $CD8^+$, and $TCR1^+$ cells were significantly higher in the probiotic-fed group than in the control group. After 14 days of the probiotic, a significantly greater number of $CD4^+$ T cells were found in the ileum of probiotic-fed chickens, and this difference was even greater after 21 days. The findings indicated that probiotics may alter the distribution of T cells in the blood and lymphoid tissues in young chickens; however, transient changes in lymphoid tissues, indicating that probiotics likely do not permanently affect mucosal immunity (77). The effects of *Saccharomyces boulardii* and *Bacillus subtilis* on cytokine expression responses via Toll-like receptors (TLRs) by intestinal epithelial cells were to decrease the expression levels of INF-γ and IL-8 and to increase the levels of serum IgA and sIgA in mucosa (78). These results indicated that *Saccharomyces boulardii* and *Bacillus subtilis* have a role in inducing mucosal innate immunity in chickens (78).

Some dietary products have the capacity to co-induce stress proteins in gut-associated cells. Oral administration of carvacrol, essential oil of oregano, was found to inhibit experimental autoimmune arthritis in mice. Upon further analysis, this compound actually co-induced the expression of heat shock protein 70 (HSP70) in cells in Peyer's patches. Subsequently, the enhanced HSP70 expression in PPs led to the activation and expansion of HSP70-specific T cells with regulatory, IL-10-producing capacities (79). Taken together, these findings suggest that dietary components may promote tolerance by various underlying mechanisms. However, the identity of such dietary factors that impact the efficacy of mucosal vaccination remains to be further elucidated.

CONCLUSION

Immune protection against infection is considered to be the most efficient when localized at the sites of entry of the infectious agent. Since most infections occur at mucosal surfaces, mucosal vaccination is an actively sought research goal in many species, including mammals and birds. Despite successes, such as with polio and rotavirus vaccination in humans, the scientific challenges in this area are still manifold. And this is, partly, due to the fact that oral administration of proteins induces tolerance, and not immune activation. Given the existing similarities between the mucosal immune systems of mammals and birds, it is possible to effectively use in birds some of the successful mucosal vaccination strategies as developed for mammals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Core-to-Core Program (Advanced Research Networks) of the Japan Society for the Promotion of Science (Establishment of international agricultural immunology research-core for a quantum improvement in food safety).

REFERENCES

1. Ohshima K, Hiramatsu K. Distribution of T-cell subsets and immunoglobulin-containing cells in nasal-associated lymphoid tissue (NALT) of chickens. *Histol Histopathol.* (2000) 15:713–20. doi: 10.14670/HH-15.713
2. Jeurissen SH, Janse EM, Koch G, De Boer GF. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* (1989) 258:119–24.
3. Savage ML, Olah I, Scott TR. Plasma cell proliferation in the chicken harderian gland. *Cell Prolif.* (1992) 25:337–44.
4. Burns RB. Specific antibody production against a soluble antigen in the Harderian gland of the domestic fowl. *Clin Exp Immunol.* (1976) 26:371–4.
5. van Ginkel FW, van Santen VL, Gulley SL, Toro H. Infectious bronchitis virus in the chicken Harderian gland and lachrymal fluid: viral load, infectivity, immune cell responses, and effects of viral immunodeficiency. *Avian Dis.* (2008) 52:608–17. doi: 10.1637/8349-050908-Reg.1
6. Fix AS, Arp LH. Quantification of particle uptake by conjunctiva-associated lymphoid tissue (CALT) in chickens. *Avian Dis.* (1991) 35:174–9.
7. van Ginkel FW, Gulley SL, Lammers A, Hoerr FJ, Gurjar R, Toro H. Conjunctiva-associated lymphoid tissue in avian mucosal immunity. *Dev Comp Immunol.* (2012) 36:289–97. doi: 10.1016/j.dci.2011.04.012S0145-305X(11)00127-3
8. Nagatake T, Fukuyama S, Kim DY, Goda K, Igarashi O, Sato S, et al. Id2-, RORgammat-, and LTbetaR-independent initiation of lymphoid organogenesis in ocular immunity. *J Exp Med.* (2009) 206:2351–64. doi: 10.1084/jem.20091436jem.20091436
9. Reese S, Dalamani G, Kaspers B. The avian lung-associated immune system: a review. *Vet Res.* (2006) 37:311–24. doi: 10.1051/vetres:2006003v6021
10. Reemers SS, van Haarlem DA, Groot Koerkamp MJ, Vervelde L. Differential gene-expression and host-response profiles against avian influenza virus within the chicken lung due to anatomy and airflow. *J Gen Virol.* (2009) 90:2134–46. doi: 10.1099/vir.0.012401-0vir.0.012401-0
11. Bienenstock J, Johnston N, Perey DY. Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab Invest.* (1973) 28:686–92.
12. Fagerland JA, Arp LH. Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. *Avian Dis.* (1993) 37:10–18.
13. Jeurissen SHM, Vervelde L, Janse EM. Structure and function of lymphoid tissues of the chicken. *Poultry Sci Rev.* (1994) 5:183–207.
14. Van Alstine WG, Arp LH. Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with Bordetella avium. *Am J Vet Res.* (1988) 49:835–39.
15. Maina JN. Development, structure, and function of a novel respiratory organ, the lung-air sac system of birds: to go where no other vertebrate has gone. *Biol Rev Camb Philos Soc.* (2006) 81:545–79. doi: 10.1017/S1464793106007111
16. Casteleyn C, Doom M, Lambrechts E, Van den Broeck W, Simoens P, Cornillie P. Locations of gut-associated lymphoid tissue in the 3-month-old chicken: a review. *Avian Pathol.* (2010) 39:143–50. doi: 10.1080/03079451003786105922993774
17. Befus AD, Johnston N, Leslie GA, Bienenstock J. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J Immunol.* (1980) 125:2626–32.
18. Kitagawa H, Hiratsuka Y, Imagawa T, Uehara M. Distribution of lymphoid tissue in the caecal mucosa of chickens. *J Anat.*(1998) 192(Pt. 2): 293–8.
19. Bar-Shira E, Sklan D, Friedman A. Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Dev Comp Immunol.* (2003) 27:147–57.
20. Ryan EJ, Daly LM, Mills KH. Immunomodulators and delivery systems for vaccination by mucosal routes. *Trends Biotechnol.* (2001) 19:293–304.
21. van Ginkel FW, Nguyen HH, McGhee JR. Vaccines for mucosal immunity to combat emerging infectious diseases. *Emerg Infect Dis.* (2000) 6:123–32. doi: 10.3201/eid0602.000204
22. De Wit JJ, Swart WA, Fabri TH. Efficacy of infectious bronchitis virus vaccinations in the field: association between the alpha-IBV IgM response, protection and vaccine application parameters. *Avian Pathol.* (2010) 39:123–31. doi: 10.1080/03079451003604639921016407
23. Hayter RB, Besch EL. Airborne-particle deposition in the respiratory tract of chickens. *Poult Sci.* (1974) 53:1507–11. doi: 10.3382/ps.0531507
24. Tell LA, Smiley-Jewell S, Hinds D, Stephens KE, Teague SV, Plopper CG, et al. An aerosolized fluorescent microsphere technique for evaluating particle deposition in the avian respiratory tract. *Avian Dis.* (2006) 50:238–44. doi: 10.1637/7427-082605R.1
25. Corbanie EA, Matthijs MG, van Eck JH, Remon JP, Landman WJ, Vervaet C. Deposition of differently sized airborne microspheres in the respiratory tract of chickens. *Avian Pathol.* (2006) 35:475–85. doi: 10.1080/03079450601028845
26. de Geus ED, Jansen CA, Vervelde L. Uptake of particulate antigens in a nonmammalian lung: phenotypic and functional characterization of avian respiratory phagocytes using bacterial or viral antigens. *J Immunol.* (2012) 188:4516–26. doi: 10.4049/jimmunol.1200092jimmunol.1200092
27. Eeckhaut V, Haesebrouck F, Ducatelle R, Van Immerseel F. Oral vaccination with a live *Salmonella Enteritidis*/Typhimurium bivalent vaccine in layers induces cross-protection against caecal and internal organ colonization by a Salmonella Infantis strain. *Vet Microbiol.* (2018) 218:7–12. doi: 10.1016/j.vetmic.2018.03.022
28. Leigh SA, Evans JD, Collier SD, Branton SL. The impact of vaccination route on *Mycoplasma gallisepticum* vaccine efficacy. *Poult Sci.* (2018) doi: 10.3382/ps/pey1884999283. [Epub ahead of print].
29. Meeusen EN, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. *Clin Microbiol Rev.* (2007) 20:489–510. doi: 10.1128/CMR.00005-07
30. Gupta SK, Deb R, Dey S, Chellappa MM. Toll-like receptor-based adjuvants: enhancing the immune response to vaccines against infectious diseases of chicken. *Expert Rev Vaccines* (2014) 13:909–25. doi: 10.1586/14760584.2014.920236
31. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol.* (2001) 2:675–80. doi: 10.1038/9060990609
32. Linghua Z, Xingshan T, Fengzhen Z. Vaccination with Newcastle disease vaccine and CpG oligodeoxynucleotides induces specific immunity and protection against Newcastle disease virus in SPF chicken. *Vet Immunol Immunopathol.* (2007) 115:216–22. doi: 10.1016/j.vetimm.2006.10.017
33. Dar A, Potter A, Tikoo S, Gerdts V, Lai K, Babiuk LA, et al. CpG oligodeoxynucleotides activate innate immune response that suppresses infectious bronchitis virus replication in chicken embryos. *Avian Dis.* (2009) 53:261–7. doi: 10.1637/8560-121808-Reg.1
34. Parvizi P, Mallick AI, Haq K, Haghighi HR, Orouji S, Thanthrige-Don N, et al. A toll-like receptor 3 ligand enhances protective effects of vaccination against Marek's disease virus and hinders tumor development in chickens. *Viral Immunol.* (2012) 25:394–401. doi: 10.1089/vim.2012.0033
35. He H, Genovese KJ, Swaggerty CL, Nisbet DJ, Kogut MH. *In vivo* priming heterophil innate immune functions and increasing resistance to Salmonella enteritidis infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides. *Vet Immunol Immunopathol.* (2007) 117:275–83. doi: 10.1016/j.vetimm.2007.03.002
36. Gomis S, Babiuk L, Allan B, Willson P, Waters E, Hecker R, et al. Protection of chickens against a lethal challenge of Escherichia coli by a vaccine containing CpG oligodeoxynucleotides as an adjuvant. *Avian Dis.* (2007) 51:78–83. doi: 10.1637/0005-2086(2007).051[0078:POCAAL]2.0.CO;2
37. Chou WK, Chen CH, Vuong CN, Abi-Ghanem D, Waghela SD, Mwangi W, et al. Significant mucosal sIgA production after a single oral or parenteral administration using *in vivo* CD40 targeting in the chicken. *Res Vet Sci.* (2016) 108:112–5. doi: 10.1016/j.rvsc.2016.08.013S0034-5288(16)30257-0
38. Tang Y, Lu J, Wu P, Liu Z, Tian Z, Zha G, et al. Inactivated vaccine with adjuvants consisting of pattern recognition receptor agonists confers protection against avian influenza viruses in chickens. *Vet Microbiol.* (2014) 172:120–8. doi: 10.1016/j.vetmic.2014.05.007S0378-1135(14)00243-0
39. Lu J, Wu P, Zhang X, Feng L, Dong B, Chu X, et al. Immunopotentiators improve the efficacy of oil-emulsion-inactivated avian influenza vaccine in chickens, ducks and geese. *PLoS ONE* (2016) 11:e0156573. doi: 10.1371/journal.pone.0156573PONE-D-15-27158
40. O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov.* (2003) 2:727–35. doi: 10.1038/nrd1176nrd1176

41. Rauw F, Gardin Y, Palya V, Anbari S, Gonze M, Lemaire S, et al. The positive adjuvant effect of chitosan on antigen-specific cell-mediated immunity after chickens vaccination with live Newcastle disease vaccine. *Vet Immunol Immunopathol.* (2010) 134:249–58. doi: 10.1016/j.vetimm.2009.10.028S0165-2427(09)00354-7
42. Men Y, Audran R, Thomasin C, Eberl G, Demotz S, Merkle HP, et al. MHC class I- and class II-restricted processing and presentation of microencapsulated antigens. *Vaccine* (1999) 17:1047–56.
43. Singh SM, Alkie TN, Nagy E, Kulkarni RR, Hodgins DC, Sharif S. Delivery of an inactivated avian influenza virus vaccine adjuvanted with poly(D,L-lactic-co-glycolic acid) encapsulated CpG ODN induces protective immune responses in chickens. *Vaccine* (2016) 34:4807–13. doi: 10.1016/j.vaccine.2016.08.009S0264-410X(16)30666-1
44. Zhao K, Rong G, Hao Y, Yu L, Kang H, Wang X, et al. IgA response and protection following nasal vaccination of chickens with Newcastle disease virus DNA vaccine nanoencapsulated with Ag@SiO2 hollow nanoparticles. *Sci Rep.* (2016) 6:25720. doi: 10.1038/srep25720srep25720
45. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol.* (2006) 6:148–58. doi: 10.1038/nri1777
46. Schiavi E, Smolinska S, O'Mahony L. Intestinal dendritic cells. *Curr Opin Gastroenterol.* (2015) 31:98–103. doi: 10.1097/MOG.0000000000000015500001574-201503000-00004
47. Staines K, Young JR, Butter C. Expression of chicken DEC205 reflects the unique structure and function of the avian immune system. *PLoS ONE* (2013) 8:e51799. doi: 10.1371/journal.pone.0051799PONE-D-11-21674
48. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* (2005) 307:254–8. doi: 10.1126/science.1102901
49. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* (2012) 483:345–9. doi: 10.1038/nature10863nature10863
50. Owen JL, Sahay B. Mohamadzadeh M. New generation of oral mucosal vaccines targeting dendritic cells. *Curr Opin Chem Biol.* (2013) 17:918–24. doi: 10.1016/j.cbpa.2013.06.013S1367-5931(13)00115-4
51. Curiel TJ, Morris C, Brumlik M, Landry SJ, Finstad K, Nelson A, et al. Peptides identified through phage display direct immunogenic antigen to dendritic cells. *J Immunol.* (2004) 172:7425–31. doi: 10.4049/jimmunol.172.12.7425
52. Mohamadzadeh M, Durmaz E, Zadeh M, Pakanati KC, Gramarossa M, Cohran V, et al. Targeted expression of anthrax protective antigen by Lactobacillus gasseri as an anthrax vaccine. *Future Microbiol.* (2010) 5:1289–96. doi: 10.2217/fmb.10.78
53. Nochi T, Kiyono H. Innate immunity in the mucosal immune system. *Curr Pharm Des.* (2006) 12:4203–13.
54. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol.* (1965) 13:238–52.
55. Bernasconi V, Norling K, Bally M, Hook F, Lycke NY. Mucosal vaccine development based on liposome technology. *J Immunol. Res.* (2016) 2016:5482087. doi: 10.1155/2016/5482087
56. Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv Drug Deliv Rev.* (2016) 99:28–51. doi: 10.1016/j.addr.2015.09.012
57. Mura S, Nicolas J, Couvreur P. Stimuli-responsive nanocarriers for drug delivery. *Nat. Mater* (2013) 12:991–1003. doi: 10.1038/nmat3776nmat3776
58. Ghaffar KA, Marasini N, Giddam AK, Batzloff MR, Good MF, Skwarczynski M, et al. Liposome-based intranasal delivery of lipopeptide vaccine candidates against group A streptococcus. *Acta Biomater.* (2016) 41:161–8. doi: 10.1016/j.actbio.2016.04.012S1742-7061(16)30165-9
59. Tada R, Muto S, Iwata T, Hidaka A, Kiyono H, Kunisawa J, et al. Attachment of class B CpG ODN onto DOTAP/DC-chol liposome in nasal vaccine formulations augments antigen-specific immune responses in mice. *BMC Res Notes* (2017) 10:68. doi: 10.1186/s13104-017-2380-810.1186/s13104-017-2380-8
60. Terech P, Weiss RG. Low molecular mass gelators of organic liquids and the properties of their gels. *Chem Rev.* (1997) 97:3133–60.
61. Akiyoshi K, Deguchi S, Moriguchi N, Yamaguchi S, Sunamoto J. Self-aggregates of hydrophobized polysaccharides in water. Formation and characteristics of nanoparticles.. *Macromolecules* (1993) 26:3062–8. doi: 10.1021/ma00064a011
62. Nishikawa T, Akiyoshi K, Sunamoto J. Macromolecular complexation between bovine serum albumin and the self-assembled hydrogel nanoparticle of hydrophobized polysaccharides. *J Am Chem Soc.* (1996) 118:6110–5. doi: 10.1021/ja953843c
63. Akiyoshi K, Nagai K, Nishikawa T, Sunamoto J. Self-aggregates of Hydrophobized Polysaccharide as a Host for Macromolecular Guests. *Chem Lett.* (1992) 21:1727–30. doi: 10.1158/1078-0432.CCR-06-1546
64. Akiyoshi K, Kobayashi S, Shichibe S, Mix D, Baudys M, Kim SW, et al. Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: complexation and stabilization of insulin. *J Control Release* (1998) 54:313–20.
65. Nishikawa T, Akiyoshi K, Sunamoto J. Supramolecular assembly between nanoparticles of hydrophobized polysaccharide and soluble protein complexation between the self-aggregate of cholesterol-bearing pullulan and alpha-chymotrypsin. *Macromolecules* (1994) 27:7654–9. doi: 10.1006/jmbi.1998.1881
66. Ayame H, Morimoto N, Akiyoshi K. Self-assembled cationic nanogels for intracellular protein delivery. *Bioconjug Chem.* (2008) 19:882–90. doi: 10.1021/bc700422s
67. Nochi T, Yuki Y, Takahashi H, Sawada S, Mejima M, Kohda T, et al. Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. *Nat Mater.* (2010) 9:572–8. doi: 10.1038/nmat2784nmat2784
68. Kong IG, Sato A, Yuki Y, Nochi T, Takahashi H, Sawada S, et al. Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by *Streptococcus pneumoniae*. *Infect Immun.* (2013) 81:1625–34. doi: 10.1128/IAI.00240-13IAI.00240-13
69. Fukuyama Y, Yuki Y, Katakai Y, Harada N, Takahashi H, Takeda S, et al. Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against Streptococcus pneumoniae in macaques. *Mucosal Immunol.* (2015) 8:1144–53. doi: 10.1038/mi.2015.5mi20155
70. Wells J. Mucosal vaccination and therapy with genetically modified lactic acid bacteria. *Annu Rev Food Sci Technol.* (2011) 2:423–45. doi: 10.1146/annurev-food-022510-133640
71. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
72. Kostadinova AI, Pablos-Tanarro A, Diks MAP, van Esch B, Garssen J, Knippels LMJ, et al. Dietary intervention with beta-lactoglobulin-derived peptides and a specific mixture of fructo-oligosaccharides and bifidobacterium breve M-16V facilitates the prevention of whey-induced allergy in mice by supporting a tolerance-prone immune environment. *Front Immunol.* (2017) 8:1303. doi: 10.3389/fimmu.2017.01303
73. Bollrath J, Powrie F. Immunology. feed your tregs more fiber. *Science* (2013) 341:463–4. doi: 10.1126/science.1242674341/6145/463
74. Fan J, Meng Q, Guo G, Xie Y, Li X, Xiu Y, et al. (2010). Effects of early enteral nutrition supplemented with arginine on intestinal mucosal immunity in severely burned mice. *Clin. Nutr.* (2013) 29:124–30. doi: 10.1016/j.clnu.2009.07.005
75. Zhang B, Lv Z, Li H, Guo S, Liu D, Guo Y. Dietary l-arginine inhibits intestinal Clostridium perfringens colonisation and attenuates intestinal mucosal injury in broiler chickens. *Br J. Nutr.* (2017) 118:321–32. doi: 10.1017/S0007114517002094
76. Tan J, Applegate TJ, Liu S, Guo Y, Eicher SD. Supplemental dietary L-arginine attenuates intestinal mucosal disruption during a coccidial vaccine challenge in broiler chickens. *Br J Nutr.* (2014) 112:1098–109. doi: 10.1017/S000711451400184600071145140001846
77. Asgari F, Madjd Z, Falak R, Bahar MA, Nasrabadi MH, Raiani M, et al. Probiotic feeding affects T cell populations in blood and lymphoid

organs in chickens. *Benef Microbes* (2016) 7:669–75. doi: 10.3920/BM2016.0014

78. Rajput IR, Ying H, Yajing S, Arain MA, Weifen L, Ping L, et al. *Saccharomyces boulardii* and *Bacillus subtilis B10* modulate TLRs and cytokines expression patterns in jejunum and ileum of broilers. *PLoS ONE* (2017) 12:e0173917. doi: 10.1371/journal.pone.0173917PONE-D-16-49094
79. Wieten L, van der Zee R, Spiering R, Wagenaar-Hilbers J, van Kooten P, Broere F, et al. A novel heat-shock protein coinducer boosts stress protein Hsp70 to activate T cell regulation of inflammation in autoimmune arthritis. *Arthritis Rheum.* (2010). 62:1026–35. doi: 10.1002/art.27344

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 August 2018
doi: 10.3389/fimmu.2018.01824

Therapeutic Effect of Intestinal Autochthonous *Lactobacillus reuteri* P16 Against Waterborne Lead Toxicity in *Cyprinus carpio*

Sib Sankar Giri[1,2‡], Saekil Yun[1‡], Jin Woo Jun[1], Hyoun Joong Kim[1], Sang Guen Kim[1], Jeong Woo Kang[1], Sang Wha Kim[1], Se Jin Han[1], V. Sukumaran[2†] and Se Chang Park[1*]*

[1] Laboratory of Aquatic Biomedicine, College of Veterinary Medicine, Research Institute for Veterinary Science, Seoul National University, Seoul, South Korea, [2] Department of Biotechnology, Periyar Maniammai University, Thanjavur, India

OPEN ACCESS

Edited by:
Tomonori Nochi,
Tohoku University, Japan

Reviewed by:
Zhenquan Jia,
University of North Carolina at Greensboro, United States
Wei Chen,
Jiangnan University, China

***Correspondence:**
V. Sukumaran
drvsukumar@gmail.com;
Se Chang Park
parksec@snu.ac.kr

†Present address:
V. Sukumaran,
Department of Zoology, Kundavai Nachiyar Government Arts College for Women (Autonomous), Thanjavur, Tamil Nadu, India

‡These authors have contributed equally to this work.

Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: 29 December 2017
Accepted: 24 July 2018
Published: 07 August 2018

Citation:
Giri SS, Yun S, Jun JW, Kim HJ, Kim SG, Kang JW, Kim SW, Han SJ, Sukumaran V and Park SC (2018) Therapeutic Effect of Intestinal Autochthonous Lactobacillus reuteri P16 Against Waterborne Lead Toxicity in Cyprinus carpio. Front. Immunol. 9:1824. doi: 10.3389/fimmu.2018.01824

Harmful effects of heavy metals are myriad. Lead (Pb) from soil and atmosphere contaminates water bodies and affects the aquatic animals. Our previous study confirmed the *in vitro* probiotic potential of *Lactobacillus reuteri* against Pb toxicity, but further investigation is necessary for gaining insights into the related protection mode. Therefore, in this study, we investigated the protective effects of the potential probiotic *L. reuteri* P16 against waterborne Pb exposure-induced toxicity in the freshwater fish *Cyprinus carpio*. Fish (average weight: 23.16 ± 0.73 g) were allocated to four groups (control, Pb only, Pb + *L. reuteri* P16, and *L. reuteri* P16 only) and Pb groups were exposed to waterborne Pb (1 mg L^{-1}) for 6 weeks. *L. reuteri* P16 (10^8 CFU g^{-1}) supplemented diet was provided twice daily. Growth performances, hemato-biochemical parameters, innate immune responses, intestinal microbiota, and Pb accumulation in tissues were measured at the end of the trial. When the fish were exposed to Pb, dietary supplementation of *L. reuteri* P16 effectively decreased mortality and accumulation of Pb in tissues, and improved the growth performance. Co-treatment with Pb and *L. reuteri* P16 alleviated Pb exposure-induced oxidative stress, reversed alterations in hemato-biochemical parameters, improved innate immune parameters, and restored intestinal enzymatic activities. Moreover, *L. reuteri* P16 supplementation reversed the changes in intestinal microbiota in Pb-exposed fish. Furthermore, Pb exposure decreased the expressions of pro-inflammatory cytokines (TNF-α, IL-1β). However, the expression of heat shock proteins (HSP70 and HSP90) increased, which might have increased the cellular stress. Interestingly, the Pb-induced alterations of gene expressions were reversed by *L. reuteri* P16 supplementation. Thus, dietary administration of the potential probiotic *L. reuteri* P16 had several beneficial effects on growth performance and immune responses, decreased Pb accumulation in tissues, and reversed alterations in hematological responses of *C. carpio*. Furthermore, it offered direct protection against Pb-induced oxidative stress. Therefore, *L. reuteri* P16 may be a novel dietary supplement for enhancing growth performance and preventing Pb-exposure-induced toxicity in fish in aquaculture and aquatic products.

Keywords: probiotics, lactic acid bacteria, Pb, common carp, oxidative stress, immune parameters, gene expression

INTRODUCTION

In recent years, contamination of aquatic environments by heavy metals is increasing rapidly. These heavy metals have caused lot of problems due to their toxicity, and it poses threat to both aquaculture and food safety (1). The natural aquatic systems are being extensively contaminated with heavy metals released from industrial and other anthropogenic activities. Among the various heavy metals, lead (Pb) is one of the most toxic and occupies the second place in Priority List of Hazardous Substances (2). Due to higher levels in the food web, fish can accumulate many metals, and the accumulation patterns in fish rely on uptake and elimination rates (3). Pb accumulation in fish tissues depends on the type of metal, exposure duration, and concentration, salinity, temperature, water quality, fish species, and metabolic activity of fish (4). Pb is mutagenic and teratogenic in humans and animals (5). Prolonged Pb exposure can produce adverse effects on pituitary function, gonadosomatic index, and oocyte growth, chromosomal aberrations, and causes neurological disorders, DNA damage, and scoliosis in freshwater fish (6–8). Furthermore, Pb showed deleterious effects on gut-associated lymphoid tissues of *Channa punctatus* (8). In addition to economic loss to aquaculture, heavy metal contamination in aquaculture poses great risks to human health because it can accumulate and enter the food chain (9). Therefore, eco-friendly and affordable strategies of controlling heavy metal (Pb) contamination in aquaculture is increasing in importance.

Until date, no treatment method for chronic Pb poisoning has been developed. Although chelation therapy has been used to promote heavy metal excretion, chelators for Pb toxicity have been reported to be deficient in terms of safety and efficacy (10). Gut microbiota of fish play important roles in metabolism, immunity, and pathogen resistance (11). Allochthonous (transient) and autochthonous (adherent) microbes normally reside in gastrointestinal (GI) tract of fish, and the latter type has been well investigated for their numerous beneficial effects on the host (11). Colonization of the GI tract of fish larvae completes within few hours after hatching. The colonization by normal or protective microbes prevents colonization by potential invaders and maintains the overall health of fish (12). Lactic acid bacteria (LAB) are classified as "generally regarded as safe" and have been widely used in aquaculture (13). LAB with potential heavy metal adsorption capacity have been isolated and identified earlier (14–16). They remove heavy metals *in vivo* and *in vitro* (14, 17, 18). Recently, Yu et al. (13) demonstrated that dietary *Lactobacillus plantarum* could alleviates the waterborne aluminum toxicity in tilapia. In another study, Zhai et al. (14) demonstrated that dietary probiotic *L. plantarum* CCFM8610 has potential to prevent cadmium-exposure induced problems in tilapia aquaculture. Therefore, LAB could be exploited further for their possible role in alleviating heavy metal toxicity in aquaculture.

Immunostimulants and other biological factors, such as probiotics, can trigger the defense system, even under stressful conditions, thereby reducing the deleterious effects caused by various biological, chemical, and physiological stresses (19). Until date, numerous studies have demonstrated that probiotics can stimulate immunity in teleosts both under *in vivo* and *in vitro* conditions. Available literature indicates that several probiotics either individually or in combination can enhance both systemic and local immunity in fish (20, 21). Probiotic activities of LAB and other bacteria isolated from the GI tract of fish have been demonstrated in several studies (22–25). However, protective effect of those autochthonous probiotic bacteria against heavy metal-induced toxicity in fish has not been reported yet. Various microorganisms have been studied and strategically used for bioremediation of water polluted with heavy metals (26, 27). Recently, it has been found that LAB, such as *L. plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, and *Bifidobacterium lactis* can bind and remove heavy metals *in vitro* from water and environment (15, 27). Yi et al. (17) demonstrated that *Leuconostoc mesenteroides* isolated from fermented kimchi has strong lead resistance and removal capacity. It has been reported that dietary supplementation of *L. plantarum* CCFM8611 (at 10^8 CFU g^{-1}) isolated from environmental samples alleviated Pb-induced oxidative stress, reversed digestive enzyme activities and innate immune status, and ameliorated the growth performance in Pb-induced fish (18). Hematological parameters are reliable indicators of physiological status of aquatic animals under stress due to metal exposure.

Recently, we have isolated Pb-resistant LAB from the gut content of carp *Cyprinus carpio* (28). Among those isolates, *L. reuteri* P16 was found to exhibit strong Pb-binding and tolerance abilities. *L. reuteri* P16 exhibited various probiotic properties, such as a high level of tolerance to both acid (pH 2.0 and 3.0) and bile (0.3% Oxgall) exposure, good adhesion to intestinal mucosa, strong inhibition of fish-pathogen growth *in vitro*, and susceptibility to several clinically effective antibiotics (28). Furthermore, "intact cells" and cell-free supernatants of P16 had stronger antioxidant abilities. Those results indicated that it is important to investigate efficacy of *L. reuteri* P16 in providing protection against toxicity caused by Pb-exposure in aquaculture. *C. carpio* is an important aquaculture species worldwide. It is the third most widely cultivated and commercially important freshwater fish species in the world. In addition, it is recognized as a good biological model because it is easy to handle, culture, and maintain in the laboratory. Therefore, this study was aimed to investigate the effect of dietary supplementation of intestinal autochthonous *L. reuteri* P16 on the growth performance, hematological, and blood biochemical parameters, and intestinal enzymatic activities of the carp *C. carpio* exposed to waterborne Pb. In addition, effects of P16 on cytokine gene expression, and Pb accumulation in tissues of carp were investigated.

MATERIALS AND METHODS

Potential Probiotic Strain

Lactobacillus reuteri P16 isolated from the gut content of *C. carpio*, exhibited *in vitro* probiotic properties, and excellent Pb-binding and Pb-tolerance capacities (28). This strain was cultured in de Man, Rosaga, and Sharpe (MRS) broth for 24 h at 37°C.

Preparation of Diet for Fish

The basal diet containing 28.8% protein, 7.16% lipid, and 12.36% ash was prepared as described in a previous study (29). The culture broth was centrifuged at 8,000 × *g* for 15 min at 4°C to precipitate the cells. The initial bacterial concentration (1.32×10^9 cells mL^{-1}) was diluted to the desired concentration using the phosphate buffer saline (pH 7.4). P16 was added to the basal diet at a final concentration of 1×10^8 CFU g^{-1} (approximately). Based on a preliminary study, P16 concentration was chosen as 1×10^8 CFU g^{-1} (unpublished data). The number of viable bacterial cells in the diet was determined *via* plate counting on MRS agar plates. The ingredients were blended thoroughly into a mixture that and then pelleted, air-dried, ground, and sieved into appropriate pellet sizes. The experimental feed was prepared weekly and stored at 4°C.

Experimental Design

Cyprinus carpio (average body length: 11.2 ± 0.8 cm; average weight: 23.16 ± 0.73 g; approximate age: 105 days) obtained from a local fish farm were acclimatized to laboratory conditions in 500 L quarantine tanks for 2 weeks at 24 ± 2°C and fed a basal diet (29). Approximately 20% of the water in all the tanks was replaced daily, and 100% of the water was replaced weekly. Basic physicochemical parameters of the water were monitored weekly; oxygen and ammonia concentrations were 6.1–7.3 and 0.03–0.06 mg L^{-1}, respectively, and pH ranged from 7.0 to 7.6. This study was conducted in accordance with the "Guidelines on the Regulation of Scientific Experiments on Animals," CPCSEA (http://cpcsea.nic.in), Govt. of India and the experimental protocols were approved by the bioethical committee of the Periyar Maniammai University (PMU/Biotech/10.01.2009).

In total, 180 fish were randomly distributed into the four experimental groups. In each independent experimental group, 45 fish were divided into three tanks (15 fish per tank), and each tank held 200 L water. The following groups were maintained:

I. Control group: the fishes were fed with basal diet and kept in Pb-free water.
II. Pb-only group: the fishes were exposed to waterborne Pb (1 mg L^{-1}) and fed with the basal diet.
III. Pb + P16 group: the fishes were exposed to waterborne Pb and fed on diet supplemented with *L. reuteri* P16.
IV. P16 group: the fishes were fed on diet containing 10^8 CFU g^{-1} of *L. reuteri* P16, and kept in Pb-free water.

The experimental feeding period selected was 6 weeks. The sublethal Pb dose (1 mg L^{-1}) was chosen based on earlier reports (14, 18). The fishes were fed experimental diets at 3–5% of body weight two times daily (09:00 and 18:00 h). For Pb-treated groups, water was exchanged with Pb-containing water every 2 days to maintain a constant Pb level. For other groups, 20% of the water was exchanged daily, and 100% of the water was exchanged once in a week. Water samples were collected daily for Pb level determination using a flame atomic absorption spectrophotometer.

Growth Performance and Sample Collection

The fishes were weighed at the beginning and end of the 6-week feeding trial. Growth performance [i.e., percent weight gain (PWG; grams per fish), specific growth rate (SGR), and feed conversion ratio (FCR)] and survival rates of fish were calculated using the following formula:

$$\mathrm{PWG} = (W_t - W_0 / W_0) \times 100\%$$

$$\mathrm{SGR} = [(\ln W_t - \ln W_0) / t] \times 100\%$$

$$\mathrm{FCR} = \mathrm{FI} / (W_t - W_0)$$

where, W_t and W_0 are the final and initial weight of the fishes, respectively, t is the duration of feeding (in days); and FI is the feed intake.

At the end of the feeding trial, the fish were starved for 24 h before sampling. Ten fish were collected from each tank (i.e., 10 fish × 3 tanks = 30 fish per group) and euthanized with 200 mg L^{-1} of MS-222 (Ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, St. Louis, MO, USA). Immediately, blood samples were collected from the caudal vein using a 2-mL syringe. Pooled blood samples were divided into two aliquots: one was centrifuged (4,000 × *g*, 10 min, 4°C) to collect serum and the other was stored in anti-coagulative tubes (EDTA-2K).

Tissue samples from gills, spleen, liver, brain, and kidney were collected from those fishes and stored in metal-free Eppendorf tubes at −20°C. Furthermore, intestinal samples from nine fish per group (3 fish × 3 tanks = 9 fish) were used for the quantification of intestinal microbiota.

Hematological Parameters

Hematological parameters, including white blood cell (WBC) count, red blood cell (RBC) count, hematocrit value (Hct, %), and hemoglobin (Hb) level, were measured using standard techniques. Serum total protein was estimated using a commercial kit (Abcam, India). Cholesterol level was measured calorimetrically using a commercial kit (Sigma-Aldrich, USA).

Blood Biochemical Parameters

Malondialdehyde (MDA) content was measured *via* barbituric acid reaction chronometry (30). Total myeloperoxidase (MPO) content in blood serum was measured using a commercial kit (Abcam, USA). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities were determined using the Hitachi 912 clinical chemistry automatic analyzer. Superoxide dismutase (SOD) activity was determined with an enzymatic assay method using a commercial kit (Randox, Crumlin, UK). Blood creatinine level was measured using commercial assay kit (Sigma-Aldrich, USA). Glutathione peroxidase (GPx) was measured with an enzymatic assay using commercial kit (Abcam, USA). Serum lysozyme activity was measured with commercial assay kit (Sigma-Aldrich). Leukocyte phagocytic activity was measured according to the method described by Dotta et al. (31).

Intestinal Enzymatic Activities and Quantification of Intestinal Microbiotia

For this experiment, whole intestines from six fish of each group were sampled aseptically and rinsed with distilled water. Intestines were homogenized and the activity of digestive enzymes was determined. Amylase and protease activities were measured as described by Pavasovic et al. (32), and lipase activity was evaluated by using a commercial kit (Sigma-Aldrich).

The entire intestinal tract was removed aseptically ($n = 9$) from the sampled fish, washed thoroughly with sterile saline (0.85% NaCl), and homogenized (Potter–Elvehjem Tissue Homogenizer, NW Kennesaw, GA, USA) to isolate the intestinal microbiological communities (33). The homogenate was serially diluted to 10^{-7} with sterile saline. Dilutions (100 μL) were spread in triplicate onto plate count agar (PCA) and MRS agar plates. MRS cultures were incubated anaerobically (5% CO_2) at 37°C for 72 h, while PCA cultures were incubated at 37°C for 24–72 h, aerobically, for determining the total count. The viability was recorded as colony-forming units (CFU) per milliliter and cell concentration was expressed as log (CFU mL^{-1}).

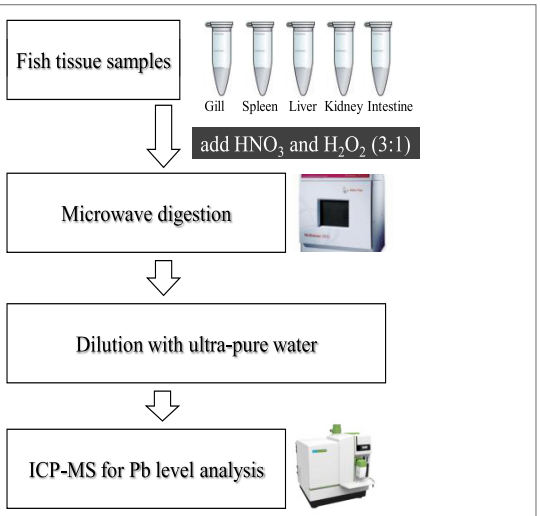


FIGURE 1 | Transfer system of tissue samples for the determination of the Pb level.

Determination of Pb Level in Tissues

Tissue samples were transferred to metal-free vessels and digested in concentrated HNO_3 and H_2O_2 (3:1; v/v) using a microwave digestion system (Microwave 3000; Anton Paar GmbH, Austria) (14). The Pb concentrations in all the tissue samples were determined using atomic absorption spectrophotometer (PerkinElmer, USA) (**Figure 1**).

Immune Gene Expression Analysis

Head-kidney were dissected from six fish per group after they were anesthetized with MS-222, and total RNA was isolated from those tissues using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction analysis of IL-1β, TNF-α, HSP70, HSP90, and the housekeeping gene β-actin were carried out following standard protocols (29). The primer sequences and thermo cycling conditions are shown in **Table 1**. After amplification, melting curve analysis was performed to verify the accuracy of each amplicon. All the samples were run in parallel with the β-actin to normalize cDNA loading. Gene expression results were analyzed using the $2^{-\Delta\Delta CT}$ method after verification that the primers amplified with an efficiency of approximately 100% (34).

Statistical Analyses

Independent data were obtained and statistical significance testing were performed. The homogeneity of variance was checked by analysis of variance, and chi-square test was used to analyze the data. Multiple comparisons were performed with Tukey's test to analyze the differences between independent experimental groups and treatments. All statistical analyses were performed using the OriginPro software (version 8; OriginLab Corporation, Northampton, MA, USA). Significance level was set at $P < 0.05$.

RESULTS

Growth Performance

Sublethal exposure to waterborne Pb resulted in the inhibition of growth performance and increased the mortality of *C. carpio* (**Table 2**). Survival in the Pb-exposed group was 91.1%, but that in other groups was 100% and this shown by plotting Kaplan–Meier survival curve (**Figure 2**). Typical symptoms include spinal deformity and blackening of the caudal region were observed

TABLE 1 | Real-time primer sequences and thermocycling conditions.

Target gene	Primer sequence (5′–3′)	Thermocycling conditions	Reference/accession no.
TNF-α	CTCAACAAGTCTCAGAACAATCAGG TCCTGGTTCCTTCTCCAATCTAGCT	95°C 30 s, 40 cycles of 95°C 5 s, 61.1°C 30 s, and 72°C 30 s	(19)
IL-1β	ATCTTGGAGAATGTGATCGAAGAG GATACGTTTTTGATCCTCAAGTGTGAAG	95°C 30 s, 40 cycles of 95°C 5 s, 61.5°C 30 s, and 72°C 30 s	(19)
HSP70	GGC AGA AAG TTT GAT GAC CCA GCA ATC TCC TTC ATA TTC ACC	95°C 30 s, 40 cycles of 95°C 5 s, 61.1°C 30 s, and 72°C 30 s	(15)
HSP90	GGAAATCTTCCTCCGAGAGC CCGAATTGACCGATCATAGA	95°C 30 s, 40 cycles of 95°C 5 s, 61.1°C 30 s, and 72°C 30 s	(28)
β-actin	GACTTCGAGCAGGAGATGG CAAGAAGGATGGCTGGAACA	95°C 30 s, 40 cycles of 95°C 5 s, 62.4°C 30 s, and 72°C 30 s	(19)

TABLE 2 | Effect of dietary supplementation on growth performance of *Cyprinus carpio* during 6-week trial.

Group	Initial weight (g)	Final weight (g)	PWG (%)	SGR (%)	FCR	Survival (%)
Control	23.26 ± 0.38[a]	32.86 ± 1.07[a]	37.61 ± 1.26[a]	0.90 ± 0.2[a]	2.04 ± 0.08[a]	100
Pb only	23.21 ± 0.71[a]	29.73 ± 1.14[b]	30.11 ± 0.82[b]	0.71 ± 0.03[b]	2.47 ± 0.13[b]	91.1
Pb + P16	23.14 ± 0.63[a]	30.91 ± 1.52[b]	33.26 ± 0.64[b]	0.83 ± 0.07[b]	2.28 ± 0.13[c]	100
P16 only	23.18 ± 0.52[a]	36.0.8 ± 0.96[c]	54.76 ± 1.77[c]	1.22 ± 0.6[c]	1.84 ± 0.6[d]	100

PWG, percent weight gain; SGR, specific growth rate; FCR, feed conversion ratio.
Values in the same column with different superscript small letters are significantly different ($P < 0.05$).
Values are presented as mean ± SD (n = 45 fish in each group).

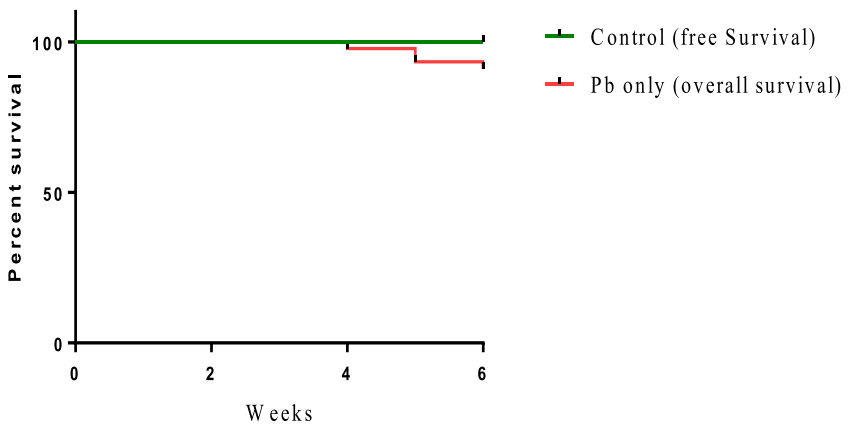


FIGURE 2 | Kaplan–Meier survival curve [cumulative survival (%) over time (weeks)] of *Cyprinus carpio* exposed waterborne Pb.

TABLE 3 | Effect of *Lactobacillus reuteri* P16 supplementation on the hematological parameters of *Cyprinus carpio*.

Group	Parameters					
	RBC ($\times 10^6$ mm^{-3})	WBC ($\times 10^3$ mm^{-3})	Hct (%)	Hb (g dL^{-1})	Total protein (g dL^{-1})	Cholesterol (mg dL^{-1})
Control	1.69 ± 0.07[ac]	4.21 ± 0.13[a]	24.03 ± 0.83[a]	5.37 ± 0.13[a]	2.87 ± 0.04[a]	58.06 ± 2.8[a]
Pb only	1.53 ± 0.08[b]	3.48 ± 0.09[b]	19.86 ± 1.07[b]	3.89 ± 0.11[b]	2.41 ± 0.07[b]	63.57 ± 2.4[b]
Pb + P16	1.58 ± 0.05[ba]	3.96 ± 0.14[ab]	21.92 ± 0.74[b]	4.92 ± 0.08[a]	2.78 ± 0.06[ba]	61.19 ± 3.3[ba]
P16 only	1.76 ± 0.09[c]	4.54 ± 0.11[a]	25.13 ± 1.28[a]	6.08 ± 0.14[c]	3.62 ± 0.11[c]	57.88 ± 3.02[a]

RBC, red blood cell; WBC, white blood cell; Hct, hematocrit; Hb, hemoglobin.
Data are presented as mean ± SD (n = 30 fish from each group). Means in the same column with different superscript small letters are significantly different ($P < 0.05$).

in fish exposed to Pb only. Final weight gain (FWG), PWG, and SGR were significantly lowered in the Pb-only group than in the control group or the group fed with *L. reuteri* P16 alone. The FCR value was significantly higher in Pb-only group, but dietary supplementation with *L. reuteri* P16 decreased the FCR value, and it was the lowest in the group fed with *L. reuteri* P16 only. Supplementation with *L. reuteri* P16 prevented death caused by Pb exposure, as revealed by 100% survival in the Pb + P16 group. Growth performance (FWG, PWG, and SGR) in the Pb + P16 group was not significantly higher than that of the Pb-only group. However, administration of *L. reuteri* P16 alone significantly increased the FG, PWG, and SGR of the fishes, when compared with the other groups. FCR was the lowest in the P16 group (**Table 2**).

Hematological Parameters

Pb exposure significantly decreased the RBC, WBC, Hct, Hb, and total protein level in blood, when compared with those in the control (**Table 3**). However, decreases of those parameters were reversed by *L. reuteri* P16 supplementation. Levels of RBC, WBC, Hb, Hct, and total protein recovered in the Pb + P16 group, and the differences with the control group were not significant. Furthermore, blood cholesterol level, which increased significantly under Pb-exposure stress, was recovered by probiotic feeding (**Table 3**). However, supplementation with *L. reuteri* P16 alone had no significant effect on these parameters, except total protein level, which was significantly increased by probiotic supplementation.

Blood Biochemical Parameters

Among biochemical parameters, levels of AST (163.1 ± 4.7 U mL^{-1}), ALT (68.41 ± 2.12 U mL^{-1}), and creatinine (0.37 ± 0.07 U mL^{-1}) increased significantly in ($P < 0.05$) in Pb-only group, but these alterations were reversed to the normal level in Pb + P16 group (**Table 4**). However, feeding with *L. reuteri* P16 alone had no significant effect on those parameters, except AST level, which

TABLE 4 | Effect of *Lactobacillus reuteri* P16 supplementation on the blood biochemical parameters of *Cyprinus carpio*.

Group	Parameters				
	ALP (IU L^{-1})	AST (U mL^{-1})	ALT (U mL^{-1})	MPO (U L^{-1})	Creatinine (mg dL^{-1})
Control	21.38 ± 1.26[a]	104.3 ± 3.4[a]	32.06 ± 1.46[a]	34.68 ± 1.83[a]	0.26 ± 0.08[ac]
Pb only	17.82 ± 1.14[b]	163.1 ± 4.7[b]	68.41 ± 2.12[b]	29.57 ± 1.24[b]	0.37 ± 0.07[b]
Pb + P16	20.87 ± 0.83[a]	107.6 ± 2.8[a]	39.6 ± 1.38[c]	32.92 ± 1.67[a]	0.29 ± 0.08[a]
P16 only	22.04 ± 1.06[a]	98.9 ± 3.1[c]	31.33 ± 1.6[a]	37.14 ± 0.92[c]	0.24 ± 0.03[c]

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MPO, myeloperoxidase.
Values in the same column with different superscript small letters are significantly different ($P < 0.05$).
Values are presented as mean ± SD ($n = 30$).

decreased significantly in this group, as compared with that in the control. Furthermore, serum ALP and MPO levels, which decreased significantly in Pb-only group, were recovered in the Pb + P16 group, but these levels were slightly lower than those in the control group. However, *L. reuteri* P16 treatment alone significantly increased the MPO level than that in the control or other groups. In case of MPO level, *L. reuteri* P16 treatment alone had no significant effect on it.

Serum Oxidative Parameters

Malondialdehyde level was increased significantly ($P < 0.05$) in the Pb-only group, but *L. reuteri* P16 treatment reversed the MDA level to the normal range (**Figure 3**). Pb exposure reduced the level of GPx and SOD in fish significantly ($P < 0.05$), but levels of GPx and SOD recovered in the Pb + P16 group. However, dietary supplementation of *L. reuteri* P16 alone had no significant effect on those parameters, except SOD level, which increased significantly than that in the others.

Lysozyme and Phagocytic Activities

Pb exposure caused a profound decline in innate immune parameters, such as serum lysozyme and phagocytic activities (**Figure 4**). However, dietary administration of *L. reuteri* P16 helped in recovering lysozyme and phagocytic activities, as evident in the Pb + P16 group. Moreover, dietary administration of *L. reuteri* P16 only markedly increased ($P < 0.05$) lysozyme (27.5 U mL^{-1}) and phagocytic activities (18.68%), when compared with those in the control or any other group (**Figure 4**).

Intestinal Enzymatic Activities

Intestinal enzymatic activities are shown in **Figure 5**. Activities of amylase, protease, and lipase declined in the Pb-exposed group, and the differences were significant only in case of amylase activity. The alterations of amylase and protease activities were significantly reversed in the Pb + P16 group. The lipase activity was slightly recovered in Pb + P16 group. Furthermore, dietary administration of *L. reuteri* P16 only resulted in significantly higher amylase (1.49 U mg^{-1} protein) and protease (3.43 U mg^{-1} protein) activities, when compared with that in the control group. However, *L. reuteri* P16 only had no significant effects on lipase activity.

Quantification of Intestinal Microbiota

Pb exposure caused a profound decline in gut microbial diversity (**Figure 6**). Population of total bacteria and LAB counts decreased

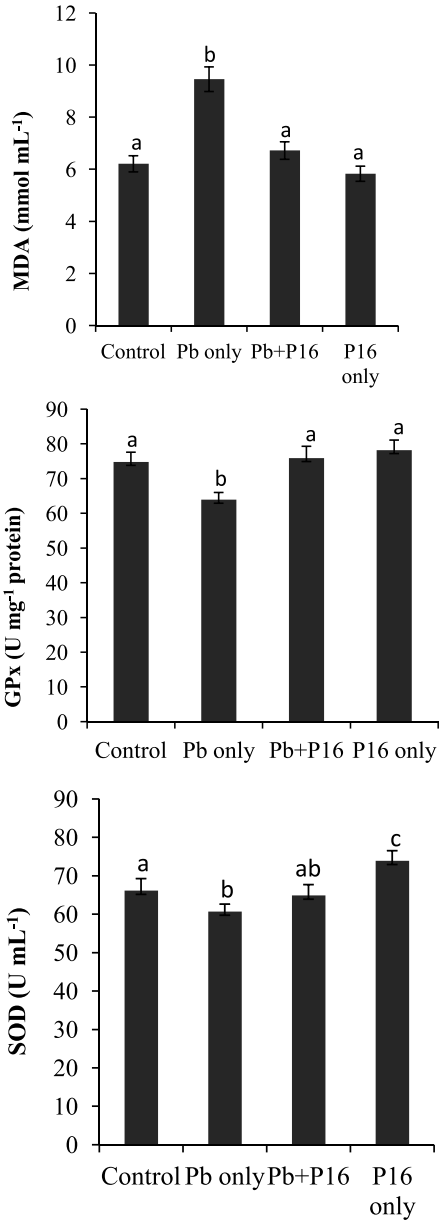


FIGURE 3 | Influence of *Lactobacillus reuteri* P16 supplementation on the oxidative parameters in the blood of *Cyprinus carpio*. Significant differences between groups are indicated with different superscript letters. Results are presented as mean ± SEM ($n = 30$).

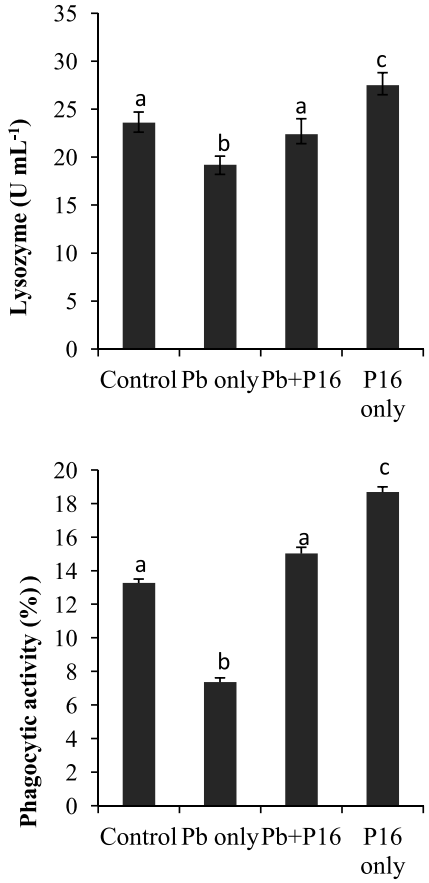


FIGURE 4 | Effect of *Lactobacillus reuteri* P16 supplementation on the serum lysozyme and leukocyte phagocytoc activities of *Cyprinus carpio*. Significant differences between groups are indicated with different superscript letters. Results are presented as mean ± SEM (n = 30).

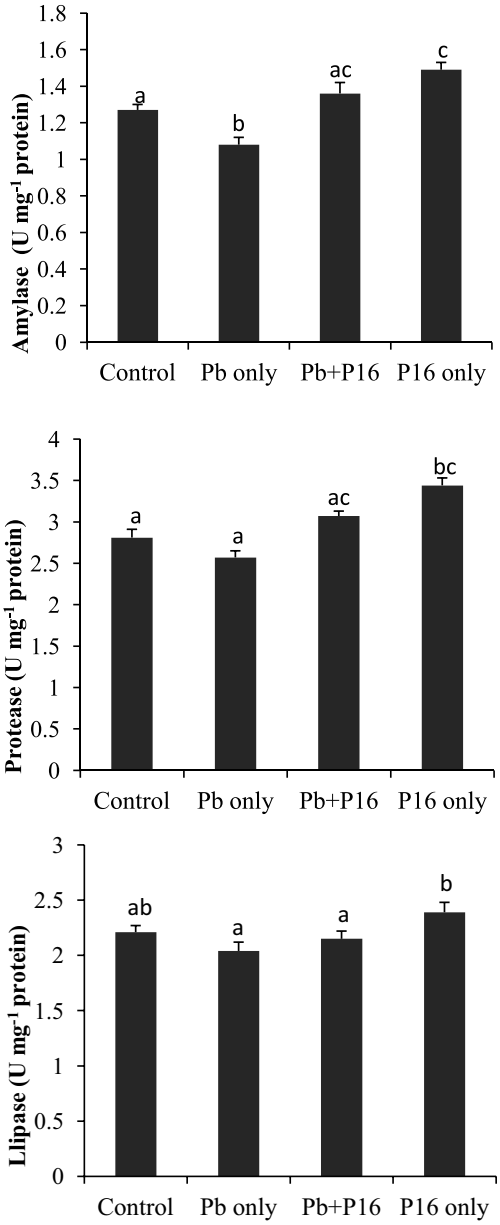


FIGURE 5 | Influence of *Lactobacillus reuteri* P16 supplementation on the intestinal enzymatic activities of *Cyprinus carpio*. Significant differences between groups are indicated with different superscript letters. Results are presented as mean ± SEM (n = 6).

significantly in Pb-only group, as compared with that of the other groups. However, probiotic treatment had considerable effect on intestinal microbiota. Alterations of bacterial population were reversed in the Pb + P16 group. Moreover, *L. reuteri* P16 treatment increased the LAB population significantly in *L. reuteri* P16-treated group.

Pb Levels in Tissues

Pb levels detected in various tissues of carps are shown in **Table 5**. Pb exposure significantly increased the level of Pb in gills (5.17 μg g^{-1}), spleen (3.86 μg g^{-1}), liver (8.92 μg g^{-1}), kidney (26.33 μg g^{-1}), and intestines (10.27 μg g^{-1}), when compared with those in the control group. Supplementation with *L. reuteri* P16 significantly decreased Pb accumulation in all tissue, except the intestine, when compared with the Pb-only group. But, the level of Pb accumulation in the Pb + P16 group was higher ($P < 0.05$) than that in the control group. Furthermore, Pb accumulation in the gills, spleen, liver, kidney, and intestine of the P16 group was 0.011, 0.05, 0.19, 0.28, and 0.09 μg g^{-1}, respectively, which were significantly lower than those in the Pb + P16 group, but no significant differences were observed with those in the control group.

Immune Gene Expression

Expression profiles of TNF-α, IL-1β, HSP70, and HSP90 in the head-kidney of the fishes at the end of the trial (**Figure 7**) showed that the expression of the pro-inflammatory cytokine TNF-α was significantly lower than that in the group exposed to Pb only, but it reversed in the Pb + P16 group. Similarly, IL-1β expression, which was lower in Pb-only group, was reversed in the Pb + P16 group. However, expression of TNF-α was significantly higher in the P16 group when compared with that in any other group.

The mRNA expression of heat shock proteins (HSP70 and HSP90) was increased drastically in Pb-only exposed group, but probiotic supplementation reversed the expression to the normal level (**Figure 7**). Interestingly, dietary administration of *L. reuteri* P16 only decreased the expression of both HSPs, but the differences remained significant in case of HSP70.

DISCUSSION

Physiological status of fishes is an important factor for determining their ability to resist pathogen attacks. Fish growth performance is affected by various external factors, such as temperature, nutrition, and toxicants. Inhibition of fish growth could be induced by physiological stress during exposure to toxic substances, because stress can reduce food consumption or assimilation (35). *L. reuteri* P16 supplementation reversed the Pb-exposure induced adverse effects on growth performance and survival of *C. carpio*. Recently, Zhai et al. (18) demonstrated that dietary supplementation with *L. plantarum* ameliorated the growth performance and prevented the death of Pb-exposed Nile tilapia (*Oreochromis niloticus*). Moreover, dietary supplementation with *L. plantarum* CCFM8610 increased the growth rate, decreased the FCR, and completely prevented the death of Cd-exposed fish (14). Also, significantly lower FCR in the P16 group reveals the fact that carp consumed dietary nutrients more efficiently when feed was supplemented with *L. reuteri*, which was consistent with numerous studies that reported significantly higher growth performances in fish fed with diet supplemented with the probiotic *Lactobacillus* (24).

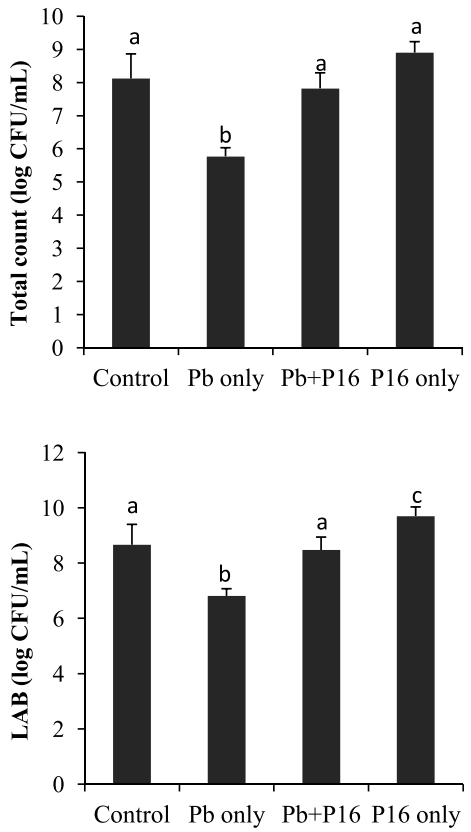


FIGURE 6 | Effect of waterborne Pb exposure and dietary *Lactobacillus reuteri* P16 on gut bacterial population of *Cyprinus carpio*. The different superscript letters indicate statistically significant differences between groups. Results are expressed as mean ± SD ($n = 6$).

Hematology has been widely used to evaluate the health status of animals exposed to environmental toxicants, and it provides information on digestive function, nutrient status, and metabolic activity of fish (36). The significant reduction in hematological parameters, such as RBC, WBC, Hct, and Hb concentration after waterborne Pb exposure was consistent with earlier reports (4). Heavy metal exposure generally induces lysis of erythrocytes in aquatic animals, leading to the depletion of Hct and Hb (37). Lead directly inhibits the synthesis of Hb by inhibiting various key enzymes involved in the heme synthesis pathway and reduces the life span of RBCs by increasing the fragility of cell membranes. The combined effect of these two processes leads to anemia (38). However, dietary *L. reuteri* P16 was effective in recovering those hematological parameters. The increased of RBC and WBC may be attributed to the effective antioxidant role of *L. reuteri* P16 (discussed below). Serum total protein, an important indicator of humoral immune system and health status of fish, was recovered in Pb + P16 group. Increase in serum protein level might be partially related with higher WBC, which is a major source of serum protein production (36). Furthermore, improved protein level in the group fed with *L. reuteri* P16 only indicated improved health status of fishes owing to probiotic supplementation.

Hepatocellular injury in fish may be attributed to stress, nutrition imbalance, diseases, or pollutants. AST and ALT levels are biological indicators of hepatic health status (39). The restoration of serum AST and ALT levels in the Pb + P16 group indicated the protective effect of *L. reuteri* P16 against Pb-induced damage

TABLE 5 | Effect of *Lactobacillus reuteri* P16 supplementation on the Pb levels in the tissues of *Cyprinus carpio*.

Group	Concentration of Pb in tissues (μg g^{-1} of wet tissue)				
	Gill	Spleen	Liver	Kidney	Intestine
Control	0.014 ± 0.001[a]	0.07 ± 0.01[a]	0.24 ± 0.02[a]	0.37 ± 0.08[a]	0.11 ± 0.02[a]
Pb only	5.17 ± ± 0.13[b]	3.86 ± 0.07[b]	8.92 ± 0.13[b]	26.33 ± 2.28[b]	10.27 ± 0.36[b]
Pb + P16	2.94 ± 0.14[c]	2.11 ± 0.08[c]	5.73 ± 0.26[c]	17.08 ± 1.17[c]	8.92 ± 0.61[b]
P16 only	0.011 ± 0.001[a]	0.05 ± 0.01[a]	0.19 ± 0.03[a]	0.28 ± 0.03[a]	0.09 ± 0.004[a]

Values in the same column with different superscript small letters are significantly different ($P < 0.05$).
Values are presented as mean ± SD ($n = 30$ fish in each group).

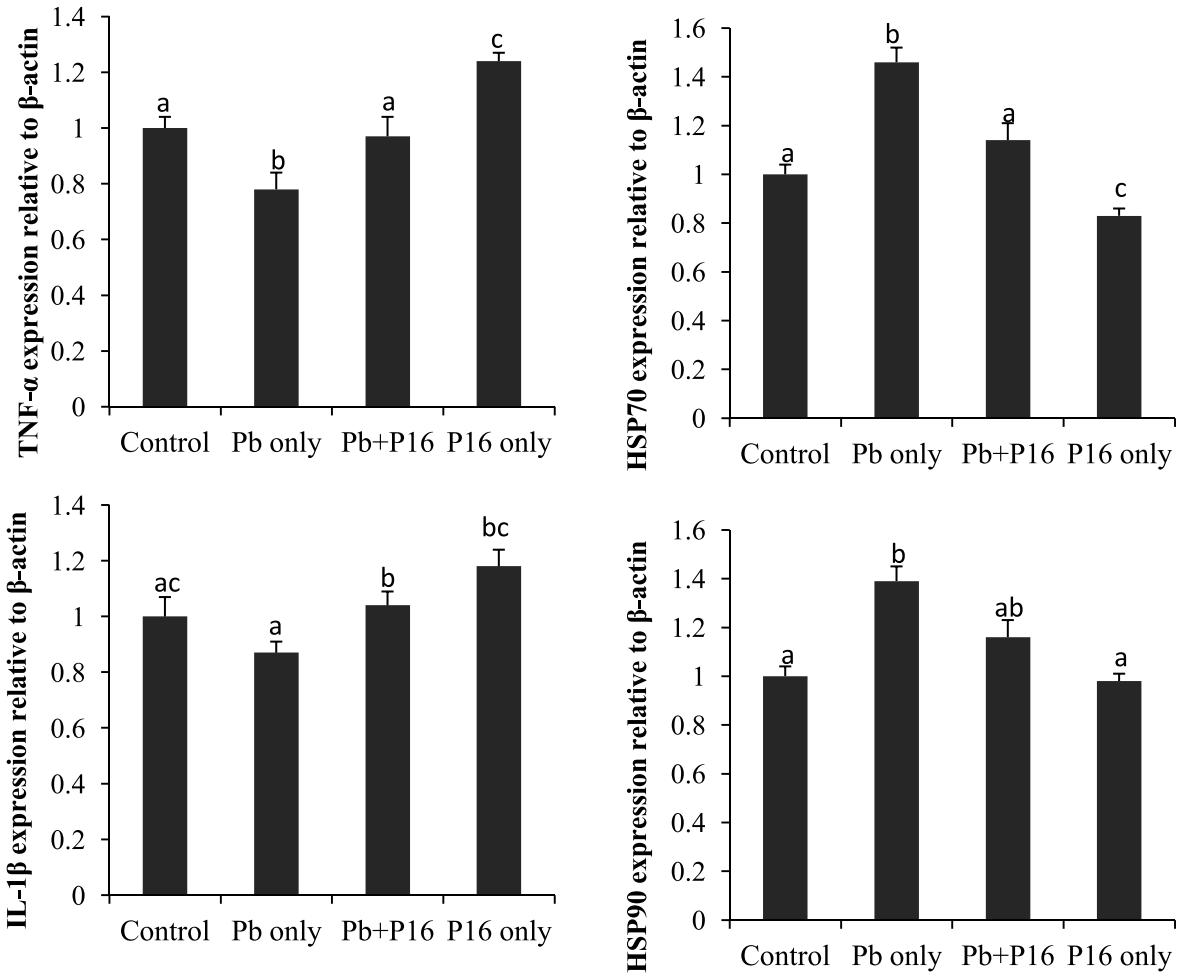


FIGURE 7 | Effects of dietary administration of *Lactobacillus reuteri* P16 on the relative mRNA expression levels of TNF-α, IL-1β, HSP70, and HSP90 in the head-kidney of *Cyprinus carpio*.

of liver and heart in *C. carpio* (40). Recently, Zhai et al. (14, 18) demonstrated that dietary administration of *L. plantarum* could restore alterations of AST and ALT in Nile tilapia. Similarly, changes in ALP activity, which an indicator of environmental stress, was restored in Pb + P16 group. However, diet-borne Pb exposure had no significant effect on ALP activity in rockfish (*Sebastes schlegelii*) (4). Cholesterol is a critical structural component of membranes and a precursor of all steroid hormones. The increase in cholesterol level in the Pb exposure group may be a sensitive indicator for metal-induced environmental stress (41). The blood cholesterol level was revered by dietary *L. reuteri* P16 supplementation. A considerable increase in the cholesterol of Nile tilapia (42) and rockfish (4) exposed to Pb was reported. Furthermore, creatinine level, which is an indicator of kidney function, is normally presents in low quantities in fishes (43). However, higher levels were present in the Pb-only group. Therefore, the higher cholesterol level in blood may be due to liver and kidney damage.

Malondialdehyde, GPx, and SOD are biomarkers of heavy metal-induced oxidative stress in aquatic animals. MDA, the main component of lipid peroxides, has strong biotoxicity and can damage the structure and function of cells (44). The first line of defense against oxidative stress consists of the antioxidant enzymes SOD, CAT, and GPx, which convert superoxide radicals into hydrogen peroxide and then into water and molecular oxygen (45). SOD comprises a group of metalloenzymes that catalyzes the dismutation of superoxide to hydrogen peroxide and plays a crucial role against the toxic effects of superoxide radicals in aerobic organisms (46). In this study, higher oxidative stress in the Pb exposure group was manifested by higher ($P < 0.05$) MDA levels and lower ($P < 0.05$) GPx and SOD levels. However, *L. reuteri* P16 supplementation reduced oxidative stress in carp. This study indicates that *L. reuteri* P16 can directly mitigate Pb-induced oxidative stress. Recently, Zhai et al. (14, 18) demonstrated that dietary supplementation of *L. plantarum* could reduce waterborne Pb-induced oxidative stress in Nile tilapia. Those results, together with those of the present study, indicate that probiotic lactobacilli have protective effects against Pb-induced oxidative damage in fish. MPOs are known to play an important role in cellular defenses against

various bacterial infections and can generates oxidants from H_2O_2 and a range of co-substrates (47). Recently, Paul et al. (8) reported that exposure to lead acetate could significantly reduce MPO levels in the macrophages of the freshwater fish *C. punctatus*. Dietary *L. reuteri* P16 supplementation was effective in recovering the MPO level in Pb-exposed fish and thereby improving host defense mechanisms.

Fish generally depend on non-specific immune responses. Lysozymes constitute the first line of defense following immune challenge to inhibit the adhesion and colonization of microorganisms (36); they split the β-1,4 glycosidic linkages between the *N*-acetyl glucosamine and *N*-acetyl muramic acid of the peptidoglycan in the bacterial cell wall, causing bacteriolysis and thereby controlling infection. By contrast, phagocytosis is responsible for early activation of the inflammatory response before antibody production and is mediated by phagocytic cells, such as neutrophils, monocytes, and macrophages, in fish (48). Significantly lower phagocytic and lysozyme activity r in the Pb-exposed group was in line with earlier reports (8, 18). But, those activities were recovered in the Pb + *L. reuteri* P16 supplementation group. Several studies have demonstrated that probiotic feeding could significantly improve various innate immune parameters in fish (22, 24, 25).

Another possible explanation for the stimulation of growth by potential probiotic *L. reuteri* P16 may be related to the induction of the expression of digestive enzymes in carp intestines by dietary P16, which boosts the natural digestive enzyme activity, and thereby the growth performance, of the host (49). This increased digestive enzymatic activity might be a means of protection against the adverse effects of Pb on fish. Very recently, Zhai et al. (18) reported that dietary administration of probiotic *L. plantarum* increased amylase and protease activity in Nile tilapia exposed to waterborne Pb. Better feed consumption (as revealed by reduced FCR) and digestion in *L. reuteri* P16 supplemented groups may be related to increased digestive enzyme activity and thereby increased appetite in fish. The gut microbiota is in direct contact with the intestinal mucosa constantly and plays a vital role in maintaining fish health (50). Pb exposure decreased LAB counts and total gut microbial populations. Pb-induced alterations in the gut microbiota may cause dysbiosis in carps, which can be related to the adverse effects on growth performance, antioxidant defense system, and hematological parameters of Pb-exposed fish. However, dietary supplementation with *L. reuteri* P16 moderately restored the gut dysbiosis. *L. reuteri* P16 supplementation increased LAB population in Pb-exposed fish. Alleviation of heavy metal-induced toxicities in fish through dietary administration of lactobacilli strains has been reported (13, 14, 18). Moreover, increased population of LAB may enhance Pb sequestration in gut, owing to its cellular accumulation and bioremoval.

Tissue-specific accumulation can be a sensitive indicator of metal exposure in aquatic toxicology. Exposure to waterborne Pb causes significant accumulation of Pb in various tissues of carp, with highest accumulation in kidney. Exposure of *S. schlegelii* to dietary Pb revealed that the highest Pb accumulation took place in the kidney (8). Similarly, varying Pb accumulation was recorded in tissues of Nile tilapia exposed to waterborne Pb (18). Differences in the extent of metal accumulation between tissues are considerably related to ecological needs and metabolic activity (51). The uptake of toxic heavy metals in aquatic animals generally occurs *via* two major routes: waterborne and diet borne. Waterborne Pb can be taken up by gills of fish, and it may contaminate food and be absorbed *via* intestines, then accumulate in tissues including the kidneys and liver (52). *L. reuteri* P16 used in this study has been reported to bind effectively to Pb *in vitro* (28). Therefore, intestinal *L. reuteri* P16 could bind secreted Pb before re-absorption from intestines, which increases Pb excretion through the feces of fish and inhibits intestinal absorption (22). This may a reason for the reduction in Pb levels in the Pb + *L. reuteri* P16 group.

The inflammatory response is a key element in the innate immune response system and is primarily mediated by cytokines (53). Very little is known about the immunotoxicology of heavy metals at the genetic level in fish. The cytokines IL-1β and TNF-α are primarily produced by monocytes and macrophages and regulate multiple aspects of the immune response. TNF-α affects tissue vasculature in inflammation and also induces acute phase proteins from the liver (54). The observed downregulation of TNF-α and IL-1β implicates NF-κB signaling pathway. However, *L. reuteri* P16 supplementation had a positive on TNF-α and IL-1β expression. In a previous study, Paul et al. (8) recorded decreased TNF-α level in sera and cell lysates of freshwater fish *C. punctatus* exposed to lead acetate. Based on the results of this study, it can be stated that Pb renders the fish in an immunocompromised and inflammatory state. Thus, TNF-α and IL-1β are downregulated, probably by the action of the heavy metal (8), but probiotic supplementation reversed these effects on the expression of those genes.

The synthesis of heat shock proteins (Hsps) increases in response to various physical and chemical stressors, including temperature and metal stress, and consequently these proteins can be good environmental stress biomarkers (55). Expression of HSPs which increased in Pb-exposure group, was further decreased in the *L. reuteri* P16 supplementation group and thus alleviated stress condition in fish. In agreement with the results of our study, Mohapatra et al. (19) demonstrated that dietary supplementation of a probiotic mixture consisting of *Bacillus subtilis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae* decreased the expression of HSP70 in *Labeo rohita*. Stress-reducing factors produced by probiotics might have lowered the HSP levels in fish, reduced Pb-induced stress, and resulted in better growth and immunity.

Interestingly, co-treatment with Pb and *L. reuteri* P16 reversed Pb-induced adverse effects on the carp species studied. The potential beneficial effects and protection by probiotics against Pb toxicity in carp has been shown in **Figure 8**. The hematological parameters indicated the ability of *L. reuteri* P16 to stimulate erythropoiesis, thereby increasing the oxygen transport, and a similar result had been reported earlier (13). Previously, we have demonstrated the excellent Pb-binding ability of this strain (28). Therefore, supplementing diets with this strain can reduce intestinal absorption of Pb and reduce Pb accumulation in other tissues. The strain *L. reuteri* P16 possess strong antioxidant abilities (28). In this study, co-treatment with Pb and *L. reuteri* P16 reduced

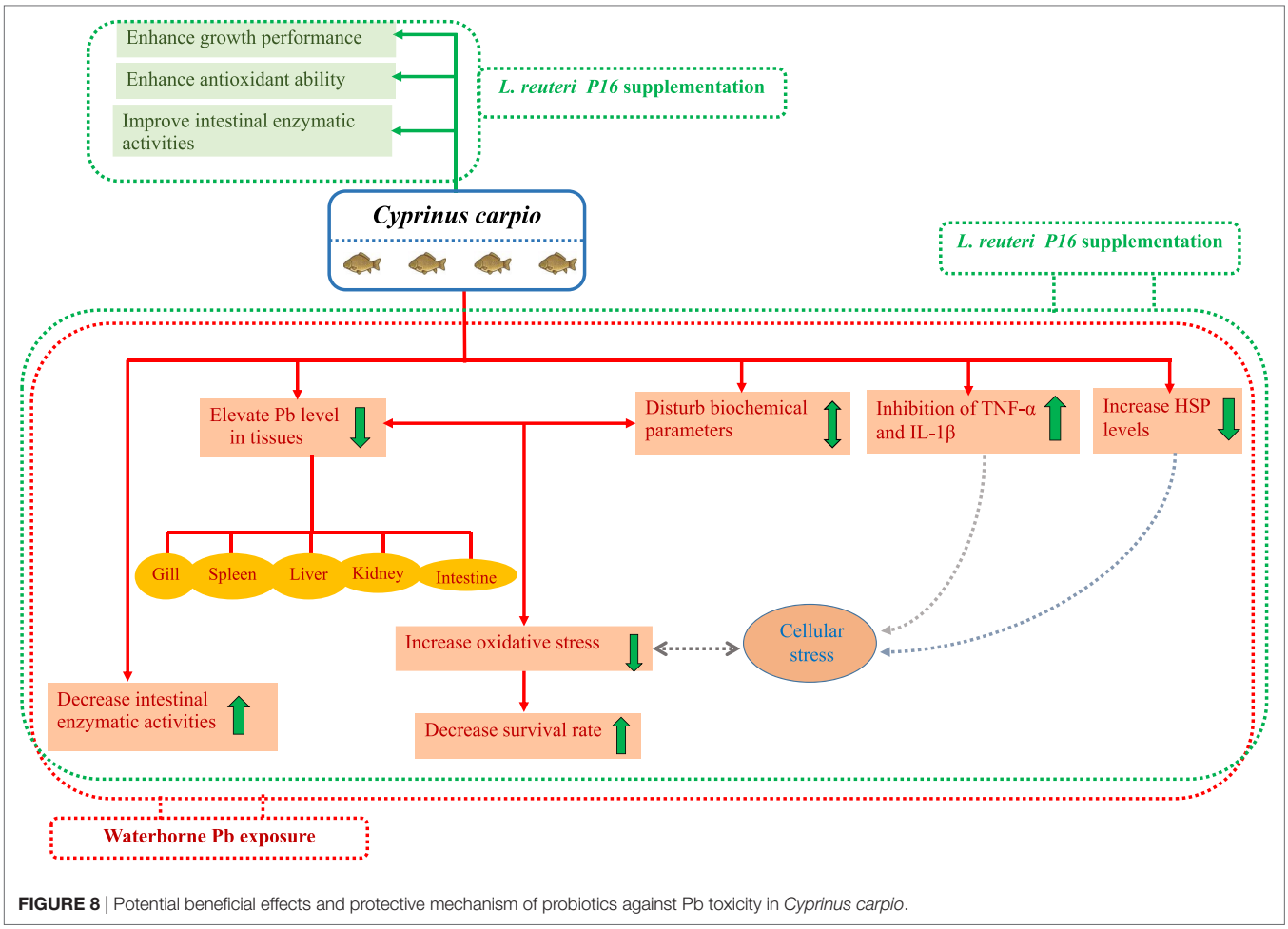


FIGURE 8 | Potential beneficial effects and protective mechanism of probiotics against Pb toxicity in *Cyprinus carpio*.

production of MDA and increased the activities of antioxidant enzymes (SOD and GPx). Thus, *L. reuteri* P16 alleviates the Pb-induced oxidative stress in carps. However, the underlying role of antioxidative activity of this strain against Pb-induced oxidative stress and dysfunction of the gut barrier need to be investigated further. In addition, co-treatment with Pb and *L. reuteri* P16 reversed the expressions of pro-inflammatory cytokines (TNF-α and IL-1β), which are associated with the NF-κB signaling pathway and subsequent decreases in reactive oxygen species production and prevents Pb-induced cellular apoptosis. However, comprehensive Pb tolerance mechanisms in LAB strains have not been investigated. Therefore, Pb stress response network in LAB strains are yet to be elucidated. Furthermore, key proteins and pathways involved in Pb tolerance of lactobacilli strains are unknown. Hence, isobaric tags for relative and absolute quantification (iTRAQ)-based comparative and functional proteomic approaches could be utilized to explore Pb-tolerance related to key proteins and pathways in *L. reuteri* strains. Moreover, at this early stage of research, we do not know how probiotics repair the metabolic damages occurring in fish exposed to heavy metals; therefore, efforts should be made in the future to incorporate multiple targets (genes, proteins, and metabolites) for exploring heavy metal defense mechanisms of probiotics in hosts.

Notably, treatment with *L. reuteri* P16 alone did not exert any adverse effects on growth performance, hematological and blood biochemical parameters, intestinal enzymatic activities, and intestinal microbiota. In addition, growth rate and feed utilization was greater in the group fed with only *L. reuteri* P16. This finding is in agreement with that of several earlier reports, which demonstrated that probiotics could boost the growth performance of fish. Furthermore, this strain exhibited higher Pb-removing antioxidant potential *in vitro* (28). Results of the present study suggest that *L. reuteri* P16 supplementation is safe for the carp species studied.

CONCLUSION

Lead has deleterious effect on physiological and immune functions in fish. Exposure of fish to sublethal concentrations resulted in significant Pb accumulation in specific tissues, whereas *L. reuteri* P16 supplementation strongly reduced this. This strain significantly increased the feed utilization, growth performance, and survival of Pb-exposed fish. *L. reuteri* P16 supplementations recovered Pb toxicity related biochemical parameters, alleviated oxidative stress, and re-established gut microbial population and intestinal enzymatic activities.

Furthermore, *L. reuteri* P16 supplementation was effective in reducing the Pb-exposure-induced expression of heat shock proteins. Overall, *L. reuteri* P16 supplementation showed considerable effectiveness in attenuating the changes caused by Pb-induced toxicity. Furthermore, administration of *L. reuteri* P16 by itself did not exert any adverse effects on fish health, suggesting that the dietary supplementation of this strain is sage. Results of the study indicate that *L. reuteri* P16 can cope with heavy metal stress owing to induction of cellular defense and repair system, but the underlying mechanisms of Pb tolerance of this strain yet to be explored. Therefore, these results suggest that *L. reuteri* P16 has potency as a novel dietary supplement to prevent safety problems induced by lead pollution in carp aquaculture. Therefore, *Lactobacillus* strains with both good Pb-binding and antioxidative capacities, such as *L. reuteri* P16, can be considered for utilization as dietary supplement for the prevention of Pb contamination aquaculture.

AUTHOR CONTRIBUTIONS

SG, VS, and SP designed the work. SG contributed significantly in lab works. SY helped him in conducting the experiments. SGK SWK, JK, and SH helped in sample collection and immunological studies. SG wrote the manuscript. All authors reviewed and contributed significantly to improve the manuscript. JJ contributed to study the immune gene expression analysis. JJ, SY, and HK contributed to statistical analysis of the data.

ACKNOWLEDGMENTS

This research was supported by the "Korea Research Fellowship Program" of the National Research Foundation of Korea (NRF), Ministry of Science and ICT (KRF: 2016H1D3A1909005), NRF research grant (NRF-2017R1C1B2004616), and Cooperative Research Program for Agriculture Science and Technology Development (Supportive managing project of Center for Companion Animals Research) by Rural Development Administration (PJ0138772018).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01824/full#supplementary-material.

REFERENCES

1. Ashraf W. Accumulation of heavy metals in kidney and heart tissues of *Epinephelus microdon* fish from the Arabian Gulf. *Environ Monit Assess* (2005) 101:311–6. doi:10.1007/s10661-005-0298-4
2. Kirillova AV, Danilushkina AA, Irisov DS, Bruslik NL, Fakhrullin RF, Zakharov YA, et al. Assessment of resistance and bioremediation ability of *Lactobacillus* strains to lead and cadmium. *Int J Microbiol* (2017) 2017:7. doi:10.1155/2017/9869145
3. Squadrone S, Prearo M, Brizio P, Gavinelli S, Pellegrino M, Scanzio T, et al. Heavy metals distribution in muscle, liver, kidney and gill of European catfish (*Silurus glanis*) from Italian Rivers. *Chemosphere* (2013) 90:358–65. doi:10.1016/j.chemosphere.2012.07.028
4. Kim J-H, Kang J-C. Toxic effects on bioaccumulation and hematological parameters of juvenile rockfish *Sebastes schlegelii* exposed to dietary lead (Pb) and ascorbic acid. *Chemosphere* (2017) 176:131–40. doi:10.1016/j.chemosphere.2017.02.097
5. Jarüp L. Hazards of heavy metal contamination. *Br Med Bull* (2003) 68:167–82. doi:10.1093/bmb/ldg032
6. Ramsdorf WA, Ferraro MV, Oliveira-Ribeiro CA, Costa JR, Cestari MM. Genotoxic evaluation of different doses of inorganic lead (Pb(II)) in *Hoplias malabaricus*. *Environ Monit Assess* (2009) 158:77–85. doi:10.1007/s10661-008-0566-1
7. Alsop D, Ng TY-T, Chowdhury MJ, Wood CM. Interactions of waterborne and dietborne Pb in rainbow trout, *Oncorhynchus mykiss*: bioaccumulation, physiological responses, and chronic toxicity. *Aquat Toxicol* (2016) 177:343–54. doi:10.1016/j.aquatox.2016.06.007
8. Paul N, Chakraborty S, Sengupta M. Lead toxicity on non-specific immune mechanisms of freshwater fish *Channa punctatus*. *Aquat Toxicol* (2014) 152:105–12. doi:10.1016/j.aquatox.2014.03.017
9. Gu Y-G, Huang H-H, Lin Q. Concentrations and human health implications of heavy metals in wild aquatic organisms captured from the core area of Daya Bay's Fishery Resource Reserve, South China Sea. *Environ Toxicol Pharmacol* (2016) 45:90–4. doi:10.1016/j.etap.2016.05.022
10. Zhai Q, Narbad A, Chen W. Dietary strategies for the treatment of cadmium and lead toxicity. *Nutrients* (2015) 7:552–71. doi:10.3390/nu7010552
11. Nayak SK. Role of gastrointestinal microbiota in fish. *Aquacult Res* (2010) 41:1553–73. doi:10.1111/j.1365-2109.2010.02546.x
12. Gómez GD, Balcázar JL. A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunol Med Microbiol* (2008) 52:145–54. doi:10.1111/j.1574-695X.2007.00343.x
13. Yu L, Zhai Q, Zhu J, Zhang C, Li T, Liu X, et al. Dietary *Lactobacillus plantarum* supplementation enhanced growth performance and alleviates aluminum toxicity in tilapia. *Ecotoxicol Environ Safety* (2017) 143:307–14. doi:10.1016/j.ecoenv.2017.05.023
14. Zhai Q, Yu L, Li T, Zhu J, Zhang C, Zhao J, et al. Effect of dietary probiotic supplementation on intestinal microbiota and physiological conditions of Nile tilapia (*Oreochromis niloticus*) under waterborne cadmium exposure. *Antonie Van Leeuwenhoek* (2017) 110:501–13. doi:10.1007/s10482-016-0819-x
15. Bhakta JN, Ohnishi K, Munekage Y, Iwasaki K, Wei MQ. Characterization of lactic acid bacteria-based probiotics as potential heavy metal sorbents. *J Appl Microbiol* (2012) 112:1193–206. doi:10.1111/j.1365-2672.2012.05284.x
16. Majlesi M, Shekarforoush SS, Reza Ghaisari HA, Nazifi S, Sajedianfard J, Eskandari MH. Effect of probiotic *Bacillus* coagulans and *Lactobacillus plantarum* on alleviation of mercury toxicity in rat. *Probiotics Antimicrobial Prot* (2017) 9:300–9. doi:10.1007/s12602-016-9250-x
17. Yi Y-J, Lim J-M, Gu S, Lee W-K, Oh E, Lee S-M, et al. Potential use of lactic acid bacteria *Leuconostoc mesenteroides* as a probiotic for the removal of Pb(II) toxicity. *J Microbiol* (2017) 55:296–303. doi:10.1007/s12275-017-6642-x
18. Zhai Q, Wang H, Tian F, Zhao J, Zhang H, Chen W. Dietary *Lactobacillus plantarum* supplementation decreases tissue lead accumulation and alleviates lead toxicity in Nile tilapia (*Oreochromis niloticus*). *Aquaculture Res* (2017) 48:5094–103. doi:10.1111/are.13326
19. Mohapatra S, Chakraborty T, Prusty AK, Pani Prasad K, Mohanta KN. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. *PLoS One* (2014) 9:e100929. doi:10.1371/journal.pone.0100929
20. Nayak SK. Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol* (2010) 29:2–14. doi:10.1016/j.fsi.2010.02.017
21. Newaj-Fyzul A, Al-Harbi AH, Austin B. Developments in the use of probiotics for disease control in aquaculture. *Aquaculture* (2014) 431:1–11. doi:10.1016/j.aquaculture.2013.08.026
22. Ringø E, Gatesoupe F-J. Lactic acid bacteria in fish: a review. *Aquacult* (1998) 160(3):177–203. doi:10.1016/S0044-8486(97)00299-8

23. Giri SS, Sukumaran V, Sen SS, Vinumonia J, Banu BN, Jena PK. Antagonistic activity of cellular components of probiotic bacteria, isolated from the gut of *Labeo rohita*, against *Aeromonas hydrophila*. *Probiotics Antimicrob Proteins* (2011) 3:214–22. doi:10.1007/s12602-011-9078-3
24. Giri SS, Sukumaran V, Oviya M. Potential probiotic bacteria *L. plantarum* VSG3 improves the growth, immunity and disease resistance of tropical freshwater fish, *Labeo rohita*. *Fish Shellfish Immunol* (2013) 34:660–6. doi:10.1016/j.fsi.2012.12.008
25. Chi C, Jiang B, Yu X-B, Liu T-Q, Xia L, Wang G-X. Effects of three strains of intestinal autochthonous bacteria and their extracellular products on the immune response and disease resistance of common carp, *Cyprinus carpio*. *Fish Shellfish Immunol* (2014) 36:9–18. doi:10.1016/j.fsi.2013.10.003
26. Coelho LM, Rezende HC, Coelho LM, de Sousa PAR, Melo DFO, Coelho NMM. Bioremediation of polluted waters using microorganisms. In: Naofumi S, editor. *Advances in Bioremediation of Wastewater and Polluted Soil*. Rijeka: IntechOpen (2015).
27. Zhai Q, Yin R, Yu L, Wang G, Tian F, Yu R, et al. Screening of lactic acid bacteria with potential protective effects against cadmium toxicity. *Food Control* (2015) 54:23–30. doi:10.1016/j.foodcont.2015.01.037
28. Giri SS, Yun S, Jun JW, Kim HJ, Kim SG, Kang JW, et al. Characterisation of lactic acid bacteria isolated from the gut of *Cyprinus carpio* that may be effective against lead toxicity. *Probiotics Antimicrobial Proteins* (2017). doi:10.1007/s12602-017-9367-6
29. Sukumaran V, Park SC, Giri SS. Role of dietary ginger *Zingiber officinale* in improving growth performances and immune functions of *Labeo rohita* fingerlings. *Fish Shellfish Immunol* (2016) 57:362–70. doi:10.1016/j.fsi.2016.08.056
30. Drape HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free Radic Biol Med* (1993) 15:353–63. doi:10.1016/0891-5849(93)90035-S
31. Dotta G, Alves de Andrade JI, Gonçalves ELT, Brum A, Mattos JJ, Maraschin M, et al. Leukocyte phagocytosis and lysozyme activity in Nile tilapia fed supplemented diet with natural extracts of propolis and Aloe barbadensis. *Fish Shellfish Immunol* (2014) 39:280–4. doi:10.1016/j.fsi.2014.05.020
32. Pavasovic A, Richardson NA, Mather PB, Anderson AJ. Influence of insoluble dietary cellulose on digestive enzyme activity, feed digestibility and survival in the red claw crayfish, *Cherax quadricarinatus* (von Martens). *Aquacult Res* (2006) 37:25–32. doi:10.1111/j.1365-2109.2005.01389.x
33. Hoseinifar SH, Mirvaghefi A, Mojazi Amiri B, Rostami HK, Merrifield DL. The effects of oligofructose on growth performance, survival and autochthonous intestinal microbiota of beluga (*Huso huso*) juveniles. *Aquaculture Nutr* (2011) 17:498–504. doi:10.1111/j.1365-2095.2010.00828.x
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* (2001) 25:402–8. doi:10.1006/meth.2001.1262
35. Hansen JA, Welsh PG, Lipton J, Suedkamp MJ. The effects of long-term cadmium exposure on the growth and survival of juvenile bull trout (*Salvelinus confluentus*). *Aquat Toxicol* (2002) 58:165–74. doi:10.1016/S0166-445X(01)00233-8
36. Giri SS, Jun JW, Sukumaran V, Park SC. Evaluation of dietary (Linn F. Muell.) as a growth and haemato-immunological modulator in *Labeo rohita*. *Fish Shellfish Immunol* (2017) 68:310–7. doi:10.1016/j.fsi.2017.07.009
37. Gabbianelli R, Lupidi G, Villarini M, Falcioni G. DNA damage induced by copper on erythrocytes of gilthead sea bream *Sparus aurata* and mollusk *Scapharca inaequivalvis*. *Arch Environ Contam Toxicol* (2003) 45:350–6. doi:10.1007/s00244-003-2171-1
38. Flora G, Gupta D, Tiwari A. Toxicity of lead: a review with recent updates. *Interdiscip Toxicol* (2012) 5:47–58. doi:10.2478/v10102-012-0009-2
39. Wan AHL, Soler-Vila A, O'Keeffe D, Casburn P, Fitzgerald R, Johnson MP. The inclusion of *Palmaria palmata* macroalgae in Atlantic salmon (*Salmo salar*) diets: effects on growth, haematology, immunity and liver function. *J Appl Phycol* (2016) 28:3091–100. doi:10.1007/s10811-016-0821-8
40. Shahsavani D, Mohri M, Kanani HG. Determination of normal values of some blood serum enzymes in *Acipenser stellatus* Pallas. *Fish Physiol Biochem* (2010) 36:39–43. doi:10.1007/s10695-008-9277-3
41. Oner M, Atli G, Canli M. Changes in serum biochemical parameters of freshwater fish *Oreochromis niloticus* following prolonged metal (Ag, Cd, Cr, Cu, Zn) exposures. *Environ Toxicol Chem* (2008) 27:360–6. doi:10.1897/07-281R.1
42. Firat O, Cogun HY, Yuzereroglu TA, Gok G, Firat O, Kargin F, et al. A comparative study on the effects of a pesticide (cypermethrin) and two metals (copper, lead) to serum biochemistry of Nile tilapia, *Oreochromis niloticus*. *Fish Physiol Biochem* (2011) 37:657–66. doi:10.1007/s10695-011-9466-3
43. Wells RMG, Mclntyre RH, Morgan AK, Davie PS. Physiological stress responses in big gamefish after capture; observations on plasma chemistry and blood factors. *Comp Biochem Physicol* (1988) 84:565–71. doi:10.1016/0300-9629(86)90366-X
44. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest* (1982) 47:412–26.
45. Ural MŞ. Chlorpyrifos-induced changes in oxidant/antioxidant status and haematological parameters of *Cyprinus carpio*: ameliorative effect of lycopene. *Chemosphere* (2013) 90(7):2059–64. doi:10.1016/j.chemosphere.2012.12.006
46. Tkachenko H, Kurhaluk N, Grudniewska J, Andriichuk A. Tissue-specific responses of oxidative stress biomarkers and antioxidant defenses in rainbow trout *Oncorhynchus mykiss* during a vaccination against furunculosis. *Fish Physiol Biochem* (2014) 40(4):1289–300. doi:10.1007/s10695-014-9924-9
47. Eiserich JP. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* (1998) 391:393–7. doi:10.1038/34923
48. Adams DO, Hamilton TA. The cell biology of macrophage activation. *Annu Rev Immunol* (1984) 2:283–318. doi:10.1146/annurev.iy.02.040184.001435
49. Bahi A, Guardiola FA, Messina C, Mahdhi A, Cerezuela R, Santulli A, et al. Effects of dietary administration of fenugreek seeds, alone or in combination with probiotics, on growth performance parameters, humoral immune response and gene expression of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* (2017) 60:50–8. doi:10.1016/j.fsi.2016.11.039
50. Pérez T, Balcázar JL, Ruiz-Zarzuela I, Halaihel N, Vendrell D, de Blas I, et al. Host-microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunol* (2010) 3:355–60. doi:10.1038/mi.2010.12
51. Canli M, Atli G. The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. *Environ Pollut* (2013) 121:129–36. doi:10.1016/S0269-7491(02)00194-X
52. Rogers J, Richards J, Wood C. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicol* (2003) 64:215–34. doi:10.1016/S0166-445X(03)00053-5
53. Han J, Ulevitch RJ. Limiting inflammatory responses during activation of innate immunity. *Nat Immunol* (2005) 6:1198–1205. doi:10.1038/ni1274
54. Denis F, Archambault D. Molecular cloning and characterization of beluga whale (*Delphinapterus leucas*) interleukin-1b and tumor necrosis factor-a. *Can J Vet Res* (2001) 65:233–40.
55. Sassi A, Darias MJ, Said K, Messaoudi I, Gisbert E. Cadmium exposure affects the expression of genes involved in skeletogenesis and stress response in gilthead sea bream larvae. *Fish Physiol Biochem* (2013) 39(3):649–59. doi:10.1007/s10695-012-9727-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 05 September 2018
doi: 10.3389/fnut.2018.00080

A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research

Sylvia Brugman[1], Wakako Ikeda-Ohtsubo[2], Saskia Braber[3], Gert Folkerts[3], Corné M. J. Pieterse[4] and Peter A. H. M. Bakker[4]*

[1] Cell Biology and Immunology Group, Animal Sciences Group, Wageningen University and Research, Wageningen, Netherlands, [2] Food and Feed Immunology Group, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [3] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, Utrecht, Netherlands, [4] Plant-Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Vida Abedi, Geisinger Health System, United States

Reviewed by:
Zhenhuan Zhao, University of Virginia, United States
Julien Diana, Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Sylvia Brugman
sylvia.brugman@wur.nl

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *07 June 2018*
Accepted: *17 August 2018*
Published: *05 September 2018*

Citation:
Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CMJ and Bakker PAHM (2018) A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research. Front. Nutr. 5:80. doi: 10.3389/fnut.2018.00080

During recent years the impact of microbial communities on the health of their host (being plants, fish, and terrestrial animals including humans) has received increasing attention. The microbiota provides the host with nutrients, induces host immune development and metabolism, and protects the host against invading pathogens (1–6). Through millions of years of co-evolution bacteria and hosts have developed intimate relationships. Microbial colonization shapes the host immune system that in turn can shape the microbial composition (7–9). However, with the large scale use of antibiotics in agriculture and human medicine over the last decades an increase of diseases associated with so-called dysbiosis has emerged. Dysbiosis refers to either a disturbed microbial composition (outgrowth of possible pathogenic species) or a disturbed interaction between bacteria and the host (10). Instead of using more antibiotics to treat dysbiosis there is a need to develop alternative strategies to combat disturbed microbial control. To this end, we can learn from nature itself. For example, the plant root (or "rhizosphere") microbiome of sugar beet contains several bacterial species that suppress the fungal root pathogen *Rhizoctonia solani*, an economically important fungal pathogen of this crop (11). Likewise, commensal bacteria present on healthy human skin produce antimicrobial molecules that selectively kill skin pathogen *Staphylococcus aureus*. Interestingly, patients with atopic dermatitis (inflammation of the skin) lacked antimicrobial peptide secreting commensal skin bacteria (12). In this review, we will give an overview of microbial manipulation in fish, plants, and terrestrial animals including humans to uncover conserved mechanisms and learn how we might restore microbial balance increasing the resilience of the host species.

Keywords: probiotics, prebiotics, comparative, microbiota, fish, livestock, plants, human

THE IMPACT OF THE MICROBIOTA ON HOST HEALTH

Each organism on earth needs to interact with the vast amount of microbes in its environment. From the moment of exposure both microbes and host interact and react to each other's presence. The current dogma holds that in early life the composition of the microbiota successively leads to the most optimal, healthy, and stable community that strengthens itself and the host. However, when disturbances occur in early life, for example due to antibiotic use, host's inability to interact properly with the microbiota, a mismatch between the environment of parent and offspring, non-optimized feeding conditions or infections, organisms might be more susceptible to disease early and later in life (**Figure 1**). In this same issue of Frontiers in Microbiology, Ikeda-Ohtsubo et al. describe what is currently known to be the most optimal microbiota in different species and will describe how microbes influence the host in more detail. In this section, we briefly highlight the impact of the microbial composition in relation to host health and continue to discuss manipulation of this composition in subsequent sections. In this review, we decided to focus on less well-reviewed species and did not include studies on rodents. For detailed reviews on (human and) mouse studies we would kindly refer to other reviews (1, 2, 13–15).

Fish

Since most fish develop from eggs, fish are exposed to the microbial world around them from the moment of hatching. Members of the commensal microbiota present on the outside of the eggs can protect against pathogens, such as Saprolegnia (an oomycete that can infect eggs as well as the skin of fish) (16). Furthermore, colonization of the skin and gills by beneficial bacteria prevents colonization of pathogens through competitive exclusion and their ability to produce antimicrobial compounds specifically targeting certain pathogens (17, 18). An important recent contribution to the field of fish microbiology was made by Sullam et al. They assessed 25 bacterial 16S rRNA data sets from the intestines of several fish species and co-referenced these to free-living and host-associated bacterial communities to understand what factors determine composition (19). In this study Aeromonadales was found to be most abundant in the gut of freshwater species, while Vibrionales dominated salt water species, suggesting that salinity influences microbiota composition. Interestingly, the microbiota of fish herbivores are closely related to those of mammals suggesting that trophic level is of importance. In comparison with free-living bacteria the authors showed that fish gut communities bear resemblance to vertebrate and invertebrate communities strongly suggesting that the intestinal habitat selects those species from the environment that feed of luminal content (specialists).

Upon colonization of the intestines, bacteria increase the renewal rate of the epithelial cell layer lining the gut, and induce a transcriptional program that includes genes involved in nutrient metabolism and immunity (20). Interestingly, several of these induced genes observed in zebrafish upon colonization, are also found when mice are colonized, indicating that the response to bacterial colonization is evolutionary very conserved. Activation of immune cell responses can in turn shape the microbiota. For example, in a small study, it was observed that upon development of adaptive immunity in zebrafish, Vibrionales were specifically repressed by T lymphocytes (8). Research into effects of host genetics on the composition of the microbiota is still in its infancy. However, recent studies performed in zebrafish reveals that host factors can act as an ecological filter, but this can be overwhelmed by other factors, including transmission of microbes among hosts (21, 22).

Several recent studies have made the case that changes in microbial composition (due to antibiotic treatment or changes in feed) might cause disturbance of the microbial community and increase the susceptibility of fish to different pathogens or chemically/feed-induced inflammation (23–26). For example, zebrafish that were pretreated with either colistin sulfate or vancomycine displayed a difference in susceptibility toward chemically-induced intestinal inflammation. Fish that harbored an abundance of Fusobacterial species due to vancomycin pretreatment showed low histological damage to the gut and decreased recruitment of neutrophils to the intestines (23). Piazzon et al. showed that vegetable oil diets fed to gilthead sea bream induced high parasite infection levels decreased growth performance, and decreased intestinal microbiota diversity, while addition of butyrate slightly decreased cumulative mortality after bacterial challenge, did not show the decrease in growth and increased intestinal microbiota diversity (26). Likewise, exposure of catfish to potassium permanganate (PP), a disinfectants used to treat external infections, disturbed the external microbiomes (skin and gills), and increased catfish mortality following experimental challenge with *Flavobacterium columnare* (Columnaris disease) (24). Since, the microbial community of fish appears to be so closely linked to disease resistance and the aquaculture industry is growing rapidly, more research on the influence of microbial populations on fish health is expected to be performed in the near future.

Plants

The microbiota associated with plants originate from the soil in which the seed germinates and seedlings start to grow (27), but also the seed itself harbors microbes that will colonize the emerging plant (28). Compared to the soil microbiome, the so called rhizosphere microbiome that is associated with the plant roots, contains much higher microbial cell densities and shows higher activity, a phenomenon known as the rhizosphere effect (29). Root exudates create this hotspot for microbial activity (30). The plant microbiome is extremely diverse and the four main associated bacterial phyla are the Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (31). The functional repertoire of plants is greatly extended by the rhizosphere microbiota, including vital functions like increasing nutrient availability, improvement of root architecture, and protection against biotic and abiotic stresses (32). Specific members of the rhizosphere microbiota can protect plants against infectious diseases and mechanisms involved are the production of antimicrobial metabolites and eliciting induced systemic resistance (ISR), in which the plant is primed for enhanced defense (33, 34). The protective effect of the root microbiome against infectious

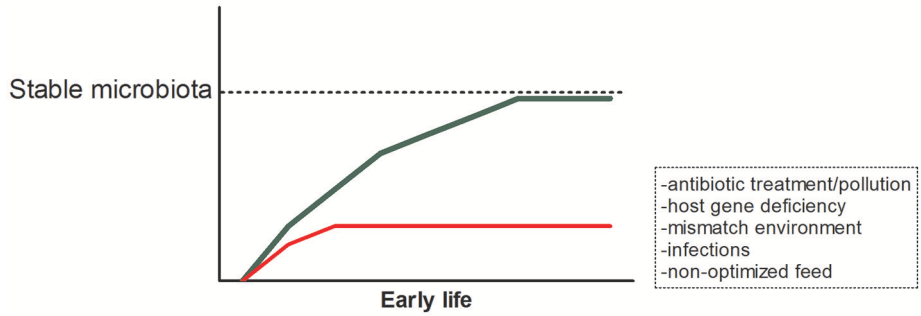


FIGURE 1 | Early life establishment of the microbiota and possible disturbances.

diseases is most obvious in so called disease suppressive soils in which susceptible plants remain healthy despite the presence of a virulent pathogen (35). It was recently demonstrated that the rhizosphere of disease resistant common bean is enriched for plant beneficial bacteria and bacterial biosynthetic genes that encode antifungal traits as compared to disease susceptible bean (36), suggesting that a first line of defense in resistant cultivars is based on the microbiome that they assemble.

Livestock

Pigs

Pork meat is the most consumed meat worldwide with 40,000 tons produced in 2017 (source: OECD, https://data.oecd.org/agroutput/meat-consumption.htm). Raising healthy piglets is of great economic importance. In neonatal piglets before weaning Firmicutes (54%), Bacteroidetes (39%), and Proteobacteria (4%) dominate the fecal microbiota (37). Weaning is a critical period in the piglets life, in which stress and sudden change in diet suppresses their health or can even lead to disease (38, 39). Pigs are generally weaned between 3 and 4 weeks of age (which is much shorter than the natural weaning which occurs around 17 weeks of age). As extensively reviewed by Gresse et al. most studies report a general decrease in diversity as well as a specific decrease in *Lactobacilli* and increase of *Clostridium*, *Prevotella,* and Proteobacteriacaea around weaning-associated with dietary changes (39). Low levels of antibiotics in feed have been used as growth promotors which also impact the microbiota around this critical weaning period and thereafter (40). Additionally, antibiotic resistance genes derived from phages within the pig microbiota pose a serious problem not only for pig health but also that of humans (41). Post-weaning diarrhea caused by Enterotoxigenic *Escherichia coli* (ETEC) as well as *Salmonella enterica* serovar Typhimurium is a major cause of death of piglets. Outgrowth of these pathogenic species coincides with the reduced diversity of the microbiota observed around weaning possibly enabling pathogens to take up the available niches. Furthermore, increased permeability of the intestines observed around weaning, supplying pathogens with an opportunity to infect (42). Preventing pathogens from colonizing, by providing beneficial bacterial at early age, might improve health of piglets.

Ruminants

In ruminants such as cows, a lot of research into microbial manipulation has been geared toward modifying the microbiota to reduce methane emission. Methane emission, partly responsible for climate change, results from digestion of plant-material by methanogenic archaea in the ruminants intestines. Changing the microbiota to increase bacteria that can utilize the methane (such as *Methanobrevibacter* species) might reduce emission (43). The intestinal community of calves changes rapidly after birth and is dynamic during the first 12 weeks of life. *Bacteroides–Prevotella* and *Clostridium coccoides–Eubacterium rectale* species dominate the calves microbiota in this period (44, 45). After weaning, the microbiota changes, and this unstable populations has to cope with a sudden change in diet. As in pigs and other mammals, weaning is considered a critical period, where numerous factors can affect the microbiota as well as health. In calves it was shown that upon weaning Bacteroidetes decreased (still remaining the dominant phyla), while Proteobacteria and Firmicutes increased (46). A change in diet and subsequent change in microbiota has been associated with development of sub-acute ruminal acidosis (SARA) (47). SARA (a reduced ruminal pH; <5.6 for more than 3 h/day) is the result of dietary shifts, leading to accumulation of volatile fatty acids generated by microbial digestive processes (48, 49).

Poultry

The ceca are the gastrointestinal organs that contain the highest microbial density and perform most of the fermentation in chicken. Mainly Firmicutes, Bacteroides, and Proteobacteria (Clostridial species) are found in the ceca of chicken [reviewed in Oakley et al. (50)]. Since eggs are separated from the hens before hatching the specific farm environment in which the eggs hatch supplies the environmental microbes for colonization. Already in the 70s and 80s of the last century it was shown that chicks receiving adult microbiota were resistant toward colonization by *Salmonella* (51). This competitive exclusion concept has helped our understanding of colonization processes of possible pathogenic (pathobionts) species, although fundamental knowledge on the mechanisms are still unclear. Other pathogens in chicken broilers are *Eimeria* and *Clostridium perfringes*. *Clostridium perfringes* is the causative agent in necrotic enteritis (NE) in poultry. Necrotic enteritis causes

tremendous losses in the poultry industry (52). As holds true for most pathobionts like *C. perfringens*, it is clearly involved in the onset of NE, but development of the disease is a multifactorial, multistep process (53). Studies report that co-infection with *Eimeria* increases the chance of NE, since *Eimeria* induce mucogenesis, providing *C. perfringens* with a substrate on which it can grow (54, 55).

Humans

The human gut microbiota, composed of trillions of individual microbes, is a complex and dynamic system crucial for the development and maturation of both systemic and mucosal immune responses. The early postnatal life is an important period for the colonization of the host microbiota impacting on host health during infancy and even throughout the entire lifespan (56–58). Colonization even starts before birth, possibly via prenatal maternal microbial transmission (59). This initial colonization does not only influence immune development, but also gut maturation, brain, and metabolic development (1).

The development and composition of the infant gut microbiota is shaped by host genetics and different environmental factors, including gestational age, delivery mode (cesarean vs. vaginal delivery), antibiotic use, stress, and diet (breast vs. formula) (60).

Dysbiosis, may drive predisposition to diseases later in life and has been linked to the pathogenesis of several gastrointestinal diseases, like irritable bowel syndrome, inflammatory bowel disease, and celiac disease, indicating that a balanced and diverse microbial community is essential for human health (61). A variety of other inflammatory or immune-mediated diseases, including diabetes, obesity, atopic diseases, and chronic kidney diseases, might largely originate from changes in gut microbiota as well (62, 63).

The microbiota and the immune system are involved in a complex crosstalk and the importance of the elaboration of gut microbiota-generated metabolites and recognition of bacterial epitopes by both intestinal epithelial and mucosal immune cells is clearly described. However, the complete mechanisms by which intestinal microbiota regulates host immunity remain undefined (64, 65).

PRO- AND PREBIOTICS

Probiotics are defined by the World Health Organization's (WHO) to be live micro-organisms that when administered in adequate amounts, confer a health benefit to the host. Prebiotics are ingredients in food such as fibers and oligosaccharides that induce the growth or activity of beneficial microorganisms. In the following section, we will give some highlights of the use of pre- and probiotics in fish, plants, live-stock animals, and humans.

Fish

Prebiotics

Prebiotics are indigestible fibers that are mainly fermented by the microbes in the intestines. The effect of mannan-oligosaccharides (MOS), derived from the cell wall of yeast (*Saccharomyces cerevisiae*) has been studied in different fish species. In common carp fingerlings, feed containing different inclusion levels of MOS (0, 0.05, 0.10, and 0.20%) were evaluated. Total intestinal bacterial counts were not affected, however, there was an increased abundance of lactic acid bacteria levels in fish fed with MOS supplemented feed at the 0.20% inclusion level after 8 weeks of feeding, which might be beneficial to the fish (66). In European sea bass, inclusion of 0.40% of MOS reduced mortality after anally inoculated *V. anguillarum* from 66 to 12.5%, compared to fish fed control diet (67). Likewise, MOS enhanced innate immune responses, led to increased gut mucus production and increased the density of eosinophils in the gut mucosa of European sea bass (68, 69). A study performed in hybrid striped bass revealed that supplementation with MOS changed the microbiota. This study, although small and DGGE based, showed that the dominant species detected in control fish is *Clostridium botulinum* which was not detected in fish fed MOS and other prebiotics, which indicates that prebiotics can reduce the abundance of a specific pathogens (70). Furthermore, 0.2% MOS supplementation in juvenile trout significantly reduced the levels of health threatening *Aeromonas/Vibrio* spp. (from 37 to 9%) (71). For an extensive review of studies on the use of MOS and Galacto-oligosaccharide (GOS) in Gilthead seabream and European seabass we refer to Carbone and Faggio (72).

Artemia (live feed for fish larvae) fed a combination of Fructo-oligosaccharide (FOS) with probiotic *Pediococcus acidilactici* (synbiotics: Pre + Pro), also increased the abundance of lactic acid bacteria in Angelfish after 7 weeks of feeding (73). Likewise, Hoseinifar et al. observed an increase in the heterotrophic aerobic bacteria and lactic acid bacteria in fish fed diets supplemented with 2 and 3% FOS (74). An increase in *Lactobacillus* levels was also observed in stellate sturgeon fed diets containing 1% FOS (75).

Some essential oils from plants might have an impact on the microbiota and host health and might therefore also be termed prebiotic. For example, when juvenile hybrid tilapia were fed 200 mg/kg Next Enhance feed 150 (NE) containing equal levels of thymol and carvacrol (essential oils of oregano) for 6 weeks, the phagocytosis activity of head kidney macrophages was enhanced as well as plasma lysozyme activity (76). Interestingly, when germ free zebrafish were colonized with the microbiota from these NE fed tilapia, they showed attenuated induction of immune response marker genes serum amyloid a, interleukin 1β, and interleukin 8, indicating that these essential oils might change the microbiota and subsequently influence host' immune responses.

Probiotics

Although most studies associate the change in microbiota levels (increased *Lactobacillus* abundance) with improved health outcomes, the mechanism by which increases in Lactobacilli ameliorate fish health still needs to be demonstrated. However, most probiotics currently used in aquaculture belong to the lactic acid bacteria group such as *Lactobacillus plantarum*, *Lactobacillus rhamnosis,* and *Lactobacillus lactis* [reviewed in Banerjee and Ray (77)]. Interestingly, *Phaeobacter inhibens* fed Artemia decrease mortality in sea bass larvae, and the probiotic fed sea bass larvae were more resistant toward *Vibrio harveyi* infection (78, 79). *Phaeobacter* produces Tropodithietic

Acid (TDA), which *in vitro* has been shown to inhibit the growth of several pathogenic bacteria, such as *Pseudomonas* and *Aeromonas*. *In vivo*, *P. inhibens* appears to specifically inhibit *Vibrio* species in the aforementioned sea bass larvae, but also in copepod cultures (78, 79).

In conclusion, pre- and probiotic supplementation of fish feed is a promising alternative for antibiotic treatment in aquaculture. However, a cautionary note on these data are the findings of Cerezuela et al. showing that diets supplemented with probiotic *Bacteroides subtilis* together with prebiotic inulin caused intestinal edema and inflammation in gilthead sea bream (80). This last example, clearly shows the need for in depth studies into the effects of known pro- and prebiotics in the different (aquaculture and model) fish species.

Plants

Prebiotics

The use of organic soil amendments to promote plant growth is common practice in agriculture (81). Apart from adding nutrients that are essential for plant growth, microbiota associated with these amendments and effects of the amendments on the resident microflora are suggested to govern beneficial effects of such additives (82). In a recent study it was demonstrated that addition of broccoli residues or crab meal amendments to soil resulted in a transition of disease conducive to suppressive soil (83). Eggplant is susceptible to wilting caused by the fungus *Verticillium dahliae*, but when grown in soil amended with green manure (broccoli residues) or the chitin containing crab shell meal, it was protected against the disease. In the amended soils bacterial genera with antifungal activity were more abundant and chitinase activity was increased (83). These results show that organic amendments can modify the soil microbiome and support microbiome activities that effectively suppress soil borne disease.

Thermal degradation of organic material by pyrolysis results in the production of biochar, a possible means to sequester carbon and to mitigate climate change (84). Soil amendment with biochar can improve soil fertility and has been reported to influence diseases caused by soil borne plant pathogens. Addition of biochar significantly impacts the soil microbiome and functions (85), suggesting that it may modulate the rhizosphere microbiome of plants grown on this soil and thereby affect disease incidence and severity. Indeed biochar amendments have been shown to reduce disease, but if this is due to the ISR eliciting activity of biochar itself or if it results from modulation of the microbiome remains to be elucidated (86).

Thus, addition of prebiotics to control plant diseases follows as yet a trial and error approach. Discovering new prebiotics may result from studies in which effects of specific plant produced compounds on beneficial microbes are recorded. Recently it was suggested that scopoletin, a compound excreted by Arabidopsis roots upon colonization by beneficial bacteria, can support the beneficial bacteria (87). Application of such plant derived prebiotics may lead to stimulation of beneficial microbes within the resident microbiota or may be used to sustain populations and activities of introduced biocontrol agents.

Probiotics

A wealth of literature is available of studies on application of so called biological control agents that can benefit plant growth by suppressing diseases, and their modes of action have been studied in detail (88, 89). The best studied bacterial biological control agents are *Bacillus* and *Pseudomonas*. When applied to seed or as a soil drench, specific strains of *Bacillus* and *Pseudomonas* spp. can protect plants against soil borne pathogens (33, 90–92) Effects of these biological control agents can be based on direct inhibition of the pathogen through the production of antimicrobial compounds, eliciting ISR, or a combination of both modes of action (33, 34). The ability of the bacteria to colonize the plant root, also referred to as rhizosphere competence, is crucial for their biocontrol efficacy (93). Inconsistency in the performance of biological control agents is often related to poor establishment of the bacteria in the rhizosphere, resulting in population densities that are below the threshold levels needed for effective biocontrol. As a result many potential biological control agents have been identified over the last decades but relatively few have been developed into commercial products. Commercialized bacterial biocontrol agents include well-studied *Bacillus* and *Pseudomonas* spp. (94). In view of the urgent need of alternatives for chemical control of plant diseases companies have great interest in developing new and innovative commercial products. Recent insights from metagenomic studies suggest that microbial consortia are involved in soil suppressiveness (11, 95, 96), and thus mixed inocula are more likely to be effective in plant protection than single inocula. Moreover, it has been hypothesized that domestication of our crop plants has resulted in degradation of their root microbiome composition and function (5, 97) and thus breeding programs that also focus on a healthy microbiome are crucial to further develop sustainable crop production.

Livestock

Pigs

Prebiotics

Especially with realization that use of in-feed antibiotics as growth promotors can greatly disturb the microbiota in post-wean piglets, studies are aimed at providing prebiotics that can restore beneficial microbes in the gut of piglets. As with most species studied, prebiotics used in pigs are GOS, FOS, MOS, and Cello-oligosaccharides (COS). It was shown that COS increased lactobacilli in jejunal and colonic contents (98). Furthermore, COS increased epithelial barrier function shown by decreased leakage of fluorescein isothiocyanate (FITC-) dextran 4 kDa in jejunum and colon and increased trans-epithelial electrical resistance (TEER) in Ussing chamber experiments. In line with this several tight junction proteins were increased (98).

Alizadeh et al. investigated the effect of GOS in early life. Piglets received a milk replacer with or without the addition of GOS for 3 or 26 days. Dietary GOS increased *Lactobacilli* and *Bifidobacteria* numbers at day 26. Addition of GOS to the diet of piglets increased defensin porcine β-defensin-2 in the colon and secretory IgA levels in saliva (99). In contrast, in another study were neonatal piglets were given formula supplemented with GOS and polydextrose (PDX) *Lactobacillus*

spp. were not increased (100). In all of these studies age of the piglets and duration of the supplementation is different, therefore it is difficult to compare the outcomes. Interestingly, a recent study evaluated the effects of maternal prebiotic consumption on offspring intestinal defenses and immune system responsiveness. The authors showed that maternal short chain FOS supplementation improved ileal cytokine secretions and increased IgA vaccine response to *Lawsonia intracellularis* in the serum and ileal mucosa (101).

In a study assessing the synbiotic effect of FOS and *Bifidobacterium animalis*, Trevisi et al. observed in 21–35 day old piglets that *B. animalis* fed together with FOS (2%) increased TLR2 expression in the lymph node, but did not reduce bacterial translocation (102).

Probiotics

In order to reverse deleterious effects of weaning on microbiota stability there have been numerous studies performed using probiotics in post-weaning piglets. The most frequently used probiotics are members of *Lactobacillus, Bifidobacterium, Enterococcus,* or *Streptococcus*. However, studies are difficult to compare and results may differ from farm to farm. For a comprehensive review on probiotic use and its challenges in pigs we refer to Barba-Vidal et al. that critically reviews the most recent literature on this topic (103).

Ruminants

Prebiotics

Similar to pigs, in calves several types of oligosaccharides have been tested for their health increasing or disease preventing activities (by prevention of pathogen binding to epithelial cells). MOS addition to the diet of 5 day old calves (4 g/calf/day up to 2 months of age) improved growth and decreased the number of coliforms in feces (104). GOS added to the diet of calves promoted *Lactobacillus* and *Bifidobacteria*, but due to its laxative effects had lower growth performance (105). Addition of short chain fructo-oligosaccharides (scFOS) to the diet of 8–10 day old calves increased butyrate production while reducing acetate production, which might have health effects, although only growth was assessed in this study (106). Few studies assessed immunological parameters to investigate whether prebiotics convey health effects. Fleige et al. assessed whether long-term lactulose feeding combined with *Enterococcus faecium* affected immune cell activation markers, cytokine responses and IgA Fc-receptor (107). Supplementation of calf feed with 3% lactulose increased the number of blood lymphocytes. Also a small increase in the expression of IgA Fc-receptor was observed in the ileal mucosa in male calves receiving 1% lactulose, but this was not significantly different in the 3% group. Furthermore, the authors report effects on CD4+ (lower in ileum in lactulose group) and CD8+ (higher in blood of females) T cells as well as decreased levels of IL10 and Interferon gamma in the ileum. Feeding calves COS increased the proportion of *C. coccoides–E. rectale* group, while it had no effect on *Bifidobacteria* and *Lactobacilli,* but did increase butyrate levels, which could have beneficial effects (108, 109).

Probiotics

To counteract low ruminal pH that might cause SARA, species that keep lactate levels stable such as *Enterococcus* and *Lactobacillus*, or that feed on lactate (Megasphaella or Propionibacteria) are used (110–113). In addition yeast is given to aid the digestion of cellulose. Besides studies that show a beneficial effects of pre- or probiotics on the health of calves, there is an equal amount not showing effects [summarized in Uyeno et al. (114)]. This discrepancy in the data might result from farm to farm differences and the health status of the calves at baseline.

Poultry

Prebiotics and probiotics

For an overview on the effects of prebiotic supplementation on the microbiota and health of chicken we refer to an excellent recent review by Pourabedin and Zhao (115). In summary, research has been done on the prebiotic effects of MOS, FOS, XOS (xylooligosaccharides; degradation products of lignocellulose materials), GOS, SMO (soybean meal oligosacchahides), and mainly found, or investigated effects on *Lactobacillus* and *Bifidobacteria*. Future research will focus more on metabolites such as short chain fatty acids (SCFAs) and other possible beneficial microbes that can be discovered as more in depth sequencing of chicken microbiota is undertaken.

Humans

Prebiotics

There is growing recognition of the role of nutritional and therapeutic strategies, including pro- and prebiotics, in targeting the composition and the metabolic activity of the gut microbiota, which can in turn impact human health.

Human milk oligosaccharides (HMO) are the first prebiotics in humans that are essential for postnatal growth and development of the gastrointestinal and immune system as demonstrated by comparing breast-fed infants with formula-fed infants (116). HMO facilitate the establishment of the microbiota, stimulate intestinal development, promote intestinal development and prevent pathogenic infections, as reviewed by Donovan and Comstock (117). Alternatives for HMO, including GOS and FOS, are used in infant formula. These non-digestible oligosaccharides (NDO) have several beneficial health effects. NDO are known to reduce the incidence of allergic manifestations (118–120), stimulate the vaccine-specific immune response (121) and protect against infections (122, 123). GOS are also effective in alleviating symptoms of chronic inflammatory diseases, like irritable bowel syndrome (124) and reduce the prevalence of diarrhea (125), while FOS can promote satiety and weight loss in obese patients (126). There is also evidence that prebiotics can impact various biomarkers of colorectal cancer (127, 128) and Abrams et al., showed that inulin-type fructans enhances mineral absorption, including calcium, and bone mineralization [Abrams et al. (129), also reviewed by Slavin (130)].

These health effects were related to their prebiotic effect, including changes in microbiota composition and stimulation of growth and activity of health-promoting *Lactobacilli* and

Bifidobacteria (131–134), but can also be caused by fermentation products of these bacteria, such as SCFAs (135–137). Moreover, oligosaccharides inhibit the adhesion of pathogens on the epithelial surface (138–140).

The impact of prebiotics on the microbiota influence immune signaling as shown by beneficial effects on the mucosal immune system and gut-associated lymphoid tissue (GALT), increased secretory IgA and mucosal Ig, increasing anti-inflammatory cytokines, decreasing pro-inflammatory cytokines and altering lymphocyte expression [reviewed by Shokryazdan et al. (141) and Wilson and Whelan (142)]. However, there are also microbiota-independent effects of NDOs. Epithelial cells and immune cells can directly interact with oligosaccharides to modulate an immune response, for example via activation of peptidoglycan recognition protein 3 (PGlyRP3) and peroxisome proliferator-activated receptor γ (PPARγ), carbohydrate receptors, such as C-type lectin or Toll-like receptors (TLRs), including TLR4, nucleotide oligomerization domain containing proteins (NODs), 2 and via galectins (143–145). Although, one should take care that, when investigating receptor-mediated signaling of oligosaccharides that the preparations are devoid of LPS contamination, since already small amounts can have effects on immune cells (146). In addition, prebiotics have stabilizing effects on the intestinal barrier and protect against barrier impairment (147–150). The structure, chain length, solubility, fermentability, and viscosity of oligosaccharides are important characteristics that possibly determine the health effect in the host (150–152). Among different dietary compounds, omega-3 (n-3) polyunsaturated fatty acids (PUFA) in the diet have demonstrated a beneficial impact on the intestinal microbiota composition and development (153–155). Polyphenols (e.g., flavonols and quercetin) are a hot topic for future nutritional strategies related to their biological activities, including antimicrobial, antioxidant, or anticarcinogenic activities, and modulation of the gut microbiota by stimulation of beneficial bacteria (156–158). Besides polyphenols, other minor food compounds, including zinc, conjugated linoleic acid, L-carnitine, choline, sphingomyelin, or ellagitannins have been reviewed by Roca-Saavedra et al. (159) to modify the intestinal microbiota and consequently, impact human health.

Probiotics

The complex microbial communities that colonize the human gastrointestinal tract are important in human health and modulation of the intestinal microbiota composition is one of the potential health-beneficial effects of probiotics (160). Probiotics are not only used to maintain and stimulate a healthy microbiota in healthy individuals, but there is increasing scientific evidence that probiotics can be used for prevention and treatment of a large number of disease states and intestinal disorders associated with an unbalanced intestinal microbiota (dysbiosis). The efficacy of probiotics have been demonstrated in diarrhea induced by antibiotics or infections, neonatal necrotizing enterocolitis, inflammatory bowel disease, and irritable bowel syndrome, *Helicobacter pylori* infection, lactose intolerance, and metabolic syndromes (161–163). Various meta-analyses and systematic reviews indicate encouraging effects of probiotics on allergy, atopic diseases, and respiratory infections (164, 165). There is strong evidence that there are interactions between the gut microbiota and the nervous system, therefore the use of pre- and probiotics in preventing or treating neurologic diseases is a topic of great interest (166, 167).

Since clinical benefits of probiotics depend on strain selection, delivery method, dosage, and duration of administration, as well as their ability to survive the stomach pH and reach the the GI tract (168) discrepancies between studies are observed.

Bifidobacterium, Lactobacillus, and *Saccharomyces* are well-known probiotics widely used and studied for improving human health. Probiotics do not always colonize the intestinal tract to exert their benefits, but can also remodel or influence the existing microbiota. Plausible mechanisms, by which probiotics are able to modify the intestinal microbiota and/or induce responses potentially beneficial to the host are: competition for nutrients (and prebiotics), reduction of the luminal pH, induction, and secretion of antimicrobial factors (e.g., bacteriocins, defensins), SCFA production, prevention of pathogen adhesion to epithelial cells, improvement of intestinal barrier function (e.g., via decreased apoptosis of epithelial cells, increased mucin production, and/or modulation of tight junction proteins), modulation of immune responses (e.g., via increasing mucosal immunity, regulating Thelper cell responses, and release of cytokines) (160, 162, 169, 170). Probiotics and/or their soluble factors can communicate with intestinal epithelial cells via TLRs, and in addition, can be transported across the intestinal epithelium by M cells and may elicit the immunomodulatory effects by activating the APCs, influencing the systemic immune responses (171).

MICROBIOTA TRANSFER

Fish

Studies performed in zebrafish and mice revealed that microbial communities are assembled in predictable ways. Rawls et al. transplanted mouse intestinal content into zebrafish and vice versa to investigate whether microbial communities are shaped by the host. It was found that the transplanted community resembled its community of origin (donor) in terms of the species that were present, but the relative abundance of these species changed to resemble the normal gut microbial community composition of the recipient host. This means that microbial communities arise in part from distinct selective pressures imposed within the gut habitat of each host (7). Recent studies using large numbers of zebrafish showed that host factors can act as an ecological filter, but this can be overwhelmed by other factors, including transmission of microbes among hosts (21, 22). This suggests that microbial management in fish must be targeted at group level and that microbial transplants in fish might be a little more challenging.

Plants

A classic experiment in studies on disease suppressive soils is the transfer of tiny amounts of suppressive soil to a disease conducive soil, leading to the transfer of disease suppressiveness. One of the first documented transfer experiments was published

in 1931 by Henry. In this study an amount of microbially active soil that adhered to the tip of a moist sterile platinum needle was transferred to 50 g of sterilized soil and resulted in almost complete suppression of *Helminthosporium* foot rot of wheat. Such transfer experiments have since then been used to study the involvement of microbes in disease suppressive soils. In disease suppressive fields plants are protected against disease, whereas in adjacent fields with similar chemical and physical soil conditions plants become diseased. The best studied example is take-all decline of wheat, in which soil that is cultivated to wheat continuously develops suppressiveness against the take-all pathogen *Gaeumannomyces graminis* var. *tritici* (35). Upon mixing take-all suppressive into conducive soil in a 1:9 ratio, the resulting mix was suppressive to the disease (172). Similar experiments have been reported for *Rhizoctonia solani* suppressive sugar beet fields (11) and *Fusarium oxysporum* suppressive strawberry fields (173). Using both cultivation dependent and sequencing based cultivation independent methods, microbes and functions involved in the control of the disease were identified in the above mentioned studies. Obviously transferring 10% suppressive soil into a conducive soil is unrealistic in practice, but elucidating the mechanisms underlying suppressiveness and especially deciphering how plants assemble their disease suppressive microbiomes will be instrumental in sustaining healthy plant microbiomes. For many suppressive soils a severe disease outbreak is needed for suppressiveness to develop, suggesting that both the plant and the pathogen need to be present to assemble a protective microbiome. Thus, it was postulated that plants can "cry for help" upon pathogen attack resulting in specific changes in their microbiomes. In *Arabidopsis thaliana* it was demonstrated that aboveground infection with the downy mildew pathogen *Hyaloperonospora arabidopsidis* results in the assemblage of a plant beneficial bacterial consortium, and effects of the disease induced changes can protect a new population of plants growing in the same soil (96). Uncovering plant cues that govern this disease induced microbiome assemblage will facilitate directed manipulation of the rhizosphere microbiome in a sensible manner.

Livestock

In ruminants, transfaunation of ruminal contents, which contains protozoa, bacteria, and methanogenic archaea as major components, has been a common treatment to improve rumen functions and milk production (174). Studies have shown that the ciliated protozoa responsible for digestion of plant materials, can be successfully transferred, but bacterial community seems to be more resistant, which may be due to high host-specific properties (175). While an early study has shown that inoculation of fecal microbiota of healthy adult broilers could reduce the number of *Salmonella infantis* in newly hatched chickens (51), fecal microbiota transplantation (FMT) from chickens with good feed efficiency has not been proved to be effective for modulating the feed efficiently of recipient chickens (176). Similarly in pigs, both positive and negative effects of FMT have been reported. Weight gain and improved innate immunity as well as low rate of diarrhea of piglets orally inoculated with fecal microbiota suspension of healthy adult have been reported (177). In contrast, McCormack et al., have reported that FMT from highly feed-efficient pigs have not been able to deliver the donor phenotype but rather had detrimental effects on recipient sows and their piglets, while increased innate host defense signaling has also been observed, which can be attributed to the altered intestinal microbiota as has been shown in the Hu's study (178). Ribeiro et al. have made an interesting attempt to transfer rumen contents of bison, which may be efficient at digesting low-quality forages, to cattle and found increased protein digestibility and nitrogen retention, while fiber digestibility was not improved (179). This kind of wild-to-domestic microbiota transplant would be an intriguing strategy to regain "extinct" microbial members through domestication, but microbial transfer between genetically different animals should be carefully conducted, since the treatment could be resulted in disturbance of original microbiota (180). In general, the major obstacle of FMT in young livestock animals for meat production may be that FMT can interrupt normal microbial acquisition such as parental transfer. Therefore, understanding the developmental timeline of intestinal microbial community assemblage of the targeted animals would be crucial for contriving future FMT strategies (181).

Humans

In order to restore a healthy balance between human host and microbes, there has been growing interest in the use of FMT, which entails stool transfer from a healthy donor into a patient's intestine. This technique durably alters the gut microbiota of the recipient. The introduced bacterial strains are easily accepted and persist in an established microbial community in the intestine, however, individual differences of microbiota resistance and donor-recipient compatibilities are indicated after FMT (182).

The microbial community for transplantation can be instilled by various methods, including nasogastric or nasointestinal tubes, endoscopy, colonoscopy, rectal tubes, sigmoidoscopy, enema, or encapsulated formulations, or a combined approach, but there is no clear consensus regarding the optimal instillation method (183). To date, most clinical experience has focused on the use of fecal transplants in patients with recurrent *Clostridium difficile* infection and FMT has become established as a highly efficacious and safe treatment method for these patients (183, 184). It is actively studied as treatment option in inflammatory bowel disease, irritable bowel syndrome and metabolic syndromes, however, evidence is still limited and more randomized controlled trials are needed (184–187). FMT may also have potential applications in a variety of other conditions associated with intestinal dysbiosis, including neuropsychiatric disorders, allergic disorders, and auto-immune disorders as reviewed by Xu et al. (188).

Future work will focus on the standardization of donor screening/selection, feces preparation, clinical application, microbiome analysis, obtaining more robust (long-term) safety data, excluding unwanted co-transfer of pathogenic microbes, the understanding of the exact microbial recovery mechanism (183, 186, 189, 190). Especially, determining what constitutes a

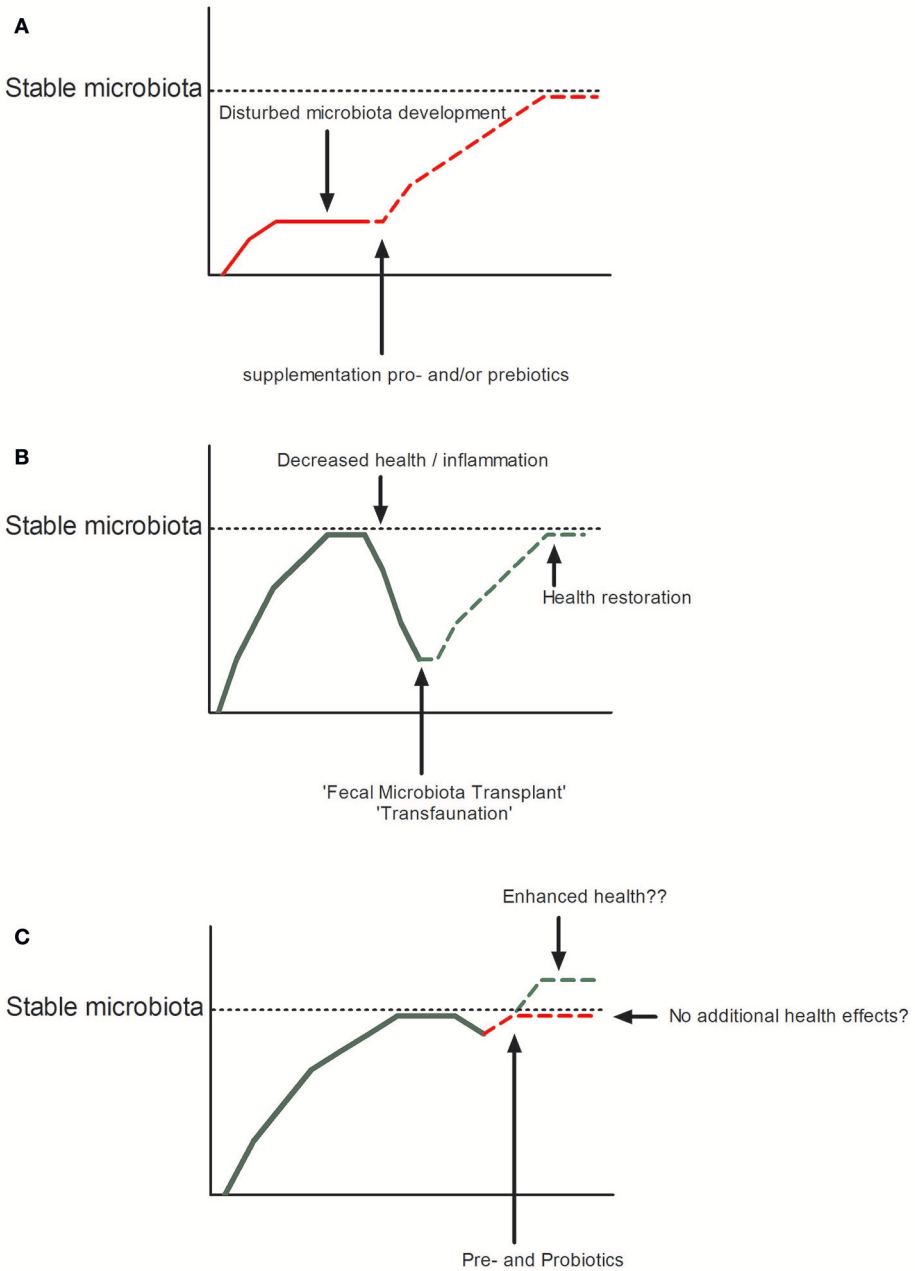


FIGURE 2 | Restoration or improvement of microbial homeostasis in disturbed (**A**: by pro- or prebiotics or **B**: Fecal Microbiota Transplant) or undisturbed states **(C)**.

healthy microbiota that can be safely transferred will still needs more fundamental research.

CONCLUSION

Antibiotic resistance is on the rise, due to many years of large-scale use of antibiotics as growth enhancers in livestock and aquaculture. This poses a threat not only to the health of our production animals but to the human population as well. Legislation preventing overuse of antibiotics has led to the rapid emergence of studies into the use of pre- and probiotics in fish, plants, livestock animals, and humans. As we have tried to illustrate in this review, a lot of progress has been made. Pre- and probiotics are used to increase early life health and help reach a stable healthy microbiota and fecal transplants have been shown to successfully restore health (**Figure 2**). Whether pre- and probiotics can enhance health when a stable microbiota is in place still remains an open question. Although from the above mentioned studies it has emerged that oligosaccharides may stimulate certain beneficial microbes to persist or even become

dominant, most of the time we do not understand the mechanism by which these species influence the health of their host, if at all. Dosing, duration, and age at which pre- and probiotics are given might all determine whether one observes effects or not. As for the effect of probiotics, are these effects transient, or do *Lactobacilli* and *Bifidobacteria* need to persist for a long time to elicit their health effects. Furthermore, what determines whether a microbe is beneficial within the genera of *Lactobacilli* and *Bifidobacteria*? This might as well be host-specific. Considering the interplay between the host and members of the microbiota selecting a "healthy microbiota" for fecal transplantation may not be as straightforward as we now believe. Depending on the genetic make-up of the individual, microbes might behave different in different hosts. Specifically, we need to make sure we are not copying that what works in one species directly to other species without understanding the effects. In fish for example, the ratio Bacteroidetes/Proteobacteria increases upon inflammation (191), while in humans and mice a decrease of this ratio is associated with inflammation (mainly due to an increase of gamma-Proteobacteria) (192, 193) and in plants Pseudomonas (a gamma-Proteobacterium) is considered to be beneficial (94). Furthermore, some experiments are performed *in vitro* in which the tissue context and immune system of the entire organism is lacking, while others are performed *in vivo* in which one is limited by the read-out parameters that can be investigated or controlled. However, the fact that we know that all hosts select certain species that can protect them from colonization or infection with pathogens is a strong lesson from nature we can exploit in our artificial rearing and culturing conditions in agriculture and human medicine. Furthermore, in an exciting new paper published in Science in 2018 Manfredo Vieira et al. (194) showed that translocation of the gut pathobiont, *Enterococcus gallinarum*, to the liver and other systemic tissues triggers autoimmune responses in a genetic background predisposing to autoimmunity in mice. This illustrates nicely, that we are still in the beginning of understanding host-microbe interaction. Therefore, more fundamental species specific research is needed to fully understand the vast network of interactions between the microbial world and their hosts. Dissecting the intimate relationship between the host and its microbial community can uncover novel mechanisms that might be exploited to restore microbial community structure in those plants and animals that suffer from dysbiosis.

AUTHOR CONTRIBUTIONS

SyB, WI-O, SaB, GF, CP, and PB all wrote the manuscript according to their expertise and added to different sections. SyB wrote the paragraphs on fish and livestock, WI-O wrote the FMT of livestock, SaB and GF wrote the paragraphs on humans, CP and PB wrote the paragraphs on plants.

ACKNOWLEDGMENTS

The authors would like to thank the Netherlands Organization for Scientific Research (NWO) for funding the Frontiers in Agricultural Immunology workshop (Lorentz Center) and the Core to Core program of the Japan Society for the promotion of science (JSPS) enabling scientific collaboration between the Netherlands and Japan.

REFERENCES

1. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. *Science* (2005) 307:1915–20. doi: 10.1126/science.1104816
2. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. *Cell* (2010) 140:859–70. doi: 10.1016/j.cell.2010.01.023
3. Dimitroglou A, Merrifield DL, Carnevali O, Picchietti S, Avella M, Daniels C, et al. Microbial manipulations to improve fish health and production–a Mediterranean perspective. *Fish Shellfish Immunol.* (2011) 30:1–16. doi: 10.1016/j.fsi.2010.08.009
4. Berendsen RL, Pieterse CM, Bakker PA. The rhizosphere microbiome and plant health. *Trends Plant Sci.* (2012) 17:478–86. doi: 10.1016/j.tplants.2012.04.001
5. Pieterse CMJ, de Jonge R, Berendsen RL. The soil-borne supremacy. *Trends Plant Sci.* (2016) 21:171–3. doi: 10.1016/j.tplants.2016.01.018
6. Andreote FD, Pereira ESMC. Microbial communities associated with plants: learning from nature to apply it in agriculture. *Curr Opin Microbiol.* (2017) 37:29–34. doi: 10.1016/j.mib.2017.03.011
7. Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* (2006) 127:423–33. doi: 10.1016/j.cell.2006.08.043
8. Brugman S, Schneeberger K, Witte M, Klein MR, van den Bogert B, Boekhorst J, et al. T lymphocytes control microbial composition by regulating the abundance of Vibrio in the zebrafish gut. *Gut Microbes* (2014) 5:737–47. doi: 10.4161/19490976.2014.972228
9. Raaijmakers JM, Mazzola M. Soil immune responses. *Science* (2016) 352:1392–3. doi: 10.1126/science.aaf3252
10. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. *Nat Rev Microbiol.* (2017) 15:630–8. doi: 10.1038/nrmicro.2017.58
11. Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, et al. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* (2011) 332:1097–100. doi: 10.1126/science.1203980
12. Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, et al. Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. *Sci Transl Med.* (2017) 9:eaah4680. doi: 10.1126/scitranslmed.aah4680
13. Fiebiger U, Bereswill S, Heimesaat MM. Dissecting the interplay between intestinal microbiota and host immunity in health and disease: lessons learned from germfree and gnotobiotic animal models. *Eur J Microbiol Immunol.* (2016) 6:253–71. doi: 10.1556/1886.2016.00036
14. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* (2010) 140:845–58. doi: 10.1016/j.cell.2010.02.021
15. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature* (2011) 474:327–36. doi: 10.1038/nature10213
16. Liu Y, de Bruijn I, Jack AL, Drynan K, van den Berg AH, Thoen E, et al. Deciphering microbial landscapes of fish eggs to mitigate emerging diseases. *ISME J.* (2014) 8:2002–14. doi: 10.1038/ismej.2014.44
17. Carbajal-Gonzalez MT, Fregeneda-Grandes JM, Suarez-Ramos S, Rodriguez Cadenas F, Aller-Gancedo JM. Bacterial skin flora variation and *in vitro* inhibitory activity against Saprolegnia parasitica in brown and rainbow trout. *Dis Aquat Org.* (2011) 96:125–35. doi: 10.3354/dao02391
18. Boutin S, Bernatchez L, Audet C, Derome N. Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against

Flavobacterium columnare and F. *psychrophilum*. *Vet Microbiol.* (2012) 155:355–61. doi: 10.1016/j.vetmic.2011.09.002
19. Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, Knight R, et al. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol Ecol.* (2012) 21:3363–78. doi: 10.1111/j.1365-294X.2012.05552.x
20. Rawls JF, Samuel BS, Gordon JI. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci USA.* (2004) 101:4596–601. doi: 10.1073/pnas.0400706101
21. Burns AR, Miller E, Agarwal M, Rolig AS, Milligan-Myhre K, Seredick S, et al. Interhost dispersal alters microbiome assembly and can overwhelm host innate immunity in an experimental zebrafish model. *Proc Natl Acad Sci USA.* (2017) 114:11181–6. doi: 10.1073/pnas.1702511114
22. Stagaman K, Burns AR, Guillemin K, Bohannan BJ. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *ISME J.* (2017) 11:1630–9. doi: 10.1038/ismej.2017.28
23. Brugman S, Liu KY, Lindenbergh-Kortleve D, Samsom JN, Furuta GT, Renshaw SA, et al. Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota. *Gastroenterology* (2009) 137:1757.e1–67.e1. doi: 10.1053/j.gastro.2009.07.069
24. Mohammed HH, Arias CR. Potassium permanganate elicits a shift of the external fish microbiome and increases host susceptibility to columnaris disease. *Vet Res.* (2015) 46:82. doi: 10.1186/s13567-015-0215-y
25. He S, Wang Q, Li S, Ran C, Guo X, Zhang Z, et al. Antibiotic growth promoter olaquindox increases pathogen susceptibility in fish by inducing gut microbiota dysbiosis. *Sci China Life Sci.* (2017) 60:1260–70. doi: 10.1007/s11427-016-9072-6
26. Piazzon MC, Calduch-Giner JA, Fouz B, Estensoro I, Simo-Mirabet P, Puyalto M, et al. Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* (2017) 5:164. doi: 10.1186/s40168-017-0390-3
27. Berg G, Smalla K. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol.* (2009) 68:1–13. doi: 10.1111/j.1574-6941.2009.00654.x
28. Meneses C, Silva B, Medeiros B, Serrato R, Johnston-Monje D. A metagenomic advance for the cloning and characterization of a cellulase from red rice crop residues. *Molecules* (2016) 21:E831. doi: 10.3390/molecules21070831
29. Bakker PA, Berendsen RL, Doornbos RF, Wintermans PC, Pieterse CM. The rhizosphere revisited: root microbiomics. *Front Plant Sci.* (2013) 4:165. doi: 10.3389/fpls.2013.00165
30. Sasse J, Martinoia E, Northen T. Feed your friends: do plant exudates shape the root microbiome? *Trends Plant Sci.* (2018) 23:25–41. doi: 10.1016/j.tplants.2017.09.003
31. Bai Y, Muller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the Arabidopsis leaf and root microbiota. *Nature* (2015) 528:364–9. doi: 10.1038/nature16192
32. Bakker P, Pieterse CMJ, de Jonge R, Berendsen RL. The soil-borne legacy. *Cell* (2018) 172:1178–80. doi: 10.1016/j.cell.2018.02.024
33. Haas D, Defago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol.* (2005) 3:307–19. doi: 10.1038/nrmicro1129
34. Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA. Induced systemic resistance by beneficial microbes. *Annu Rev Phytopathol.* (2014) 52:347–75. doi: 10.1146/annurev-phyto-082712-102340
35. Weller DM, Raaijmakers JM, Gardener BB, Thomashow LS. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol.* (2002) 40:309–48. doi: 10.1146/annurev.phyto.40.030402.110010
36. Mendes LW, Raaijmakers JM, de Hollander M, Mendes R, Tsai SM. Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. *ISME J.* (2018) 12:212–24. doi: 10.1038/ismej.2017.158
37. Pajarillo EA, Chae JP, Balolong MP, Kim HB, Seo KS, Kang DK. Pyrosequencing-based analysis of fecal microbial communities in three purebred pig lines. *J Microbiol.* (2014) 52:646–51. doi: 10.1007/s12275-014-4270-2
38. Kim HB, Isaacson RE. The pig gut microbial diversity: understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Vet Microbiol.* (2015) 177:242–51. doi: 10.1016/j.vetmic.2015.03.014
39. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends Microbiol.* (2017) 25:851–73. doi: 10.1016/j.tim.2017.05.004
40. Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, et al. Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J.* (2014) 8:1566–76. doi: 10.1038/ismej.2014.12
41. Johnson TA, Looft T, Severin AJ, Bayles DO, Nasko DJ, Wommack KE, et al. The in-feed antibiotic carbadox induces phage gene transcription in the swine gut microbiome. *mBio* (2017) 8:e00709-17. doi: 10.1128/mBio.00709-17
42. Brown DC, Maxwell CV, Erf GF, Davis ME, Singh S, Johnson ZB. The influence of different management systems and age on intestinal morphology, immune cell numbers and mucin production from goblet cells in post-weaning pigs. *Vet Immunol Immunopathol.* (2006) 111:187–98. doi: 10.1016/j.vetimm.2005.12.006
43. Danielsson R, Dicksved J, Sun L, Gonda H, Muller B, Schnurer A, et al. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front Microbiol.* (2017) 8:226. doi: 10.3389/fmicb.2017.00226
44. Uyeno Y, Sekiguchi Y, Kamagata Y. rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. *Lett Appl Microbiol.* (2010) 51:570–7. doi: 10.1111/j.1472-765X.2010.02937.x
45. Uyeno Y, Sekiguchi Y, Tajima K, Takenaka A, Kurihara M, Kamagata Y. An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of *Holstein heifers*. *Anaerobe* (2010) 16:27–33. doi: 10.1016/j.anaerobe.2009.04.006
46. Meale SJ, Li S, Azevedo P, Derakhshani H, Plaizier JC, Khafipour E, et al. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front Microbiol.* (2016) 7:582. doi: 10.3389/fmicb.2016.00582
47. Khafipour E, Li S, Plaizier JC, Krause DO. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl Environ Microbiol.* (2009) 75:7115–24. doi: 10.1128/AEM.00739-09
48. Kleen JL, Hooijer GA, Rehage J, Noordhuizen JP. Subacute ruminal acidosis (SARA): a review. *J Vet Med A Physiol Pathol Clin Med.* (2003) 50:406–14. doi: 10.1046/j.1439-0442.2003.00569.x
49. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KM. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J Dairy Sci.* (2005) 88:1399–403. doi: 10.3168/jds.S0022-0302(05)72807-1
50. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. *FEMS Microbiol Lett.* (2014) 360:100–12. doi: 10.1111/1574-6968.12608
51. Rantala M, Nurmi E. Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *Br Poult Sci.* (1973) 14:627–30. doi: 10.1080/00071667308416073
52. Lee KW, Lillehoj HS, Jeong W, Jeoung HY, An DJ. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. *Poult Sci.* (2011) 90:1381–90. doi: 10.3382/ps.2010-01319
53. Antonissen G, Eeckhaut V, Van Driessche K, Onrust L, Haesebrouck F, Ducatelle R, et al. Microbial shifts associated with necrotic enteritis. *Avian Pathol.* (2016) 45:308–12. doi: 10.1080/03079457.2016.1152625
54. Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathol.* (2004) 33:537–49. doi: 10.1080/03079450400013162
55. Collier CT, Hofacre CL, Payne AM, Anderson DB, Kaiser P, Mackie RI, et al. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. *Vet Immunol Immunopathol.* (2008) 122:104–15. doi: 10.1016/j.vetimm.2007.10.014
56. Ahern PP, Faith JJ, Gordon JI. Mining the human gut microbiota for effector strains that shape the immune system. *Immunity* (2014) 40:815–23. doi: 10.1016/j.immuni.2014.05.012

57. Geuking MB, Koller Y, Rupp S, McCoy KD. The interplay between the gut microbiota and the immune system. *Gut Microbes* (2014) 5:411–8. doi: 10.4161/gmic.29330
58. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. *Science* (2016) 352:539–44. doi: 10.1126/science.aad9378
59. Gomez de Aguero, M, Ganal-Vonarburg, SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. *Science* (2016) 351:1296–302. doi: 10.1126/science.aad2571
60. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* (2012) 489:220–30. doi: 10.1038/nature11550
61. Passos M, Moraes-Filho JP. Intestinal microbiota in digestive diseases. *Arq Gastroenterol.* (2017) 54:255–62. doi: 10.1590/s0004-2803.201700000-31
62. Collado MC, Rautava S, Isolauri E, Salminen S. Gut microbiota: a source of novel tools to reduce the risk of human disease? *Pediatr Res.* (2015) 77:182–8. doi: 10.1038/pr.2014.173
63. Mahmoodpoor F, Rahbar Saadat Y, Barzegari A, Ardalan M, Zununi Vahed S. The impact of gut microbiota on kidney function and pathogenesis. *Biomed. Pharmacother.* (2017) 93:412–9. doi: 10.1016/j.biopha.2017.06.066
64. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
65. Lee WJ, Hase K. Gut microbiota-generated metabolites in animal health and disease. *Nat Chem Biol.* (2014) 10:416–24. doi: 10.1038/nchembio.1535
66. Momeni-Moghaddam P, Keyvanshokooh S, Ziaei-Nejad S, Parviz Salati A, Pasha-Zanoosi H. Effects of mannan oligosaccharide supplementation on growth, some immune responses and gut lactic acid bacteria of common carp (*Cyprinus carpio*) fingerlings. *Vet Res Forum* (2015) 6:239–44.
67. Torrecillas S, Makol A, Caballero MJ, Montero D, Dhanasiri AK, Sweetman J, et al. Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. *J Fish Dis.* (2012) 35:591–602. doi: 10.1111/j.1365-2761.2012.01384.x
68. Torrecillas S, Makol A, Caballero MJ, Montero D, Robaina L, Real F, et al. Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish Shellfish Immunol.* (2007) 23:969–81. doi: 10.1016/j.fsi.2007.03.007
69. Torrecillas S, Makol A, Benitez-Santana T, Caballero MJ, Montero D, Sweetman J, et al. Reduced gut bacterial translocation in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Fish Shellfish Immunol.* (2011) 30:674–81. doi: 10.1016/j.fsi.2010.12.020
70. Burr G, Hume M, Ricke S, Nisbet D, Gatlin, DIII. *In vitro* and *in vivo* evaluation of the prebiotics GroBiotic-A, inulin, mannanoligosaccharide, and galactooligosaccharide on the digestive microbiota and performance of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). *Microb Ecol.* (2010) 59:187–98. doi: 10.1007/s00248-009-9597-6
71. Dimitroglou A, Merrifield DL, Moate R, Davies SJ, Spring P, Sweetman J, et al. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Anim Sci.* (2009) 87:3226–34. doi: 10.2527/jas.2008-1428
72. Carbone D, Faggio C. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish Shellfish Immunol.* (2016) 54:172–8. doi: 10.1016/j.fsi.2016.04.011
73. Azimirad M, Meshkini S, Ahmadifard N, Hoseinifar SH. The effects of feeding with synbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult Artemia on skin mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish (*Pterophyllum scalare*). *Fish Shellfish Immunol.* (2016) 54:516–22. doi: 10.1016/j.fsi.2016.05.001
74. Hoseinifar SH, Soleimani N, Ringo E. Effects of dietary fructo-oligosaccharide supplementation on the growth performance, haemato-immunological parameters, gut microbiota and stress resistance of common carp (*Cyprinus carpio*) fry. *Br J Nutr.* (2014) 112:1296–302. doi: 10.1017/S0007114514002037
75. Akrami R, Iri Y, Rostami HK, Razeghi Mansour M. Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, lactobacillus bacterial population and hemato-immunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile. *Fish Shellfish Immunol.* (2013) 35:1235–9. doi: 10.1016/j.fsi.2013.07.039
76. Ran C, Hu J, Liu W, Liu Z, He S, Dan BC, et al. Thymol and carvacrol affect hybrid tilapia through the combination of direct stimulation and an intestinal microbiota-mediated effect: insights from a germ-free zebrafish model. *J Nutr.* (2016) 146:1132–40. doi: 10.3945/jn.115.229377
77. Banerjee G, Ray AK. The advancement of probiotics research and its application in fish farming industries. *Res Vet Sci.* (2017) 115:66–77. doi: 10.1016/j.rvsc.2017.01.016
78. Makridis P, Papandroulakis N, Divanach P. Use of Phaeobacter sp. probiotic bacteria for the rearing of sea bass larvae (*Dicentrarchus labrax*). *Commun Agric Appl Biol Sci.* (2013) 78:259–61.
79. Rasmussen BB, Grotkjaer T, D'Alvise PW, Yin G, Zhang F, Bunk B, et al. *Vibrio anguillarum* is genetically and phenotypically unaffected by long-term continuous exposure to the antibacterial compound tropodithietic acid. *Appl Environ Microbiol.* (2016) 82:4802–10. doi: 10.1128/AEM.01047-16
80. Cerezuela R, Meseguer J, Esteban MA. Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* (2013) 34:843–8. doi: 10.1016/j.fsi.2012.12.026
81. Larkin RP. Soil health paradigms and implications for disease management. *Annu Rev Phytopathol.* (2015) 53:199–221. doi: 10.1146/annurev-phyto-080614-120357
82. Ling N, Zhu C, Xue C, Chen H, Duan Y, Peng C, et al. Insight into how organic amendments can shape the soil microbiome in long-term field experiments as revealed by network analysis. *Soil Biol Biochem.* (2016) 99:137–49. doi: 10.1016/j.soilbio.2016.05.005
83. Inderbitzin P, Ward J, Barbella A, Solares N, Izyumin D, Burman P, et al. Soil microbiomes associated with verticillium wilt-suppressive broccoli and chitin amendments are enriched with potential biocontrol agents. *Phytopathology* (2018) 108:31–43. doi: 10.1094/PHYTO-07-17-0242-R
84. Lehmann J, Rillig MC, Thies J, Masiello CA, Hockaday WC, Crowley D. Biochar effects on soil biota–a review. *Soil Biol Biochem.* (2011) 43:1812–36. doi: 10.1016/j.soilbio.2011.04.022
85. Jenkins JR, Viger M, Arnold EC, Harris ZM, Ventura M, Miglietta F, et al. Biochar alters the soil microbiome and soil function: results of next-generation amplicon sequencing across Europe. *GCB Bioenergy* (2017) 9:591–612. doi: 10.1111/gcbb.12371
86. Elad Y, David DR, Harel YM, Borenshtein M, Kalifa HB, Silber A, et al. Induction of systemic resistance in plants by biochar, a soil-applied carbon sequestering agent. *Phytopathology* (2010) 100:913–21. doi: 10.1094/PHYTO-100-9-0913
87. Stringlis IA, Yu K, Feussner K, De Jonge R, Van Bentum S, Van Verk MC, et al. MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc Natl Acad Sci USA* (2018) 115:E5213–22. doi: 10.1073/pnas.1722335115
88. Compant S, Duffy B, Nowak J, Clement C, Barka EA. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol.* (2005) 71:4951–9. doi: 10.1128/AEM.71.9.4951-4959.2005
89. Timmusk S, Behers L, Muthoni J, Muraya A, Aronsson AC. Perspectives and challenges of microbial application for crop improvement. *Front Plant Sci.* (2017) 8:49. doi: 10.3389/fpls.2017.00049
90. Ongena M, Jacques P, Delfosse P, Thonart P. Unusual traits of the pyoverdin-mediated iron acquisition system in Pseudomonas putida strain BTP1. *Biometals* (2002) 15:1–13. doi: 10.1023/A:1013157824411
91. Ongena M, Jacques P, Toure Y, Destain J, Jabrane A, Thonart P. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl Microbiol Biotechnol.* (2005) 69:29–38. doi: 10.1007/s00253-005-1940-3
92. Ongena M, Jacques P. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* (2008) 16:115–25. doi: 10.1016/j.tim.2007.12.009
93. Lugtenberg BJ, Dekkers L, Bloemberg GV. Molecular determinants of rhizosphere colonization by Pseudomonas. *Annu Rev Phytopathol.* (2001) 39:461–90. doi: 10.1146/annurev.phyto.39.1.461

94. Fravel DR. Commercialization and implementation of biocontrol. *Annu Rev Phytopathol.* (2005) 43:337–59. doi: 10.1146/annurev.phyto.43.032904.092924
95. Chapelle E, Mendes R, Bakker PA, Raaijmakers JM. Fungal invasion of the rhizosphere microbiome. *ISME J.* (2016) 10:265–8. doi: 10.1038/ismej.2015.82
96. Berendsen RL, Vismans G, Yu K, Song Y, de Jonge R, Burgman WP, et al. Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* (2018) 12:1496–507. doi: 10.1038/s41396-018-0093-1
97. Perez-Jaramillo JE, Mendes R, Raaijmakers JM. Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol Biol.* (2016) 90:635–44. doi: 10.1007/s11103-015-0337-7
98. Jiao LF, Ke YL, Xiao K, Song ZH, Hu CH, Shi B. Effects of cello-oligosaccharide on intestinal microbiota and epithelial barrier function of weanling pigs. *J Anim Sci.* (2015) 93:1157–64. doi: 10.2527/jas.2014-8248
99. Alizadeh A, Akbari P, Difilippo E, Schols HA, Ulfman LH, Schoterman MH, et al. The piglet as a model for studying dietary components in infant diets: effects of galacto-oligosaccharides on intestinal functions. *Br J Nutr.* (2016) 115:605–18. doi: 10.1017/S0007114515004997
100. Hoeflinger JL, Kashtanov DO, Cox SB, Dowd SE, Jouni ZE, Donovan SM, et al. Characterization of the intestinal Lactobacilli community following galactooligosaccharides and polydextrose supplementation in the neonatal piglet. *PLoS ONE* (2015) 10:e0135494. doi: 10.1371/journal.pone.0135494
101. Le Bourgot C, Le Normand L, Formal M, Respondek F, Blat S, Apper E, et al. Maternal short-chain fructo-oligosaccharide supplementation increases intestinal cytokine secretion, goblet cell number, butyrate concentration and *Lawsonia intracellularis* humoral vaccine response in weaned pigs. *Br J Nutr.* (2017) 117:83–92. doi: 10.1017/S0007114516004268
102. Trevisi P, De Filippi S, Minieri L, Mazzoni M, Modesto M, Biavati B, et al. Effect of fructo-oligosaccharides and different doses of *Bifidobacterium animalis* in a weaning diet on bacterial translocation and Toll-like receptor gene expression in pigs. *Nutrition* (2008) 24:1023–9. doi: 10.1016/j.nut.2008.04.008
103. Barba-Vidal E, Martin-Orue SM, Castillejos L. Review: are we using probiotics correctly in post-weaning piglets? *Animal* (2018). doi: 10.1017/S1751731118000873. [Epub ahead of print].
104. Ghosh S, Mehla RK. Influence of dietary supplementation of prebiotics (mannanoligosaccharide) on the performance of crossbred calves. *Trop Anim Health Prod.* (2012) 44:617–22. doi: 10.1007/s11250-011-9944-8
105. Castro JJ, Gomez A, White BA, Mangian HJ, Loften JR, Drackley JK. Changes in the intestinal bacterial community, short-chain fatty acid profile, and intestinal development of preweaned Holstein calves. 1. Effects of prebiotic supplementation depend on site and age. *J Dairy Sci.* (2016) 99:9682–702. doi: 10.3168/jds.2016-11006
106. Grand E, Respondek F, Martineau C, Detilleux J, Bertrand G. Effects of short-chain fructooligosaccharides on growth performance of preruminant veal calves. *J Dairy Sci.* (2013) 96:1094–101. doi: 10.3168/jds.2011-4949
107. Fleige S, Preissinger W, Meyer HH, Pfaffl MW. The immunomodulatory effect of lactulose on *Enterococcus faecium* fed preruminant calves. *J Anim Sci.* (2009) 87:1731–8. doi: 10.2527/jas.2007-0494
108. Duncan SH, Louis P, Flint HJ. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl Environ Microbiol.* (2004) 70:5810–7. doi: 10.1128/AEM.70.10.5810-5817.2004
109. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett.* (2009) 294:1–8. doi: 10.1111/j.1574-6968.2009.01514.x
110. Ghorbani GR, Morgavi DP, Beauchemin KA, Leedle JA. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. *J Anim Sci.* (2002) 80:1977–85. doi: 10.2527/2002.8071977x
111. Nocek JE, Kautz WP, Leedle JA, Allman JG. Ruminal supplementation of direct-fed microbials on diurnal pH variation and *in situ* digestion in dairy cattle. *J Dairy Sci.* (2002) 85:429–33. doi: 10.3168/jds.S0022-0302(02)74091-5
112. Klieve AV, Hennessy D, Ouwerkerk D, Forster RJ, Mackie RI, Attwood GT. Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. *J Appl Microbiol.* (2003) 95:621–30. doi: 10.1046/j.1365-2672.2003.02024.x
113. Nocek JE, Socha MT, Tomlinson DJ. The effect of trace mineral fortification level and source on performance of dairy cattle. *J Dairy Sci.* (2006) 89:2679–93. doi: 10.3168/jds.S0022-0302(06)72344-X
114. Uyeno Y, Shigemori S, Shimosato T. Effect of probiotics/prebiotics on cattle health and productivity. *Microb Environ.* (2015) 30:126–32. doi: 10.1264/jsme2.ME14176
115. Pourabedin, M., and Zhao, X. (2015). Prebiotics and gut microbiota in chickens. *FEMS Microbiol Lett.* 362:fnv122. doi: 10.1093/femsle/fnv122
116. Vael C, Desager K. The importance of the development of the intestinal microbiota in infancy. *Curr Opin Pediatr.* (2009) 21:794–800. doi: 10.1097/MOP.0b013e328332351b
117. Donovan SM, Comstock SS. Human milk oligosaccharides influence neonatal mucosal and systemic immunity. *Ann Nutr Metab.* (2016) 69(Suppl 2):42–51. doi: 10.1159/000452818
118. Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, Boehm G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *J Nutr.* (2008) 138:1091–5. doi: 10.1093/jn/138.6.1091
119. Gourbeyre P, Desbuards N, Gremy G, Tranquet O, Champ M, Denery-Papini S, et al. Perinatal and postweaning exposure to galactooligosaccharides/inulin prebiotics induced biomarkers linked to tolerance mechanism in a mouse model of strong allergic sensitization. *J Agric Food Chem.* (2013) 61:6311–20. doi: 10.1021/jf305315g
120. Verheijden KA, Braber S, Leusink-Muis T, Thijssen S, Boon L, Kraneveld AD, et al. Regulatory T cell depletion abolishes the protective effect of dietary galacto-oligosaccharides on eosinophilic airway inflammation in house dust mite-induced asthma in mice. *J Nutr.* (2016). 146, 831–837. doi: 10.3945/jn.115.224402
121. Vos AP, Knol J, Stahl B, M'Rabet L, Garssen, J. Specific prebiotic oligosaccharides modulate the early phase of a murine vaccination response. *Int. Immunopharmacol.* (2010) 10:619–25. doi: 10.1016/j.intimp.2010.02.014
122. Arslanoglu S, Moro GE, Boehm G. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. *J Nutr.* (2007) 137:2420–4. doi: 10.1093/jn/137.11.2420
123. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Long-term safety and impact on infection rates of postnatal probiotic and prebiotic (synbiotic) treatment: randomized, double-blind, placebo-controlled trial. *Pediatrics* (2008) 122:8–12. doi: 10.1542/peds.2007-1192
124. Silk DB, Davis A, Vulevic J, Tzortzis G, Gibson GR. Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome. *Aliment Pharmacol Ther.* (2009) 29:508–18. doi: 10.1111/j.1365-2036.2008.03911.x
125. Drakoularakou A, Tzortzis G, Rastall RA, Gibson GR. A double-blind, placebo-controlled, randomized human study assessing the capacity of a novel galacto-oligosaccharide mixture in reducing travellers' diarrhoea. *Eur J Clin Nutr.* (2010) 64:146–52. doi: 10.1038/ejcn.2009.120
126. Parnell JA, Reimer RA. Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. *Am J Clin Nutr.* (2009) 89:1751–9. doi: 10.3945/ajcn.2009.27465
127. Qamar TR, Syed F, Nasir M, Rehman H, Zahid MN, Liu RH, et al. Novel combination of prebiotics galacto-oligosaccharides and inulin-inhibited aberrant crypt foci formation and biomarkers of colon cancer in wistar rats. *Nutrients* (2016) 8:E465. doi: 10.3390/nu8080465
128. Qamar TR, Iqbal S, Syed F, Nasir M, Rehman H, Iqbal MA, et al. Impact of novel prebiotic galacto-oligosaccharides on various biomarkers of colorectal cancer in wister rats. *Int J Mol Sci.* (2017) 18:E1785. doi: 10.3390/ijms18091785
129. Abrams SA, Griffin IJ, Hawthorne KM, Liang L, Gunn SK, Darlington G, et al. A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents. *Am J Clin Nutr.* (2005) 82:471–6. doi: 10.1093/ajcn/82.2.471
130. Slavin J. Fiber and prebiotics: mechanisms and health benefits. *Nutrients* (2013) 5:1417–35. doi: 10.3390/nu5041417
131. Bouhnik Y, Raskine L, Simoneau G, Paineau D, Bornet F. The capacity of short-chain fructo-oligosaccharides to stimulate faecal bifidobacteria: a

dose-response relationship study in healthy humans. *Nutr J.* (2006) 5:8. doi: 10.1186/1475-2891-5-8
132. Brunser O, Gotteland M, Cruchet S, Figueroa G, Garrido D, Steenhout P. Effect of a milk formula with prebiotics on the intestinal microbiota of infants after an antibiotic treatment. *Pediatr Res.* (2006) 59:451–6. doi: 10.1203/01.pdr.0000198773.40937.61
133. Scholtens PA, Alliet P, Raes M, Alles MS, Kroes H, Boehm G, et al. Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. *J Nutr.* (2008) 138:1141–7. doi: 10.1093/jn/138.6.1141
134. Scholtens S, Wijga AH, Smit HA, Brunekreef B, de Jongste JC, Gerritsen J, et al. Long-chain polyunsaturated fatty acids in breast milk and early weight gain in breast-fed infants. *Br J Nutr.* (2009) 101:116–21. doi: 10.1017/S0007114508993521
135. Meijer K, de Vos P, Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? *Curr Opin Clin Nutr Metab Care* (2010) 13:715–21. doi: 10.1097/MCO.0b013e32833eebe5
136. McLoughlin RF, Berthon BS, Jensen ME, Baines KJ, Wood LG. Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. *Am J Clin Nutr.* (2017) 106:930–45. doi: 10.3945/ajcn.117.156265
137. Postler TS, Ghosh S. Understanding the holobiont: how microbial metabolites affect human health and shape the immune system. *Cell Metab.* (2017) 26:110–30. doi: 10.1016/j.cmet.2017.05.008
138. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect Immun.* (2006) 74:6920–8. doi: 10.1128/IAI.01030-06
139. Jantscher-Krenn E, Bode L. Human milk oligosaccharides and their potential benefits for the breast-fed neonate. *Minerva Pediatr.* (2012) 64:83–99.
140. Douellou T, Montel MC, Thevenot Sergentet D. Invited review: anti-adhesive properties of bovine oligosaccharides and bovine milk fat globule membrane-associated glycoconjugates against bacterial food enteropathogens. *J Dairy Sci.* (2017) 100:3348–59. doi: 10.3168/jds.2016-11611
141. Shokryazdan P, Faseleh Jahromi M, Navidshad B, Liang JB. Effects of prebiotics on immune system and cytokine expression. *Med Microbiol Immunol.* (2017) 206:1–9. doi: 10.1007/s00430-016-0481-y
142. Wilson B, Whelan K. Prebiotic inulin-type fructans and galacto-oligosaccharides: definition, specificity, function, and application in gastrointestinal disorders. *J Gastroenterol. Hepat.* (2017) 32(Suppl 1):64–8. doi: 10.1111/jgh.13700
143. Zenhom M, Hyder A, de Vrese M, Heller KJ, Roeder T, Schrezenmeir J. Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinal Caco-2 cells via activation of PPARgamma and peptidoglycan recognition protein 3. *J Nutr.* (2011) 141:971–7. doi: 10.3945/jn.110.136176
144. Bode L. The functional biology of human milk oligosaccharides. *Early Hum Dev.* (2015) 91:619–22. doi: 10.1016/j.earlhumdev.2015.09.001
145. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, van Neerven RJJ. Cow's milk and immune function in the respiratory tract: potential mechanisms. *Front Immunol.* (2018) 9:143. doi: 10.3389/fimmu.2018.00143
146. Perdijk O, van Neerven RJJ, Meijer B, Savelkoul HFJ, Brugman S. Induction of human tolerogenic dendritic cells by 3'-sialyllactose via TLR4 is explained by LPS contamination. *Glycobiology* (2018) 28:126–30. doi: 10.1093/glycob/cwx106
147. Zhong Y, Cai D, Cai W, Geng S, Chen L, Han T. Protective effect of galactooligosaccharide-supplemented enteral nutrition on intestinal barrier function in rats with severe acute pancreatitis. *Clin Nutr.* (2009) 28:575–80. doi: 10.1016/j.clnu.2009.04.026
148. Searle LE, Cooley WA, Jones G, Nunez A, Crudgington B, Weyer U, et al. Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*. *J Med Microbiol.* (2010) 59:1428–39. doi: 10.1099/jmm.0.022780-0
149. Bhatia S, Prabhu PN, Benefiel AC, Miller MJ, Chow J, Davis SR, et al. Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells. *Mol Nutr Food Res.* (2015) 59:566–73. doi: 10.1002/mnfr.201400639
150. Akbari P, Fink-Gremmels J, Willems R, Difilippo E, Schols HA, Schoterman MHC, et al. Characterizing microbiota-independent effects of oligosaccharides on intestinal epithelial cells: insight into the role of structure and size: structure-activity relationships of non-digestible oligosaccharides. *Eur J Nutr.* (2017) 56:1919–30. doi: 10.1007/s00394-016-1234-9
151. Garcia-Rodenas CL, Bergonzelli GE, Nutten S, Schumann A, Cherbut C, Turini M, et al. Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. *J Pediatr Gastroenterol Nutr.* (2006) 43:16–24. doi: 10.1097/01.mpg.0000226376.95623.9f
152. Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, et al. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am J Clin Nutr.* (2009) 90:1236–43. doi: 10.3945/ajcn.2009.28095
153. Kaliannan K, Wang B, Li XY, Kim KJ, Kang JX. A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Sci Rep.* (2015) 5:11276. doi: 10.1038/srep11276
154. Pusceddu MM, El Aidy S, Crispie F, O'Sullivan O, Cotter P, Stanton C, et al. Correction: N-3 Polyunsaturated Fatty Acids (PUFAs) reverse the impact of early-life stress on the gut microbiota. *PLoS ONE* (2015) 10:e0142228. doi: 10.1371/journal.pone.0142228
155. Pusceddu MM, El Aidy S, Crispie F, O'Sullivan O, Cotter P, Stanton C, et al. N-3 Polyunsaturated Fatty Acids (PUFAs) reverse the impact of early-life stress on the gut microbiota. *PLoS ONE* (2015) 10:e0139721. doi: 10.1371/journal.pone.0139721
156. Etxeberria U, Fernandez-Quintela A, Milagro FI, Aguirre L, Martinez JA, Portillo MP. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *J Agric Food Chem.* (2013) 61:9517–33. doi: 10.1021/jf402506c
157. Ozdal T, Sela DA, Xiao J, Boyacioglu D, Chen F, Capanoglu E. The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. *Nutrients* (2016) 8:78. doi: 10.3390/nu8020078
158. Tomas-Barberan FA, Selma MV, Espin JC. Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Curr Opin Clin Nutr Metab Care* (2016) 19:471–6. doi: 10.1097/MCO.0000000000000314
159. Roca-Saavedra P, Mendez-Vilabrille V, Miranda JM, Nebot C, Cardelle-Cobas A, Franco CM, et al. Food additives, contaminants and other minor components: effects on human gut microbiota-a review. *J Physiol Biochem.* (2018) 74:69–83. doi: 10.1007/s13105-017-0564-2
160. Gerritsen J, Smidt H, Rijkers GT, de Vos WM. Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr.* (2011) 6:209–40. doi: 10.1007/s12263-011-0229-7
161. Ismail IH, Licciardi PV, Tang ML. Probiotic effects in allergic disease. *J Paediatr Child Health* (2013) 49:709–15. doi: 10.1111/jpc.12175
162. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br J Nutr.* (2014) 111:387–402. doi: 10.1017/S0007114513002560
163. Sanchez B, Delgado S, Blanco-Miguez A, Lourenco A, Gueimonde M, Margolles A. Probiotics, gut microbiota, and their influence on host health and disease. *Mol. Nutr. Food Res.* (2017) 61:1600240. doi: 10.1002/mnfr.201600240
164. Liu S, Hu P, Du X, Zhou T, Pei X. *Lactobacillus rhamnosus* GG supplementation for preventing respiratory infections in children: a meta-analysis of randomized, placebo-controlled trials. *Indian Pediatr.* (2013) 50:377–81. doi: 10.1007/s13312-013-0123-z
165. Inoue Y, Shimojo N. Microbiome/microbiota and allergies. *Semin Immunopathol.* (2015) 37:57–64. doi: 10.1007/s00281-014-0453-5
166. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann Gastroenterol.* (2015) 28:203–9.
167. Umbrello G, Esposito S. Microbiota and neurologic diseases: potential effects of probiotics. *J Transl Med.* (2016) 14:298. doi: 10.1186/s12967-016-1058-7
168. Goulet O. Potential role of the intestinal microbiota in programming health and disease. *Nutr Rev.* (2015) 73(Suppl 1):32–40. doi: 10.1093/nutrit/nuv039
169. Morrow LE, Gogineni V, Malesker MA. Probiotic, prebiotic, and synbiotic use in critically ill patients. *Curr Opin Crit Care* (2012) 18:186–91. doi: 10.1097/MCC.0b013e3283514b17

170. Bron PA, Kleerebezem M, Brummer RJ, Cani PD, Mercenier A, MacDonald TT, et al. Can probiotics modulate human disease by impacting intestinal barrier function? *Br J Nutr.* (2017) 117:93–107. doi: 10.1017/S0007114516004037
171. Fong FL, Shah NP, Kirjavainen P, El-Nezami H. Mechanism of action of probiotic bacteria on intestinal and systemic immunities and antigen-presenting cells. *Int Rev Immunol.* (2016) 35:179–88. doi: 10.3109/08830185.2015.1096937
172. Raaijmakers JM, Bonsall RF, Weller DM. Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. *Phytopathology* (1999) 89:470–5. doi: 10.1094/PHYTO.1999.89.6.470
173. Cha JY, Han S, Hong HJ, Cho H, Kim D, Kwon Y, et al. Microbial and biochemical basis of a Fusarium wilt-suppressive soil. *ISME J.* (2016) 10:119–29. doi: 10.1038/ismej.2015.95
174. DePeters EJ, George LW. Rumen transfaunation. *Immunol Lett.* (2014) 162:69–76. doi: 10.1016/j.imlet.2014.05.009
175. Weimer PJ, Stevenson DM, Mantovani HC, Man SL. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. *J Dairy Sci.* (2010) 93:5902–12. doi: 10.3168/jds.2010-3500
176. Siegerstetter SC, Petri RM, Magowan E, Lawlor PG, Zebeli Q, O'Connell NE, et al. Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-related changes in feed-efficiency-associated fecal microbiota from chickens. *Appl. Environ. Microbiol.* 84:e02330-17. doi: 10.1128/AEM.02330-17
177. Hu L, Geng S, Li Y, Cheng S, Fu X, Yue X, et al. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front Microbiol.* (2017) 8:2663. doi: 10.3389/fmicb.2017.02663
178. McCormack UM, Curiao T, Buzoianu SG, Prieto ML, Ryan T, Varley P, et al. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. *Appl Environ Microbiol.* (2017) 83:e00380-17. doi: 10.1128/AEM.00380-17
179. Ribeiro GO, Oss DB, He Z, Gruninger RJ, Elekwachi C, Forster RJ, et al. Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. *Sci Rep.* (2017) 7:1276. doi: 10.1038/s41598-017-01269-3
180. Diao H, Yan HL, Xiao Y, Yu B, Zheng P, He J, et al. Modulation of intestine development by fecal microbiota transplantation in suckling pigs. *RSC Adv.* (2018) 8:8709–20. doi: 10.1039/C7RA11234C
181. Donaldson EE, Stanley D, Hughes RJ, Moore RJ. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. *PeerJ* (2017) 5:e3587. doi: 10.7717/peerj.3587
182. Li SS, Zhu A, Benes V, Costea PI, Hercog R, Hildebrand F, et al. Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science* (2016) 352:586–9. doi: 10.1126/science.aad8852
183. Kelly CR, Kahn S, Kashyap P, Laine L, Rubin D, Atreja A, et al. Update on fecal microbiota transplantation 2015: indications, methodologies, mechanisms, and outlook. *Gastroenterology* (2015) 149:223–37. doi: 10.1053/j.gastro.2015.05.008
184. van Nood E, Speelman P, Nieuwdorp M, Keller J. Fecal microbiota transplantation: facts and controversies. *Curr Opin Gastroenterol.* (2014) 30:34–9. doi: 10.1097/MOG.0000000000000024
185. Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. *Gastroenterology* (2013) 145:946–53. doi: 10.1053/j.gastro.2013.08.058
186. Chehoud C, Dryga A, Hwang Y, Nagy-Szakal D, Hollister EB, Luna RA, et al. Transfer of viral communities between human individuals during fecal microbiota transplantation. *mBio* (2016) 7:e00322. doi: 10.1128/mBio.00322-16
187. Shen Z, Zhu C, Quan Y, Yuan W, Wu S, Yang Z, et al. Update on intestinal microbiota in Crohn's disease 2017: mechanisms, clinical application, adverse reactions, and outlook. *J Gastroenterol Hepatol.* (2017) 32:1804–12. doi: 10.1111/jgh.13861
188. Xu MQ, Cao HL, Wang WQ, Wang S, Cao XC, Yan F, et al. Fecal microbiota transplantation broadening its application beyond intestinal disorders. *World J Gastroenterol.* (2015) 21:102–11. doi: 10.3748/wjg.v21.i1.102
189. Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *J Crohns Colitis* (2014) 8:1569–81. doi: 10.1016/j.crohns.2014.08.006
190. Jorgensen SMD, Hansen MM, Erikstrup C, Dahlerup JF, Hvas CL. Faecal microbiota transplantation: establishment of a clinical application framework. *Eur J Gastroenterol Hepatol.* (2017) 29:e36–e45. doi: 10.1097/MEG.0000000000000958
191. Legrand T, Catalano SR, Wos-Oxley ML, Stephens F, Landos M, Bansemer MS, et al. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Front Microbiol.* (2017) 8:2664. doi: 10.3389/fmicb.2017.02664
192. Brugman S, Nieuwenhuis EE. Mucosal control of the intestinal microbial community. *J Mol Med.* (2010) 88:881–8. doi: 10.1007/s00109-010-0639-9
193. Litvak Y, Byndloss MX, Tsolis RM, Baumler AJ. Dysbiotic Proteobacteria expansion: a microbial signature of epithelial dysfunction. *Curr Opin Microbiol.* (2017) 39:1–6. doi: 10.1016/j.mib.2017.07.003
194. Manfredo Vieira S, Hiltensperger M, Kumar V, Zegarra-Ruiz D, Dehner C, Khan N, et al. Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science* (2018) 359:1156–61. doi: 10.1126/science.aar7201

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 13 September 2018
doi: 10.3389/fimmu.2018.02088

Immunomodulation by Processed Animal Feed: The Role of Maillard Reaction Products and Advanced Glycation End-Products (AGEs)

Malgorzata Teodorowicz[1], *Wouter H. Hendriks*[2,3], *Harry J. Wichers*[4] *and Huub F. J. Savelkoul*[1]*

[1] Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, Netherlands, [2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, Netherlands, [3] Department of Nutrition, Faculty of Veterinary Sciences, Utrecht University, Utrecht, Netherlands, [4] Food and Biobased Research, Wageningen University & Research, Wageningen, Netherlands

The immune system provides host protection to infection with pathogenic organisms, while at the same time providing tolerance upon exposure to harmless antigens. Thus, an impaired immune function is associated with increased susceptibility to infections with increased disease severity and thereby necessitating the therapeutic use of antibiotics. Livestock performance and feed efficiency, in addition to their health status, are dependent on the microbial load of their gut, the barrier function of the intestinal epithelium and the activity of the mucosal immune system, all of which can be modulated by dietary components. The majority of feeds that are consumed in pets and livestock have been processed. Processing promotes a non-enzymatic reaction between proteins and sugars called Maillard reaction (MR). Maillard reaction products (MRPs) and advanced Maillard reaction products (AGEs) determine taste, smell, and color of many food products therefore the MR is highly relevant for the feed industry. MRPs interact with different types of immune receptors, including the receptor for advanced glycation end products (RAGE) and immunomodulatory potential of feed proteins can be modified by Maillard reaction. This MR has become an important concern since MRPs/AGEs have been shown to contribute to increasing prevalence of diet-related chronic inflammatory states in the gut with negative health consequences and performance. The immunomodulatory effects of dietary MRPs and AGEs in livestock and pet animals are far less well-described, but widely considered to be similar to the relevant concepts and mechanisms obtained in the human field. This review will highlight immunological mechanisms underlying initiation of the innate and adaptive immune responses by MRPs/AGEs present in animal feeds, which are currently not completely understood. Bridging this knowledge gap, and taking advantage of progress in the human field, will significantly improve nutritional quality of feed and increase the prevention of diet-mediated inflammation in animals.

Keywords: Maillard products, advanced glycation end products, RAGE, animal feed, immunity

OPEN ACCESS

Edited by:
Willem Van Eden, Utrecht University, Netherlands

Reviewed by:
Claudio Nicoletti, Università degli Studi di Firenze, Italy
Julio Villena, Centro de Referencia para Lactobacilos, Argentina

***Correspondence:**
Huub F. J. Savelkoul
huub.savelkoul@wur.nl

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *06 June 2018*
Accepted: *24 August 2018*
Published: *13 September 2018*

Citation:
Teodorowicz M, Hendriks WH, Wichers HJ and Savelkoul HFJ (2018) Immunomodulation by Processed Animal Feed: The Role of Maillard Reaction Products and Advanced Glycation End-Products (AGEs). Front. Immunol. 9:2088. doi: 10.3389/fimmu.2018.02088

INTRODUCTION

Protein Quality of Animal Feeds

Dietary proteins are a source of amino acids and the ability to absorb amino acids and use them for protein synthesis determines their quality and their required dose to meet the requirement (1). The efficiency with which individual amino acids are utilized for e.g., development, growth, immunocompetence, and lactation depends not only on the concentration of amino acids and their bioavailability in foods, but also on the relative proportions to each other. Protein quality and synthesis decrease when the amino acids in the food are imbalanced or even absent, e.g., tryptophan is the least abundant amino acid in foods, while lysine is often the limiting essential amino acid in animal feeds (2, 3). Proteins are essential in foods, not only for its nutritional value, but they also determine food structure, perception, and immunomodulatory capacity. These functional characteristics are dependent on physico-chemical conditions like pH, ionic strength, temperature, or pressure, and the individual behavior of protein and their amino acids are largely unknown and unpredictable. There is a need to better understand these parameters in food production as they, have a significant effect on food quality. Often enzymes are part of these dietary proteins and these include proteases, hydrolyzing large protein molecules into smaller peptides, and peptidases, releasing single amino acids from terminal ends of proteins and peptides. Mainly proteases are used in processing to improve visual appeal, taste, yield, nutritional value, and physical properties of dietary proteins. Processing technologies affect the quality of protein foods and thereby also its safety and for this reason novel processing technologies, e.g., high pressure processing, pulsed electric field, radiofrequency and cold plasma, have been developed.

Amino acids contained within the dietary protein can undergo crosslinking and glycation reactions, including the "Maillard reaction" (MR) or glycation, which is named after the French physician and chemist, Louis Camile Maillard, who in 1912 described this reaction for the first time (4). It was, however, John Hobbs in 1953 who proposed the mechanism for the chemistry of the Maillard reaction as know it today (5). The MR is also known as the non-enzymatic browning reaction and typically involves amino acids (e.g., lysine, arginine) and reducing sugars (e.g., fructose, glucose) that progresses via a series of chemical rearrangements resulting in the formation of MR products (MRPs). MRPs include Schiff bases, Amadori products and consequently advanced glycation end products (AGEs) as the final products. The advanced products are produced via either an oxidative or non-oxidative pathway. Pentosidine and N-(carboxymethyl)lysine (CML) are examples of glycoxidation (glycation and oxidation) and pyrraline is a product of a non-oxidative pathway (6). The MR is positively correlated with the temperature and the duration of heating of proteins in the presence of reducing compounds predominantly sugars. Higher temperatures increase the reactions where long-term thermal processing with relatively lower temperatures may result in comparable results. The type and concentration of available sugars in the environment are linked to the progression of the reaction with, for instance, glucose-rich products showing a slower glycation rate in comparison with those rich in fructose (6). Of the 20 amino acids naturally occurring in food proteins, lysine due to its ε-amino acids and arginine because of its guanidine side group are the most susceptible amino acids but also histidine and tryptophan can be involved in the MR as well as the α-amino or N-terminal amino group of any amino acid or peptide, respectively (7).

As lysine is the most reactive amino acid, modification produces modified lysine derivatives (8), including formation of a Schiff's base, which is a reversible but unstable compound. Subsequently, this base rearranges into the Amadori compound, ε-N-deoxyketosyllysine (9). The latter reaction is irreversible and can proceed further into advanced Maillard reaction products, and ultimately give rise to melanoidins, leading to brown coloring of processed feeds. Because amino groups are involved in several steps of the Maillard reaction, a strong decrease of the availability of amino acids can occur. Amadori products and AGEs resulting from the MR may absorbed, metabolized by gastrointestinal bacteria to other components or leave unaltered via the feces. Once absorbed AGEs may be metabolized to other components, retained in the body or leave unaltered via the urine. Melanoidins are only partially digested and absorbed by the intestines and may be retained in the kidneys. In contrast to high molecular weight melanoidins, low molecular weight non-absorbed melanoidins are degraded in the intestines (10).

Food Processing

Food processing has been a routine procedure for increasing the taste, safety, texture, longevity, and bioavailability of nutritional components (11). Humans have long been utilizing different methods such as salting, fermenting, smoke processing, and heating in order to get to conserve food and obtain desirable product features. Among all the above-mentioned procedures, thermal processing is the most commonly used method of our modern time. Ever since the exploration of fire, cooking raw materials improved the taste and digestibility of food. High temperatures have been widely used by the food industry with a number of these processes involving product temperature reaches up to 250°C to changes the appearance and occasionally the structure of the food. Food processing may result in products of similar composition, but with markedly different physiological effects, due to differences in structure and physicochemical properties. In general, food processing has a major influence on its nutritional components and modifies biological properties of biomolecules resulting in various effects on consumer's body once ingested (2, 12). Some of these modifications have a direct and instant effect on the user such as improved olfaction and gustation as well as digestibility of dietary components (e.g., starch, amino acids). Other modifications may show their influence even years after processed foods have been consumed. The latter is mainly the results of the accumulation of modified components in the body and their gradual effects on cells and tissues over time. The accumulation of AGE-modified proteins correlates with the pathogenesis of several chronic inflammatory diseases including diabetes, rheumatoid arthritis,

and Alzheimer's disease (13–17). The focus of this review is on immunomodulatory effects of dietary MRPs and AGEs in livestock and pets as this is far less well-known than in the human situation and have therefore used relevant knowledge from the human field. Immunomodulatory activity of MRPs and AGEs in feeds are based on concepts and mechanisms that are equally relevant and applicable in humans and animals.

Advanced Glycation End Products (AGEs)

The final glycated products of the MR are commonly referred to as glycation end-products (AGEs). In laboratory animals and humans, consumption of large quantities of such AGEs were shown to induce pathological alterations and therefore AGEs are increasingly subject of safety studies (18). Advanced glycation end products are either produced endogenously (*in vivo*) due to physiological processes such as oxidative stress and aging or are formed exogenously (in food) following the progression of the MR (19). Glycation changes protein structures and this may result in malformation and malfunction of affected proteins. Small endogenously formed glycated and misfolded proteins are targets for intracellular degradation by the 20S proteasome of the Ubiquitin-proteasome-system (UPS) (20). However, these small proteins when oxidized further and following cross-linking reactions, may form larger structures. The formation of big and bulky glycated proteins blocks the activity of proteasomes and makes them resistant to degradation leading to the accumulation of such molecules in the cells and tissues (13). Another mechanism for eliminating AGE-modified proteins is via the lysosomal system. Cellular receptors recognize these modified proteins (especially from exogenous sources) and internalize them into endosomes. The AGE-containing endosomes are then fused to cytosolic lysosomes where lysosomal proteases process and break down these modified proteins (21). The degraded peptides are then cleared from the body through the urinary system with a, hitherto, poorly defined mechanism.

ADVANCED MAILLARD REACTION PRODUCTS AND THEIR POSSIBLE EFFECTS ON HEALTH

MR and Damaged Dietary Proteins

The most common form of dietary AGEs are protein-bound, while some are either in free state of bound to peptides. These protein-AGEs are enzymatically hydrolyzed into small fractions that can either be absorbed in the small intestine of remain unable to be absorbed, and thus is absorption related to digestibility of the dietary AGEs (22). The abundance of α-dicarbonyl groups in processed feed and their relevance in the formation of AGEs, determines the digestibility, nutritional value and health impact of protein-AGEs (23).

The MR can occur at temperatures similar to that of the human body (24). As an example, a fraction of hemoglobin (HbA0) reacts with glucose under *in vitro* conditions, yielding the MR product HbA1c and further reaction products including Schiff's base and Amadori compounds. In diabetics high concentrations of HbA1c are present and these consist of α-amino-1-deoxyfructose at the N-terminal valine amino acid in the β-chain (24).

The concentration, nutritional value and digestibility of amino acids (esp. lysine) in feed ingredients and diets may be reduced due to heat treatment of feed ingredients (10). Feeding broiler chicken or weanling piglets a heat-damaged soybean meal diet, a decrease in body weight and carcass weight was observed compared to feeding untreated soybean meal. These negative effects of heat damage on performance, however, were partially mitigated by adding crystalline amino acids to the diets (25). Heat damage also may cause losses in vitamins, e.g., loss of vitamin B6 and thiamine when storing milk powder at 70°C (26).

The structural and functional properties of proteins can be modified due to covalent interactions and the cross-linkage of proteins during the formation of such AGEs. The resulting resistance to digestion, delays the turnover rate of these proteins and this accumulation of AGEs may hinder tissue repair (27). Moreover, these Ages bind to receptors widely expressed on tissue cells and as a consequence oxidative stress, vasoconstriction, excessive collagen deposition, and inflammatory responses are stimulated (27–30). The development of chronic systemic inflammation (metaflammation) can be the consequence of prolonged exposure to AGEs and metaflammation is observed in many cancers in both humans and dogs (31). Canine diets should therefore limit stimulation of the AGE/metaflammation axis resulting in less carcinogenic activity. Such diets offer opportunities to be tested for AGE and metaflammation accumulation that result in lower prevalence and incidence of cancer in dogs.

A low MRP diet in a mouse model resulted in decreased body weight, lowering of insulin concentration during fasting, increased HDL levels in plasma, and reduction of a high-fat diet-induced insulin resistance (32). Also in human diabetics, complications associated with impaired wound healing were improved when MRPs were avoided in the diet (3, 32).

Advanced glycation end products have been associated with the etiology of age-related diseases in humans, such as atherosclerosis, nephropathy, retinopathy, osteoarthritis, neurodegenerative diseases, and diabetes mellitus. Also in dogs such age-related diseases occur, with many similarities to humans (33). In aging dogs with e.g., diabetes mellitus, increased tissue levels of AGEs were found (34), but also in conditions like carteracts, osteoarthritis (35), neurodegenerative canine cognitive dysfunction syndrome (28), vascular dysfunction, and atherosclerosis (29, 30). The limited exposure to dietary AGEs and a reduced AGEs pool during calorie restriction could explain the widely described beneficial effects on aging and related complications (36–40).

Adverse Effects of MR on Bioavailability of Lysine and Other Dietary Compounds

The MR importantly results in blockage of lysine thereby reducing the biological availability of the lysine amino acid but also together with the crosslinking hinder hydrolysis of the protein by digestive enzymes. This has has been demonstrated

in animal feed ingredients, like dairy products (41), dried grains used as the feed for pigs, soybeans (42), carrots (43), peas (44), and maize (45). The thermally induced reduction in lysine bioavailability in the presence of sugar, depends on the level and duration of temperature application, water activity and pH of the environment during processing (46). Furosine (ε-N-(furoylmethyl)-L-lysine), which is related to the early stage of the MR, is an indicator of the formation and presence of Maillard products such as fructoselysine, lactuloselysine, and lysinoalanine and of the losses in available lysine.

Next to the impaired bioavailability of lysine, MRPs have a strong mineral chelating power affecting the availability of minerals such as calcium, iron, and phosphorus (47, 48).

Rérat et al.(49) used an *in vivo* pig model to study feeding of a protein source with a high level of MR-induced blocked lysine on the kinetics of digestion, nutrient absorption and excretion, including amino acids but also minerals. The pigs were fed with non-processed milk (MR free) or skimmed milk processed to obtain about 50% of lysine blockage due to the early stages of Maillard reaction. Consumption of processed skimmed milk induced a lower absorption of milk sugars glucose and galactose. This was in part due to the loss of milk-derived lactose as this was converted into lactuloselysine and lactulose. In addition, reduced amounts of lysine, cysteine, and alanine were found due to absorption and these appeared in the portal blood in pigs fed the processed skimmed milk suggesting that lactuloselysine was not bioavailable. The fecal excretion of amino acids was higher in the group of pigs fed processed skimmed milk confirming the impaired digestibility of proteins modified via MR (49). Thermal processing of milk was shown to induce damage of casein resulting in a decreased bioavailability of lysine and this lysine blockage was reflected in the lower growth rate of kittens fed with heated casein (50). A decreased protein digestibility results in a diet high in the MRPs and consumption led to 47% higher excretion of fecal nitrogen, 12% lower absorption of nitrogen, and a 6% lower nitrogen digestibility in a group of adolescent males (51). The MRP-rich diet was also shown to affect the absorption of phosphorus, resulting in a decrease of the phosphorus balance (52). Some MRPs can directly inhibit brush border enzymes as shown for glucose-lysine reaction compound (2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine) *in vitro* but also *in vivo* in a rat study (53, 54).

Moreover, fructoselysine, an Amadori adduct of glucose to lysine, was detected in the portal blood and urine of pigs fed with processed skimmed milk in amounts corresponding to 18.6% of the ingested quantity. This suggested that the galactose residue of lactuloselysine is released by enzymes in the gut lumen and/or in the epithelial brush border and subsequently transported through the intestinal barrier (49). Both early and advanced glycation end products were detected in the blood of rats after feeding a MR-rich diet. Approximately 26.0 to 29.0% of ingested dietary CML in rats was excreted in urine, compared to 15.0 to 22.0% in feces (55). In humans, protein-bound fructolysine urinary excretion ranged from 1.4 to 3.5% of the ingested amount (56). In contrast, about 10% of diet-derived AGEs were absorbed in healthy subjects, and two thirds of these AGEs remained in the body while one-third of the absorbed AGEs was excreted into the urine within 3 days. Low molecular weight AGEs are water-soluble and are not substrates for liver detoxifying enzymes and therefore rapidly excreted. Consumption of high molecular weight pentosidine led to excretion of only 2% AGE resulting in accumulation, endothelial perturbation and vascular disease (57).

Influence of Cross-Linking Immunogenicity of Proteins

The MR but also heat treatment alone during thermal processing of food can result in cross-linking of the proteins. Methylglyoxal, a common intermediate in the MR *in vivo* and *in vitro*, has been shown to be involved in the formation of cross-linked aggregates via lysine, arginine, or cysteine. Normal physiological concentrations of methylglyoxal are sufficient to induce these reactions resulting in different fluorescent products that resemble proteins characteristic for aging and diabetes development (58–60). Furthermore, dehydroalanine may react with lysine and cysteine residues to form cross-linked products such as lysinoalanine (LAL) and lanthionine (61). LAL was found in the urine, plasma, liver and kidneys of rats fed with heat-modified casein (55). However, the study of Hellwig and colleagues suggests that LAL is broken down during the digestion process into larger peptides (61). Next to the formation of LAL, MR, and/or denaturation of proteins during the heat treatment may initiate hydrophobic interactions between the proteins and formation of new disulfide-bonded aggregates (62). The cross-linking of proteins decreases their digestibility (63) but also affects the immunogenicity and allergenicity of proteins (64–67). Roth-Walter and colleagues demonstrated an impaired uptake of aggregated β-lactoglobulin and α-lactalbumin by intestinal epithelial cells. In a mouse model, protein aggregation was shown to increase the uptake into Peyer's patches. Compared to non-aggregated proteins, this uptake promoted significantly higher mucosal Th2-associated antibody responses and cytokine production profiles (64). Also exposure to cross-linked β-lactoglobulin was shown in mice to elicit a stronger allergic sensitization probably due to enhanced resistance to gastrointestinal proteolysis, retrogradee protein transport to Peyer's patches, and an altered uptake and processing in antigen-presenting cells (65). Liu and colleagues showed that agglomeration of whey proteins during heating is positively correlated with the decreasing water activity and progress of the MR. Moreover, the formation of aggregates was associated with the formation of ligands binding to the cell-bound but also sRAGE (the soluble form of receptor for advanced glycation end-products), which reflects increased immunoreactivity of MR-modified agglomerates (66, 67).

IMMUNE-RELATED EFFECTS

Influence of AGEs on Immune System

In general, AGEs affect biological procedures in three levels; the first effect is an alteration in signal transduction pathways, which happens following the AGE-receptor interaction. Secondly, via altered signaling, AGEs induce or inhibit the production of certain cytokines, hormones, and free radicals. Finally, as a result of AGEs effects and increased pro-oxidative activities,

the proteins in the target tissues modify leading to functional deregulations (68).

There is substantial evidence on the association of MRPs with immunity stimulation and immune system responses (69, 70). The interaction begins with the recognition of MRP's conformational epitopes by the pattern recognition receptors (PRRs) and subsequently a downstream signaling to the nucleus for mainly, NF-kB activation and consequent cellular responses (71). Multiple PRRs have the potential recognition ability and binding affinity for MRPs but their interaction may lead to various responses (72, 73). Both early and advanced glycation products are related to an increase in oxidative stress along with inflammatory response (70, 74). As a consequence of interactions of AGE to the RAGE receptor, increased production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 occurs in endothelial cells and monocytes (75). An increase in free radicals and oxidative stress aggravates the inflammatory state (auto-amplifying) and may eventually affect long-lived proteins such as collagen and elastin (76).

MRP and AGEs that are absorbed in the gut and arrive in the mucosal tissue are being confronted with the local mucosal immune system. This exposure induces immune activation leading to local effects in the gut, including induction of tissue damage and inflammation, and the start of an activated immune response leading to T-cell activation and (IgA) antibody production. Besides a local activation of the immune system in the gut, activated immune cells can also travel to the mesenteric lymph node and arrive through the portal vein into the liver and systemic circulation, leading to consequences in the entire body. One such example of a systemically activated immune response is allergy. Dietary AGEs (dAGEs) have various allergenicity and immunogenicity properties in terms of food allergy (77, 78). The mechanism for initiation and progression of the reactions are still under debate. However, it was shown that depending on the structure and type of glycated proteins, these molecules might increase or attenuate allergic reactions. An increase in allergenicity of roasted peanut with an accompanied increase in IgE reactivity was reported (79, 80). Besides, Hilmenyuk et al. demonstrated that mature DCs loaded with AGE-modified proteins induced a higher T helper 2 (Th2) response (81) what promotes an allergic reaction. In addition, the cross-linking between the proteins as a result of heating makes them resistant to proteolysis and also may increases the allergenicity properties of the products. That was confirmed by increased sensitizing capacity of glycated β-Lactoglobulin when compared to the native form of the molecule (65), which could be because of neo-allergen generation. In contrast, heating may also denature the proteins and consequently, destroy or mask the conformational epitopes that leads to a reduced allergenicity (82).

Cellular Receptors for AGEs

A series of cell surface receptors present on antigen presenting cells (APCs) have an affinity to bind and interact with AGEs. According to the glycoprotein structure of AGEs, these receptors mostly have carbohydrate recognition domains (CRDs) and/or domains to interact with available peptides. There is a wide range of cellular receptor with such characteristics, however, not all of them bind to AGEs. In addition, the AGE receptors do not bind to modified proteins with similar affinity, which may lead to dissimilar responses. Taking into account the studies related to AGEs, six membrane-associated receptors are considered valuable.

RAGE

RAGE or receptor for advanced glycation end products is a multi-ligand receptor and is a member of the immunoglobulin (Ig) superfamily. This receptor is expressed by different cell types including monocytes/macrophages (83), endothelial cells (84), and dendritic cells (85). They recognize a wide range of molecules such as AGEs and amyloid-β peptides and are involved in activation, migration, and maturation of different cells. The presence of excessive amounts of AGEs or large increases in inflammatory conditions will up regulate RAGE expression and activation. Following their activation, Reactive Oxygen Species (ROS) generation and inflammatory responses are exerted which may lead to chronic inflammatory disorders if the stimulation persists. Three variants of the RAGE protein were described: full-length RAGE, N-truncated RAGE, and soluble RAGE (sRAGE) and all of these share various common core operational domains. The latter form (sRAGE), which has a molecular weight of ∼46–50 kDa is secreted extracellular and contains the extracellular ligand-binding domain and regulates RAGE levels by negative feedback mechanisms (86).

Galectin-3

AGE-R3/Galectin-3 is a member of lectin family, which along with two other components (AGE/R1/OST-48 and AGE-R/80K-H) forms the AGE-R complex. This complex and mainly the extracellular ∼32 kDa Galectin-3 subunit, was shown to bind with high affinity to AGE-BSA on macrophages (87). This multiple function receptor interacts with glycoproteins via its carbohydrate recognition domains (CRDs) and N-terminus domain. It is also a major regulator of biological processes including acute and chronic inflammation (88, 89). According to this capacity of Galectin-3 for interacting with glycoproteins such as AGEs and also its importance in immune responses.

SR-AI

Scavenger receptor class A1 (SR-AI) with the molecular weight of ∼77 kDa (as monomer), belongs to the family of macrophage scavenger receptors (MSR) and consists of six different domains. Likewise other receptors of this family, SR-AI is mainly involved in mediating phagocytosis of microorganisms. Being generally expressed on monocytes and macrophages as well as DCs, these membrane-bound PRRs have versatile functions and have a wide range of ligands. In addition to microbial ligands, they bind to modified molecules including glycated proteins such as AGEs with high affinity and facilitate their endocytosis. They are one of the key role players of innate immunity responses and are involved in, for instance, macrophage polarization and pathogenesis of diseases such as atherosclerosis (90).

CD36

CD36 (SR-BIII) is a glycoprotein belonging to the scavenger receptor family and is present on macrophages. Similar to other

members of this family, the 88 kDa CD36 has a large repertoire of ligands and binds to microbial ligands as well as modified self-molecules. This multi-function receptor participates in activities such as phagocytosis, antigen presentation and apoptotic cell clearance and contributes to inflammatory responses (91). Several studies showed CD36 as an AGE-binding receptor, which facilitates the cellular uptake of these glycated molecules (92).

DC-SIGN

DC-specific ICAM3-grabbing non-integrin (DC-SIGN; CD209) is a member of the type II transmembrane receptor family. It is present on the cell membrane of dendritic cells and mediates their adhesion process to T lymphocytes (93). DC-SIGN is abundantly expressed on DCs and contains a C-terminal lectin domain as it a member of the C-type lectin family (94). DC-SIGN binds to several ligands and exerts a variety of responses based on them. For instance, it was shown that following the DC-SIGN interaction with mannose-containing molecules, production of IL-10, IL-12, and IL-6 increased where after binding to fucose-containing ligands only IL-10 production was upregulated (95). This potential ability to bind to carbohydrates makes this receptor a candidate to bind modified glycoproteins and specifically AGEs.

MMR

The type I transmembrane mannose receptor (MR; CD206) is another member of the C-type lectin family with 8 carbohydrate recognition domains (CRDs) (96). With no capacity to bind galactose, MR preferentially binds to mannose and fucose and with a lower affinity to glucose (97). It is also shown that MR (CD206) acts as pattern recognition receptor (PRR) and enhances the uptake and presentation of antigens by DCs (98). This potential capacity to bind to carbohydrates and aiding DCs in antigen internalization makes this mannose receptor a candidate to bind AGEs.

Antigen Presenting Cells

T-lymphocytes (T cells), as the effector cells of the adaptive immune system, are not able to recognize free antigens (99). Dendritic cells (DCs), macrophages, and B-lymphocytes have the ability to internalize antigens and present antigen-derived peptides to T cells on their major histocompatibility complex class II (MHC II) molecules. These cells are known as professional antigen-presenting cells (APCs) because displaying endogenously obtained peptides is one of the main integral parts of their function (100). DCs and macrophages (as mononuclear blood cells) are the primary immune cell types that establish the link between innate immunity and adaptive immune system. The APCs in general internalize and process complex antigens and subsequently, via different pathways, display the peptides on their MHC-binding groove. Following this presentation and based on the type and nature of the antigen, proliferation and differentiation of T lymphocytes begin and various forms of immune responses are generated. DCs mainly play a role in introducing antigens to naïve T cells where macrophages and B cells are involved in activating T cells in cell-mediated and humoral responses respectively (101). The whole process of antigen recognition, presentation, and activation is essential for a proper immune response to different perturbations and APCs play a key role in this regard.

When AGEs interact with receptors expressed on APC, these AGEs can be internalized and presented in the context of MHC class II molecules to specific T-cells. In a mouse model, AGE-modified ovalbumin was phagocytosed much more efficiently by scavenger receptor class A types I and II (SR-AI/II) expressed on myeloid dendritic cells compared to non-modified native OVA. This enhanced antigen presentation led to the increased activation of ovalbumin-specific CD4+ helper T cells (81). An enhanced uptake of FITC-labeled AGE-modified ovalbumin was observed in human DCs, and this was mediated by the AGE-binding mannose receptor, scavenger receptor, and also by macro-pinocytosis by these cells. As a result, the resulting T cell activation led to an increased Th2 cytokine production (IL-5, IL-4, and IL-6), compared to the non-glycated ovalbumin-loaded DCs that induced a significant Th1 (IFN-γ) or regulatory T-cell cytokine production profile (IL-10) (102). A schematic representation of how feed derived GE affect several aspects of the metabolism are depicted in **Figure 1**.

ADVANCED GLYCATION END PRODUCTS IN DIFFERENT ANIMAL FEEDS

AGEs in Milk and Dried Whey Proteins

The dairy industry generate large volumes of liquid cheese whey that are processed to produce different whey products, including whey protein concentrate, whey protein isolate, and several individual whey proteins. These processed whey products are widely used as additives or supplements to animal feeds including sows and neonatal piglets, young ruminants (calves), dogs, cats, poultry and aquaculture (103–106). In pig industry, early weaning is becoming a common practice to boost efficient and economic production systems. The sudden change from sow's milk to solid feed and accompanied change in husbandry conditions, provide strong stressors to the piglet and its health. To minimize the impact increasingly milk replacers are being provided stabilizing gut microbiota, preventing intestinal dysfunction and improve performance (107). Cow's milk because of its balanced nutrient composition makes it a suitable feed for neonatal piglets. Even dairy cattle are fed whey proteins being a side-product of the cheese manufacturing industry. Liquid, solid, or condensed whey or products derived thereof are applied (108). Moreover, lactose and dried whey are used as supplements in poultry diets (104, 105). Lactose is not absorbed from intestine in poultry due to their inability to secrete lactase. Instead, lactose is fermented to lactic acid and volatile fatty acids (VFA), and these products promote the colonization of the intestinal tract by Lactobacilli. Elevated VFA concentrations are considered beneficial as they induce a decrease of the caecal pH and alter the oxidation and reduction potentials, and together this may suppress the growth of potentially pathogenic bacteria (104, 105). Therefore, supplementation of the diet with dried whey proteins sounds to be an effective method in enhancing the productivity of broilers.

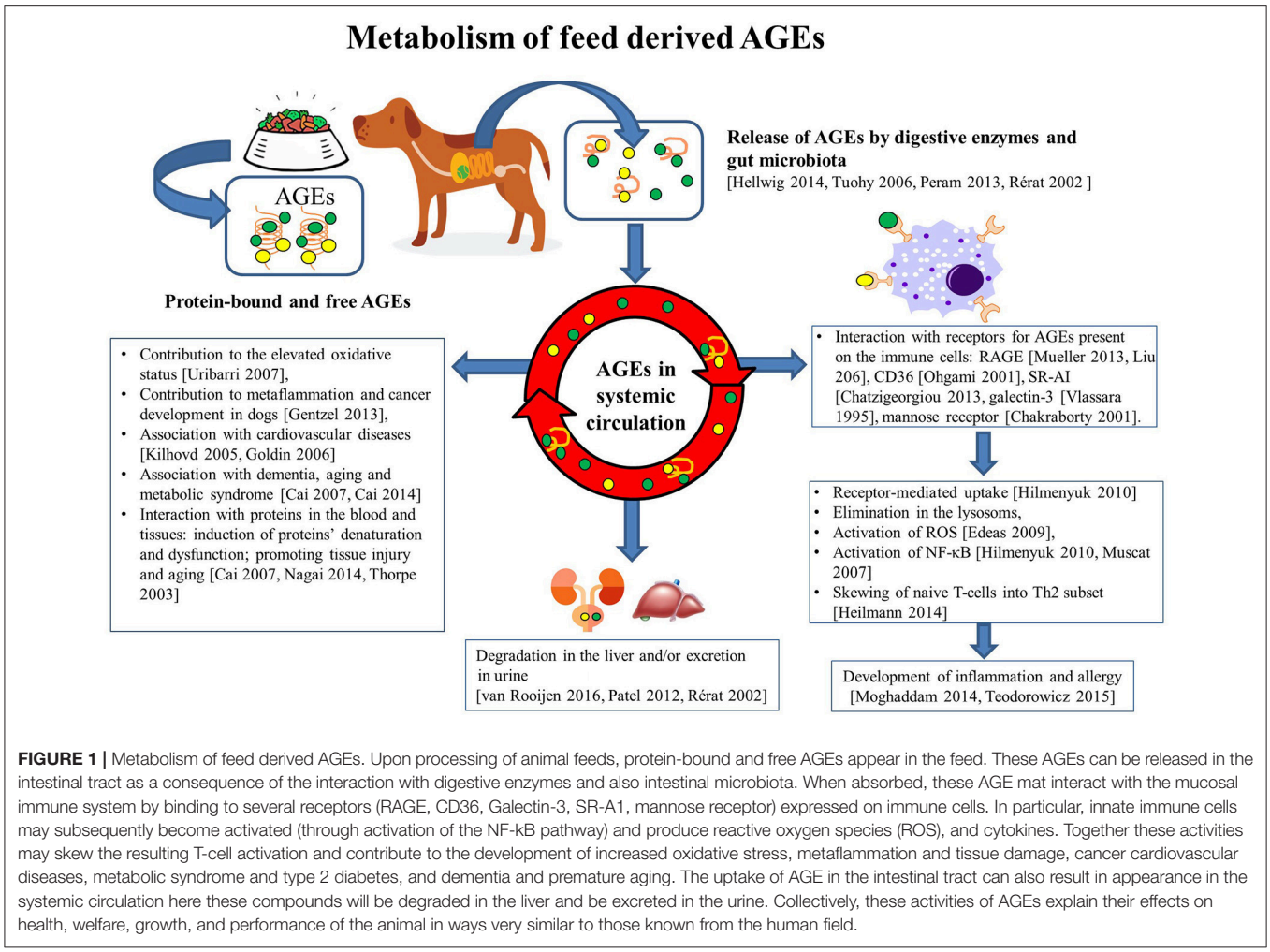


FIGURE 1 | Metabolism of feed derived AGEs. Upon processing of animal feeds, protein-bound and free AGEs appear in the feed. These AGEs can be released in the intestinal tract as a consequence of the interaction with digestive enzymes and also intestinal microbiota. When absorbed, these AGE mat interact with the mucosal immune system by binding to several receptors (RAGE, CD36, Galectin-3, SR-A1, mannose receptor) expressed on immune cells. In particular, innate immune cells may subsequently become activated (through activation of the NF-kB pathway) and produce reactive oxygen species (ROS), and cytokines. Together these activities may skew the resulting T-cell activation and contribute to the development of increased oxidative stress, metaflammation and tissue damage, cancer cardiovascular diseases, metabolic syndrome and type 2 diabetes, and dementia and premature aging. The uptake of AGE in the intestinal tract can also result in appearance in the systemic circulation here these compounds will be degraded in the liver and be excreted in the urine. Collectively, these activities of AGEs explain their effects on health, welfare, growth, and performance of the animal in ways very similar to those known from the human field.

The combination of proteins, sugar, and high temperatures during thermal processing of milk and whey proteins makes milk and other dairy products prone to glycation and creation of AGEs. In milk, whey proteins are the most heat vulnerable proteins and are subject to glycation (109). Intensive heating has been already shown to promote the formation of Maillard reaction products in whey. Intensive dry heating at a lower water activity (aw= 0.23) favors the protein aggregation and the occurrence of Maillard reaction of whey proteins (66, 67). Moreover, dry heating promoted aggregation of whey proteins and thereby the formation of sRAGE-binding ligands which influence the immunogenicity of the food compounds (66, 67). RAGE is considered an innate immunity related pattern recognition receptor that recognizes mainly conformational secondary structures, such as β-sheets and fibrils, rather than the primary amino acid sequence of proteins (110). This may suggest that thermally induced increase in the content of β-sheets also favors the aggregation of whey proteins and at the same time the binding to sRAGE (66, 67). Effects of protein denaturation during the heating are specially known for whey proteins (111). Accordingly, investigating an interaction between these AGE-modified proteins and cellular receptors on immune cells (as a crucial encounter and interaction point) seems worthwhile.

AGEs in Pet Foods

Pet animals, including dogs and cats, are most often fed commercial foods that are highly processed and which they consume during their entire live. The main processing procedures for these foods rely on heat treatments (e.g., extrusion, sterilization, drying) to improve their nutrient digestibility, shelf life, and safety. As a result, the proportion of reactive lysine is on average, 73% (range 39–100%) of total lysine, while foods for growing dogs may supply less lysine content than the animals require and is recommended. As a consequence higher AGEs contents in plasma from dogs suffering from canine diabetes mellitus and impaired renal function compared with healthy control animals fed for prolonged periods with these processed foods (3).

The ingredients used to formulate pet foods and the types of processing appear to be key factors for the MRP concentrations in the final product. On average and on a dry matter basis, higher MRP and AGEs amounts occur in canned foods than in pelleted and extruded foods. van Rooijen et al. (112) calculated that the content of CML and HMF that are present in commercial pet foods are, on average, within the range reported in processed human food products. However, the average daily intake (mg/kg body weight$^{0.75}$) of HMF was 122 times higher for dogs and 38

times higher for cats than the calculated average intake for adult humans, while the average daily intake of CML was comparable to the intake of adult humans (112). This study also highlighted the importance of measuring the reactive lysine content in foods for growing dogs used as weaning diets.

The important questions remains whether the MRP and AGEs contents reported in pet foods are physiologically relevant in these animals and this depends, in part, on the bioavailability of these MRP components. The observed increase in urinary excretion with increasing dietary intake indicates that dietary MRPs are absorbed from the gastro-intestinal tract of adult cats and excreted in the urine. The observed decrease in urinary recovery with increasing intake suggests a limiting factor in digestion, absorption, metabolism or urinary excretion. Whether such absorbed dietary MRP affect the long term health of pet animals has, hitherto, not been studied.

AGEs in Pig Feed

Proteins are the main macronutrient in swine feed and, thus, understanding the absorption and the utilization by the animal is important for successful swine production and the sustainability of this sector. Storage and in particular processing conditions largely determine the nutritional value of important feed ingredients and this is most likely dependent on the combination of heating and humidity parameters that induces the formation of MRP (113). Many different processing conditions of feed ingredients are generally used in swine diets (e.g., soybean meal, dried distillers grain, corn, maize gluten feed). Heat processing to improve nutritional quality and to remove solvents that are commonly used during oil extraction is commonly required when producing oilseed ingredients (soybean meal, canola meal, sunflower meal, and cottonseed meal). These procedures comprise varying degrees of heat with the risk to be deleterious to protein quality, especially when applying high temperature regimens. As discussed above lysine, being the most important limiting amino acid in swine feed ingredients, is the most reactive amino acids in the MR. Consequently, lysine is commonly added to diets of swine in crystalline form to ensure that the balance of absorbed and available amino acids closely aligns with the requirements for protein synthesis to ensure optimal performance.

AGEs in Cattle Feed

Conventionally fed cows regularly consume concentrated and processed feed containing MRP and AGEs as a consequence of heating and these compounds can be measured in the milk. Organically fed cows only get non-heated feed such as grass or silage. Thus, organic milk is supposed to contain fewer glycated proteins and the contents and composition can now be measured (114). Dietary MRPs like pyrraline can be found in the urine originating from blood clearance, and therefore it was speculated that cows excrete AGEs in milk during lactation and thereby expose the suckling newborn. The milk yield of dairy cows has increased significantly over the past decades (115) and thus, a cow needs a ration with a higher energy density and more nitrogen compared to its natural food sources. Thus, the use of processed molasses, soybean meal, and rapeseed meal are increasingly used with the risk of exposure to elevated MRP levels (116). Dietary MRPs, are able to influence the rumen microbiota (117). In addition, also the digestibility of the roughages used to formulate dairy rations, is often low and therefore MRP-containing concentrates that can be absorbed are necessary to ensure dairy productivity. Processed milk proteins using hydrolysis but also heat-induced glycation, display anti-oxidant and anti-inflammatory activities and thereby enhanced functional properties. Raw beef and pork naturally have a small proportion of protein-bound AGEs, while that is much more in raw chicken breasts. Therefore, there is no strong influence of the protein content on the total amount of protein-bound AGEs in beef. The commercial processing strategy, will thus largely determine the final amount of protein-bound AGEs (118).

AGE and Lameness in Dairy Cattle

One of the most prominent and serious health and welfare problems in dairy cattle worldwide is lameness (or laminitis), mostly due to injury or inflammatory disease in the hoof (119, 120). The prevalence of lameness of dairy cows ranges from 2 to 55% throughout the world depending on area, and has dramatically increased in herds over the past 20 years (121). Apart from the fact that lameness is considered to be a crucial welfare issue, lameness has also a significant economic impact due to a loss in milk production (122–124), reduction in fertility (125–127), and hence an increased risk of culling (128, 129). Laminitis can be defined as a diffuse aseptic inflammation of the dermis of the claw (*Pododermatitis aseptica diffusa*) and is considered to be an important cause of lameness (130–132). It is well-documented that laminitis-related claw lesions including hemorrhage of the sole and the white line along with sole ulcers, are considered to be the most important causes of lameness in dairy cows (119, 133, 134). Many predisposing factors are associated with the occurrence of laminitis including farm management, housing, genetics, breeding, and nutrition (135, 136). Although nutrition is widely related to the development of laminitis, the mechanisms underlying the characteristics of ration and/or feeding management and how these contribute to laminitis occurrence have not yet been extensively studied.

Also in equines, laminitis can occur and the role of obesity and insulin resistance are well known factors that are closely related to the development of laminitis (137–139). The role of insulin was experimentally confirmed by inducing laminitis in clinically normal horses by prolonged infusions of insulin and glucose to maintain physiological levels of plasma glucose (140). The presence of dietary AGEs was suggested to be involved in the development of insulin-induced laminitis. Also in cattle, the combination of dietary AGEs and insulin resistance in development of laminitis was suggested (141). It has been shown that insulin resistance is often seen in early lactating cows (142, 143), which may be exacerbated by high intakes of rapidly fermentable carbohydrates or starch (144). Interestingly, high prevalence of laminitis lesions, i.e., hemorrhage of the sole and white line are

often observed during early lactation (145–148). It can be argued that the occurrence of laminitis during early lactation may be related to insulin resistance and the formation of AGEs.

Advanced glycation end products are derived from glucose through intermediates such as glyoxal, 3-deoxyglucosone and methylglyoxal (149, 150) and it has been postulated that methylglyoxal is a major source of intracellular and plasma AGEs (151). It has been shown that under *in vitro* conditions, the enzymes glyoxalase I and II act in concert to convert methylglyoxal into D-lactate, thereby, preventing the formation of AGEs. In bovines and equines suffering from a systemic acidosis induced by the feeding of high amounts of fermentable carbohydrates and subsequent acidosis of either the rumen or cecum, bovine plasma levels of D- lactate may increase up to 25 mMol/L (152, 153). Unfortunately, the end product D-lactate exerts a negative feedback on the activity of glyoxalase I but this notion may be of interest in relation to the development of laminitis. Apart from the potential that AGEs may be derived from methylglyoxal that originate from the animals intermediary metabolism, methylglyoxal can also be formed during the anaerobic fermentation of rapidly fermentable carbohydrates (154, 155). It was already mentioned that methylglyoxal is converted to D-lactate under physiological conditions. Therefore, it can be speculated that under practical feeding conditions of cows and horses, the fermentation of rapidly fermentable carbohydrates results in the accumulation of both D-lactate and methylglyoxal. Methylglyoxal is toxic to cells (156), which ultimately results in the lysis of bacteria (155) and the subsequent release of lipopolysaccharides, which are implicated in the etiology of laminitis in both bovines (132) and equines (157, 158). Alternatively, methylglyoxal may be absorbed by the rumen epithelium and across the epithelium of the gastro-intestinal tract of bovine and equine and subsequently triggers the formation of AGEs.

DISCUSSION

Thermal processing of food alters the chemical and biological characteristics of the food components. An example of biochemical changes as a result of heating is the creation of MRPs and AGEs. The MR modified proteins are created in presence of sugars and heat. These molecules are present in various forms in a heterogeneous mixture that justifies their diverse bioactivities.

Feed components and formulation, but also feed processing determine intestinal health and disease resistance. Protein feeds that contain MRP and AGE can cause expansion of intestinal microbiota and together with potentially gut barrier damaging compounds can compromise epithelial barrier function and cause immune stimulation resulting in lower growth, performance and ultimately, in development of disease.

Animals reared and kept in industrial systems are subjected to immunological stress, that together with the pathogen load, the husbandry environment, the feed composition and regimen, and the installed vaccination program determine the immune status. As a consequence, inflammation can occur associated with the release of pro-inflammatory cytokines, the mobilization of nutritional reserves, suppressed nutrient absorption in the gut, and body fluid loss like diuresis and diarrhea. Therefore, inflammation will come at a significant nutrient cost. By activation of the adaptive immune response, specific antibodies will be produced that will consume a relatively small nutrient cost. Thus, dietary immunomodulators and/or vaccines that enhance immune responsiveness and minimize immunological stress will positively affect health, growth, and performance.

The dietary glycated proteins (dAGEs) have shown to have immunomodulatory properties (159, 160). There is substantial evidence to support the association of these glycated proteins with several chronic disorders, which are principally caused due to the accumulation of AGE-modified proteins in cells and tissues (161–164). A probable mechanism for such an immune-stimulatory effect could be the interaction between the AGEs and the antigen presenting cells including macrophages and DCs (165, 166). The pattern recognition receptors present on the cell membrane of these cells recognize the modified proteins, form complexes, and initiate internalization. As a result, activation of the NF-kB transcription factor occurs, leading to the production of pro-inflammatory cytokines and also induction of oxidative stress. Additionally, since macrophages and DCs are APCs, peptides derived from the processed antigens will be presented to CD4+ T-cells on their MHC-II molecules. The combination of presented antigens and secreted cytokine will activate the T-cells and induce cellular responses leading to a chronic inflammatory state if the stimulation persists or the inhibitory mechanisms are inefficient for resolving the homeostasis.

Due to the large diversity of AGEs that are formed during Maillard reactions, various cellular receptors were shown to have binding affinity for these AGEs. Among these receptors, RAGE is the most referred and studied one and sRAGE is the soluble variant of this receptor (167–169). The transmembrane and intracellular signaling domains, which are present in RAGE but not sRAGE, are crucial for transducing the signal to the nucleus and activation of NF-kB (170). However, as sRAGE still carries the ligand-binding domain (V domain), it has a similar binding affinity to AGE-modified proteins as RAGE itself (171). *In vivo*, sRAGE plays a decoy role and binds to circulating AGEs, thereby regulating the interaction between membrane-associated RAGE and the AGEs (110). This interaction between plasma sRAGE and AGEs decreases the risk of undesirable inflammatory response since unlike RAGE, these complexes are assumed to be degraded (172). Indeed, lower plasma levels of sRAGE were reported in patients with chronic inflammation (173). Likewise RAGE, Galectin-3 interacts with the AGEs and contributes to subsequent cell signaling and also uptake of these modified proteins (174). Since this receptor lacks the transmembrane domain, Galectin-3 links to other members of AGE-R complex namely AGE/R1/OST-48 and AGE-R/80K-H. The available CRD on Galectin-3 has an affinity for lactose (175), which may explain the potential interaction with food or feed-derived AGEs. Activation of Galectin-3 leads to an alteration in biological processes including the immune responses and inflammation (176, 177). Furthermore, SR-AI and SR-BIII (CD36) are two members of the macrophage scavenger receptor family that were

shown to bind to the glycated proteins (178, 179). These two receptors basically facilitate the endocytosis of the AGEs and are abundantly present on phagocytes (180). The cellular responses following the interaction of these receptors and the AGEs are involved in multiple functions including immune (allergic or/and inflammatory) response (181).

As mentioned before, RAGE is the most recognized receptor for AGEs and the majority of the available studies have focused on this receptor. The structure of the protein is important for driving its biological activities and any modification in this assembly leads to an altered function. As a result of the heat, the hydrogen bonds and polar hydrophobic interactions in secondary and tertiary structures of the proteins are distorted (109). Therefore, the alpha-helixes and beta-sheets are disrupted and the molecule loses its natural folding and 3-dimensional shape. Furthermore, the denatured proteins undergo crosslinking and agglomeration (182). Two or more denatured molecules covalently attach to each other and form new structures that possibly do not match the characteristics of any of the parent molecules. These new molecules are usually rather bulky with altered conformational epitopes that might be recognized by the cellular receptors. Liu et al. showed the association of RAGE binding with whey protein agglomeration (170). In general, the RAGE receptor binding is higher in heated and glycated proteins as they went through structural alterations and became more potent for receptor binding when compared to the unheated samples (183). Generally, glycated proteins (AGEs) have a higher affinity for binding to sRAGE, CD36, SR-AI, and Galectin-3 (16, 183–185).

CONCLUSION

In conclusion, during the heating of foods and feeds, protein denaturation, glycation, and agglomeration can occur. These modifications in protein structures, increased their binding affinity to cellular receptors that are mainly present on antigen-presenting cells: namely sRAGE, CD36, SR-AI, and Galectin-3.Although both heated and glycated proteins show an increased receptor binding capacity, the effect of glycation is generally more prominent when compared to the heated one. Despite the proven relation of the MR to affect the protein quality by impairing the bioavailability of amino acids and minerals, decreasing the digestibility and increasing the immunoreactivity of proteins, more information on the physiological and immunological effects of the consumption of MRPs rich diets by animals is urgently needed to the benefit of the health, welfare, growth, and performance of animals.

AUTHOR CONTRIBUTIONS

MT and HS wrote the manuscript. HW and WH reviewed the manuscript.

REFERENCES

1. Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Nat Acad Sci USA*. (1994) 91:4766–70.
2. Morris A, Barnett A, Burrows O. Effect of processing on nutrient content of foods. *Cajarticles* (2004) 37:160–4.
3. Tessier FJ. The Maillard reaction in the human body. The main discoveries and factors that affect glycation. *Pathol Biol.* (2010) 58:214–9. doi: 10.1016/j.patbio.2009.09.014
4. Maillard LC. Action des acides amines sur les sucres: formation des melanoidines par voie methodique. *Compt Rend.* (1912) 154:66–8.
5. Hodge JE. Chemistry of browning reactions in model systems. *J Agric Food Chem.* (1953) 1:928–43.
6. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetalogia* (2001) 41:129–46. doi: 10.1007/s0012500 51591
7. van Rooijen C, Bosch G, van der Poel AF, Wierenga PA, Alexander L, Hendriks WH. The Maillard reaction and pet food processing: effects on nutritive value and pet health *Nutr Res Rev.* (2013) 26:130–48. doi: 10.1017/S0954422413000103
8. Kasper M, Funk RHW. Age-related changes in cells and tissues due to advanced glycation end products (AGEs). *Arch Gerontol Geriatrics* (2001) 32:233–43. doi: 10.1016/S0167-4943(01)00103-0
9. Toda M, Heilmann M, Ilchmann A, Vieths S. The Maillard reaction and food allergies: is there a link? *Clin Chem Lab Med.* (2014) 52:61–7. doi: 10.1515/cclm-2012-0830
10. Almeida FN. *Effects of the Maillard Reactions on Chemical Composition and Amino Acid Digestibility of Feed Ingredients and on Pig Growth Performance.* PhD Thesis, University of Illinois (2013).
11. O'Brien J, Morrissey PA, Ames JM. Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit Rev Food Sci Nutr.* (1989) 28:211–48.
12. Bender AE. Nutritional effects of food processing. *Int J Food Sci Technol.* (1966) 1:261–89.
13. John WG, Lamb EJ. The maillard or browning reaction in diabetes. *Eye* (1993) 7:230–7.
14. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Ann Rev Med.* (1995) 46:223–34.
15. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *New Engl J Med.* (1988) 318:1315–21.
16. Goldin A, Beckman JA, Schmidt A, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* (2006) 114:597–605. doi: 10.1161/CIRCULATIONAHA.106.621854
17. Uribarri J. (Ed.). *Dietary AGEs and Their Role in Health and Disease.* London: CRC Press; Taylor & Francis Group (2017).
18. Chuyen NV, Arai H, Nakanishi T, Utsunomiya N. Are food advanced glycation end products toxic in biological systems? *Ann NY Acad Sci.* (2005) 1043:467–73. doi: 10.1196/annals.1333.053
19. Chuang PY, Yu Q, Fang W, Uribarri J, He JC. Advanced glycation endproducts induce podocyte apoptosis by activation of the FOXO4 transcription factor. *Kidney Int.* (2007) 72:965–76. doi: 10.1038/sj.ki.5002456
20. Jung T, Catalgol B, Grune T. The proteasomal system. *Mol Aspects Med.* (2009) 30:191–296. doi: 10.1016/j.mam.2009.04.001
21. Grimm S, Horlacher M, Catalgol B, Hoehn A, Reinheckel T, Grune T. Cathepsins D and L reduce the toxicity of advanced glycation end products. *Free Radic Biol Med.* (2012) 52:1011–23. doi: 10.1016/j.freeradbiomed.2011.12.021
22. Finot PA. The absorption and metabolism of modified amino acids in processed foods. *J AOAC Int.* (2005) 88:894–903.
23. Kellow NJ, Coughlan MT. Effect of diet-derived advanced glycation end products on inflammation. *Nutr Rev.* (2015) 73:737–59. doi: 10.1093/nutrit/nuv030
24. Uribarri J, Cai W, Sandu O, Peppa M, Goldberg T, Vlassara H. Diet-derived advanced glycation end products are major contributors to the body's AGE

pool and induce inflammation in healthy subjects. *Ann N Y Acad Sci.* (2005) 101:461–6. doi: 10.1196/annals.1333.052
25. Almeida FN, Htoo JK, Thomson J, Stein HH. Effects of balancing crystalline amino acids in diets containing heat-damaged soybean meal or distillers dried grains with solubles fed to weanling pigs. *Animal* (2014) 8:1594–602. doi: 10.1017/S175173111400144X
26. Ford JE, Hurrel RF, Finot PA. Storage of milk powders under adverse conditions. *Br J Nutr.* (1983) 49:355–64.
27. Vlassara H, Bucala R, Striker LJ. Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest.* (1994) 70:138–51.
28. Ramasamy R, Vannucci J, Yan D, Herold K, Yan F, Schmidt AM. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* (2005) 15:16–28R. doi: 10.1093/glycob/cwi053
29. De Groot J, Verzijl N, Wenting-van Wijk MJG, Jacobs KMG, Van El B, Van Roermund PM, et al. Accumulation of advanced glycation end products as a molecular mechanism for aging as a risk factor in osteoarthritis. *Arthritis Rheum.* (2004) 50:1207–15. doi: 10.1002/art.20170
30. Chiers K, Vandenberge V, Ducatelle R. Accumulation of advanced glycation end products in canine atherosclerosis. *J Comp Pathol.* (2010) 143:65–9. doi: 10.1016/j.jcpa.2009.12.006
31. Gentzel JB. Does contemporary canine diet cause cancer? A review. *Vet World* (2013) 6:632–9. doi: 10.14202/vetworld.2013.632-639
32. Tessier FJ, Birlouez-Aragon I. Health effects of dietary Maillard reaction products: the results of ICARE and other studies. *Amino Acids* (2012) 42:1119–31. doi: 10.1007/s00726-010-0776-z
33. Nass N, Bartling B, Navarrete Santos A, Scheubel RJ, Borgermann J, Silber RE, et al. Advanced glycation end products, diabetes and ageing. *Z Gerontol Geriatr.* (2007) 40:319–56. doi: 10.1007/s00391-007-0484-9
34. Comazzi S, Bertazzolo W, Bonfanti U, Spagnolo V, Sartorelli P. Advanced glycation end products and sorbitol in blood from differently compensated diabetic dogs. *Res Vet Sci.* (2008) 84:341–6. doi: 10.1016/j.rvsc.2007.05.013
35. Bras ID, Colitz CMH, Kusewitt DF, Chandler H, Lu P, Gemensky-Metzler AJ, et al. Evaluation of advanced glycation end-products in diabetic and inherited canine cataracts. *Graefes Arch Clin Exp Ophthalmol.* (2007) 245:249–57. doi: 10.1007/s00417-006-0293-7
36. Uribarri J, Cai W, Peppa M, Goodman S, Ferrucci L, Striker G, et al. Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *J Gerontol A Biol Sci Med Sci.* (2007) 62:427–33. doi: 10.1093/gerona/62.4.427
37. Baynes JW, Gillery P. Frontiers in research on the Maillard reaction in aging and chronic disease. *Clin Chem Lab Med.* (2014) 52:1–3. doi: 10.1515/cclm-2013-0551
38. Robert L, Labat-Robert J. Role of the Maillard reaction in aging and age-related diseases. Studies at the cellular-molecular level. *Clin Chem Lab Med.* (2014). 52:5–10. doi: 10.1515/cclm-2012-0763
39. Cai W, Uribarri J, Zhu L, Chen X, Swamy S, Zhao Z, et al. Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans. *Proc Natl Acad Sci USA.* (2014) 111:4940–5. doi: 10.1073/pnas.1316013111
40. Monnier VM, Taniguchi N. Advanced glycation in diabetes, aging and age-related diseases: conclusions. *Glycoconj J.* (2016) 33:691–2. doi: 10.1007/s10719-016-9711-1
41. Mehta BM, Deeth HC. Blocked lysine in dairy products: formation, occurrence, analysis, and nutritional implications. *Compreh Rev Food Sci Food Saf.* (2016) 15:206–18. doi: 10.1111/1541-4337.12178
42. Žilić S, BoŽović I, Savić S, Šobajić, S. Heat processing of soybean kernel and its effect on lysine availability and protein solubility. *Cent Eur J Biol.* (2006) 1:572–83.
43. Wellner A, Huettl C, Henle T. Formation of Maillard reaction products during heat treatment of carrots. *J Agric Food Chem.* (2011) 59:7992–8. doi: 10.1021/jf2013293
44. Hendriks WH, Moughan PJ, Boer H, van der Poel AFB. Effects of extrusion on the dye-binding, fluorodinitrobenzene-reactive and total lysine content of soyabean meal and peas. *Animal Feed Sci Technol.* (1994) 48:99–109.
45. Žilić S, Ataç Mogol B, Akillioglu G, Serpen A, Babić M, Gökmen V. Effects of infrared heating on phenolic compounds and Maillard reaction products in maize flour. *J Cereal Sci.* (2013) 58:1–7. doi: 10.1016/j.jcs.2013.05.003
46. van Boekel MA. Kinetic aspects of the Maillard reaction: a critical review. *Mol Nutr Food Res.* (2001) 45:150–9. doi: 10.1002/1521-3803(20010601)45:3<150::AID-FOOD150>3.0.CO;2-9
47. Navarro MP. Impact of Maillard reaction products on mineral bioavailability. In: Vaquero MP, García-Arias, T, Carbajal, A, Sánchez-Múniz FJ, Ediors. *Bioavailability of Micronutrients and Minor Dietary Compounds. Metabolic and Technological Aspects.* Kerala: Research Signpost (2003). p. 133–45.
48. Delgado-Andrade C, Seiquer I, Navarro MP. Comparative effects of glucose-lysine versus glucose-methionine Maillard reaction products consumption: *in vitro* and *in vivo* calcium availability. *Mol Nutr Food Res.* (2005) 49:679–84. doi: 10.1002/mnfr.200400100
49. Rérat A, Calmes R, Vaissade P, Finot PA. Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig. Significance for man. *Eur J Nutr.* (2002) 41:1–11. doi: 10.1007/s003940200000
50. Larsen JA, Calvert CC, Rogers OR. Processing of dietary casein decreases bioavailability of lysine in growing kittens. *J Nutr.* (2002) 132:1748–50S. doi: 10.1093/jn/132.6.1748S
51. Seiquer I, Diaz-Alguacil J, Delgado-Andrade C, Lopez-Frias M, Munoz Hoyos A, Galdo G, et al. Diets rich in maillard reaction products affect protein digestibility in adolescent males aged 11-14 y. *Am J Clin Nutr.* (2006) 83:1082–8. doi: 10.1093/ajcn/83.5.1082
52. Delgado-Andrade C, Seiquer I, García MM, Galdó G, Navarro MP. Increased Maillard reaction products intake reduces phosphorus digestibility in male adolescents. *Nutrition* (2011) 27:86–91. doi: 10.1016/j.nut.2009.10.009
53. Oste R. Effect of maillard reaction products on protein digestion. *Prog Clin Biol Res.* (1989) 304:329–42.
54. Oste R, Sjodin P. Effect of maillard reaction products on protein digestion. *In vivo* studies on rats *J Nutr.* (1984) 114:2228–34.
55. Somoza V, Wenzel E, Weiss C, Clawin-Radecker I, Grubel N, Erbersdobler HF. Dose-dependent utilisation of casein-linked lysinoalanine, n(epsilon)-fructoselysine and n(epsilon)-carboxymethyllysine in rats. *Mol Nutr Food Res.* (2006) 50:833–41. doi: 10.1002/mnfr.200600021
56. Henle T. Protein-bound advanced glycation endproducts (ages) as bioactive amino acid derivatives in foods. *Amino Acids* (2005) 29:313–22. doi: 10.1007/s00726-005-0200-2
57. Sharma C, Kaur A, Thind SS, Singh B, Raina, S. Advanced glycation End-products (AGEs): an emerging concern for processed food industries. *J Food Sci Technol.* (2015) 52:7561–76. doi: 10.1007/s13197-015-1851-y
58. Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ. Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin *J Biol Chem.* (1994) 269:32299–305.
59. Nagaraj RH, Shipanova IN, Faust FM. Protein cross-linking by the Maillard reaction. Isolation, characterization, and *in vivo* detection of a lysine-lysine cross-link derived from methylglyoxal. *J Biol Chem.* (1996) 271:19338–45.
60. Chellan P, Nagaraj RH. Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes. *Arch Biochem Biophys.* (1999) 368:98–104.
61. Hellwig M, Matthes R, Peto A, Löbner J, Henle T. N-ε-fructosyllysine and N-ε-carboxymethyllysine, but not lysinoalanine, are available for absorption after simulated gastrointestinal digestion. *Amino Acids* (2014) 46:289–99. doi: 10.1007/s00726-013-1501-5
62. Patel HA, Singh H, Anema SG, Creamer LK Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. *J Agric Food Chem.* (2006) 54:3409–20. doi: 10.1021/jf052834c
63. Peram MR, Loveday SM, Ye A, Singh H. *In vitro* gastric digestion of heat-induced aggregates of beta-lactoglobulin. *J Dairy Sci.* (2013) 96:63–74. doi: 10.3168/jds.2012-5896
64. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through peyer's patches. *Allergy* (2008) 63:882–90. doi: 10.1111/j.1398-9995.2008.01673.x

65. Stojadinovic M, Pieters R, Smit J, Velickovic TC. Cross-linking of beta-lactoglobulin enhances allergic sensitization through changes in cellular uptake and processing. *Toxicol Sci.* (2014) 140:224–35. doi: 10.1093/toxsci/kfu062
66. Liu F, Teodorowicz M, Wichers HJ, van Boekel MA, Hettinga KA. Generation of Soluble Advanced Glycation End Products Receptor (sRAGE)-binding ligands during extensive heat treatment of whey protein/lactose mixtures is dependent on glycation and aggregation. *J Agric Food Chem.* (2016) 64:6477–86. doi: 10.1021/acs.jafc.6b02674
67. Liu F, Teodorowicz M, van Boekel MA, Wichers HJ, Hettinga KA. The decrease in the IgG-binding capacity of intensively dry heated whey proteins is associated with intense maillard reaction, structural changes of the proteins and formation of rage-ligands. *Food Funct.* (2016) 7:239–49. doi: 10.1039/c5fo00718f
68. Dobson CM. Protein folding and misfolding. *Nature* (2003) 426:884–90. doi: 10.1038/nature02261
69. Edeas M, Attaf D, Mailfert AS, Nasu M, Joubet R. Maillard reaction, mitochondria and oxidative stress: potential role of antioxidants. *Pathol Biol.* (2010) 58:220–5. doi: 10.1016/j.patbio.2009.09.011
70. Kierdorf K, Fritz G. RAGE regulation and signaling in inflammation and beyond. *J Leukocyte Biol.* (2013) 94:55–68. doi: 10.1189/jlb.1012519
71. Muscat S, Pelka J, Hegele J, Weigle B, Münch G, Pischetsrieder M. Coffee and Maillard products activate NF-kappaB in macrophages via H_2O_2 production. *Mol Nutr Food Res.* (2007) 51:525–35. doi: 10.1002/mnfr.200600254
72. Sarabia-Sainz HM, Mata Haro V, Sarabia Sainz JA, Vázquez-Moreno L, Montfort GR. Maillard neoglycans as inhibitors for *in vitro* adhesion of F4+ enterotoxigenic *Escherichia coli* to piglet intestinal cells. *Acta Biochim Pol.* (2017) 64:679–86. doi: 10.18388/abp.2017_2199
73. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KM. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J Dairy Sci.* (2005) 88:1399–403. doi: 10.3168/jds.S0022-0302(05)72807-1
74. Yan SF, Yan SD, Ramasamy R, Schmidt AM. Tempering the Wrath of RAGE: an emerging therapeutic strategy against diabetic complications, neurodegeneration and inflammation. *Ann Med.* (2009) 41:408–22. doi: 10.1080/07853890902806576
75. Zebeli Q, Metzler-Zebeli BU, Ametaj BN. Meta-analysis reveals threshold level of rapidly fermentable dietary concentrate that triggers systemic inflammation in cattle. *J Dairy Sci.* (2012) 95:2662–72. doi: 10.3168/jds.2011-5080
76. Cai W, He JC, Zhu L, Chen X, Wallenstein S, Striker GE, et al. Reduced oxidant stress and extended lifespan in mice exposed to a low glycotoxin diet association with increased AGER1 Expression. *Am J Pathol.* (2007) 170:1893–902. doi: 10.2353/ajpath.2007.061281
77. Teodorowicz M, Jansen APH, Roovers MHWM, Ruinemans-Koerts J, Wichers HJ, Savelkoul HFJ. Maillard-type neoallergens present in processed soy extract may cause an allergic reaction in soy allergic patients. *Clin Translat Allergy* (2015) 5:P21. doi: 10.1186/2045-7022-5-S3-P21
78. Gupta RK, Gupta K, Sharma A, Das M, Ansari IA, Dwivedi PD. Maillard reaction in food allergy: Pros and cons. *Crit Rev Food Sci Nutr.* (2018) 58:208–16. doi: 10.1080/10408398.2016.1152949
79. Vissers YM, Blanc F, Skov PS, Johnson PE, Rigby NM, Przybylski-Nicaise L, et al. Effect of heating and glycation on the allergenicity of 2S Albumins (Ara h 2/6) from peanut. *PLoS ONE* (2011) 6:e23998. doi: 10.1371/journal.pone.0023998
80. Maleki SJ, Chung SY, Champagne ET, Raufman JP. The effects of roasting on the allergenic properties of peanut proteins. *J Allergy Clin Immunol.* (2000) 106:763–8. doi: 10.1067/mai.2000.109620
81. Hilmenyuk T, Bellinghausen I, Heydenreich B, Ilchmann A, Toda M, Grabbe S, et al. Effects of glycation of the model food allergen ovalbumin on antigen uptake and presentation by human dendritic cells. *Immunology* (2010) 129:437–45. doi: 10.1111/j.1365-2567.2009.03199.x
82. Gruber P, Vieths S, Wangorsch A, Nerkamp J, Hofmann T. Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J Agricult Food Chem.* (2004) 52:4002–7. doi: 10.1021/jf035458+
83. Ohashi K, Takahashi HK, Mori S, Liu K, Wake H, Sadamori H, et al. Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction. *Clin Immunol.* (2010) 134:345–53. doi: 10.1016/j.clim.2009.10.008
84. Pollreisz A, Hudson BI, Chang JS, Qu W, Cheng B, Papapanou PN, et al. Receptor for advanced glycation endproducts mediates pro-atherogenic responses to periodontal infection in vascular endothelial cells. *Atherosclerosis* (2010) 212:451–6. doi: 10.1016/j.atherosclerosis.2010.07.011
85. Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP, et al. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J Immunol.* (2005) 174:7506–15. doi: 10.4049/jimmunol.174.12.7506
86. Teodorowicz M, van Neerven J, Savelkoul H. Food processing: the influence of the maillard reaction on immunogenicity and allergenicity of food proteins. *Nutrients* (2017) 9:E835. doi: 10.3390/nu9080835
87. Vlassara H, Li YM, Imani F, Wojciechowicz D, Yang Z, Liu FT, et al. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (age): a new member of the age-receptor complex. *Mol Med.* (1995) 1:634–46.
88. Henderson NC, Sethi T. The regulation of inflammation by galectin-3. *Immunol Rev.* (2009) 230:160–71. doi: 10.1111/j.1600-065X.2009.00794.x
89. Liu F-T, Rabinovich GA. Galectins: regulators of acute and chronic inflammation. *Ann N Y Acad Sci.* (2010) 1183:158–82. doi: 10.1111/j.1749-6632.2009.05131.x.
90. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol.* (2013) 13:621–34. doi: 10.1038/nri3515
91. Silverstein RL, Febbraio M. CD36, a Scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci Signal.* (2009) 2:re3. doi: 10.1126/scisignal.272re3
92. Ohgami N, Nagai R, Ikemoto M, Arai H, Kuniyasu A, Horiuchi S, et al. CD36, a member of the class b scavenger receptor family, as a receptor for advanced glycation end products. *J Biol Chem.* (2001) 276:3195–202. doi: 10.1074/jbc.M006545200
93. Geijtenbeek TBH, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell–specific ICAM-3 receptor that supports primary immune responses. *Cell* (2000) 100:575–85. doi: 10.1016/S0092-8674(00)80693-5
94. Geijtenbeek TBH, Engering A, van Kooyk Y. DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. *J Leukocyte Biol.* (2002) 71:921–31. doi: 10.1189/jlb.71.6.921
95. Williams R. Dendritic cells: one SIGN, different paths. *Nat Rev Immunol.* (2009) 9:676. doi: 10.1038/nri2640
96. Martinez-Pomares L, Linehan SA, Taylor PR, Gordon, S. Binding properties of the mannose receptor. *Immunobiology* (2001) 204:527–35. doi: 10.1078/0171-2985-00089
97. Largent BL, Walton KM, Hoppe CA, Lee YC, Schnaar RL. Carbohydrate-specific adhesion of alveolar macrophages to mannose-derivatized surfaces. *J Biol Chem.* (1984) 259:1764–9.
98. Engering AJ, Cella M, Fluitsma D, Brockhaus M, Hoefsmit EC, Lanzavecchia A, et al. The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur J Immunol.* (1997) 27:2417–25.
99. Waithman J, Moffat JM, Patterson NL, van Beek AE, Mintern JD. Antigen Presentation. In: *Reference Module in Biomedical Sciences*. Elsevier (2014). doi: 10.1016/B978-0-12-801238-3.00118-5
100. Kurtz J. Specific memory within innate immune systems. *Trends Immunol.* (2005) 26:186–92. doi: 10.1016/j.it.2005.02.001
101. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad, M, Ley, K. Development of monocytes, macrophages and dendritic cells. *Science* (2010) 327:656–61. doi: 10.1126/science.1178331
102. Heilmann M, Wellner A, Gadermaier G, Ilchmann A, Briza P, Krause M, et al. Ovalbumin modified with pyrraline, a Maillard reaction product, shows enhanced T-cell immunogenicity. *J Biol Chem.* (2014) 289:7919–28. doi: 10.1074/jbc.M113.523621
103. Yadav JS, Yan S, Pilli S, Kumar L, Tyagi RD, Surampalli RY. Cheese whey: a potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnol Adv.* (2015). 33(6 Pt 1):756–74. doi: 10.1016/j.biotechadv.2015.07.002

104. Gülşen N, Coşkun B, Umucalilar HD, Inal F, Boydak, M. Effect of lactose and dried whey supplementation on growth performance and histology of the immune system in broilers. *Arch Tierernahr*. (2002) 56:131–9. doi: 10.1080/00039420214186
105. Alloui MN, Szczurek W. Effects of different dietary levels of whey lactose as a prebiotic disaccharide on the productive performances and selected indices of the caecal micro-environment in broiler chickens. *Ann Anim Sci*. (2017) 17:1107–22. doi: 10.1515/aoas-2017-0021
106. Abdel-Tawwab M, Abbass FE. Dry whey meal as a protein source in practical diets for Nile tilapia, *Oreochromis niloticus* fingerlings. *J. Appl. Aquacult.* (2016) 28:276–84. doi: 10.1080/10454438.2016.1215686
107. Maxwell CV. Future of feed/food industry: re-inventing animal feed. In: Lyons TP and Jacques KA, editors. *Nutritional Biotechnology in the Feed and Food Industries*. Nottingham: Nottingham University Press (2004). p. 11–25.
108. Pieper R, Scharek-Tedin L, Zetzsche A, Röhe I, Kröger S, Vahjen W, et al. Bovine milk-based formula leads to early maturation-like morphological, immunological, and functional changes in the jejunum of neonatal piglets. *J Anim Sci*. (2016) 94:989–99. doi: 10.2527/jas.2015-9942
109. Mulcahy EM, Fargier-Lagrange M, Mulvihill DM, O'Mahony JA. Characterisation of heat-induced protein aggregation in whey protein isolate and the influence of aggregation on the availability of amino groups as measured by the ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods. *Food Chem.* (2017) 229:66–74. doi: 10.1016/j.foodchem.2017.01.155
110. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest.* (2001) 108:949–55. doi: 10.1172/JCI14002
111. Brodkorb A, Croguennec T, Bouhallab S, Kehoe JJ. Heat-Induced Denaturation, Aggregation and Gelation of Whey Proteins. In: McSweeney PLH, O'Mahony JA, editors. *Advanced Dairy Chemistry*; *Volume 1B: Proteins: Applied Aspects*. New York, NY: Springer (2016). p. 155–78.
112. van Rooijen C, Bosch G, van der Poel AF, Wierenga PA, Alexander L, Hendriks WH. Quantitation of Maillard reaction products in commercially available pet foods. *J Agric Food Chem.* (2014) 62:8883–91. doi: 10.1021/jf502064h
113. Friedman M. Food browning and its prevention: an overview. *J Agric Food Chem.* (1996) 44:631–53.
114. Schwarzenbolz U, Hofmann T, Sparmann N, Henle T. Free Maillard reaction products in milk reflect nutritional intake of glycated proteins and can be used to distinguish "organic" and "conventionally" produced milk. *J Agric Food Chem*. (2016) 64:5071–8. doi: 10.1021/acs.jafc.6b01375
115. Tamime AY. Dairy technology-principles of milk properties and processes. *Int J Dairy Technol.* (2007) 60:154. doi: 10.1111/j.1471-0307.2007.00279.x
116. Haskell MJ, Rennie LJ, Bowell VA, Bell MJ, Lawrence AB. Housing system, milk production, and zero-grazing effects on lameness and leg injury in dairy cows. *J Dairy Sci.* (2006) 89:4259–66. doi: 10.3168/jds.S0022-0302(06)72472-9
117. Tuohy KM, Hinton DJ, Davies SJ, Crabbe MJ, Gibson GR, Ames JM. Metabolism of maillard reaction products by the human gut microbiota - implications for health. *Mol Nutr Food Res.* (2006) 50:847–57. doi: 10.1002/mnfr.200500126
118. Bach A, Dinares M, Devant M, Carre X. Associations between lameness (2007) and production, feeding and milking attendance of Holstein cows milked with an automatic milking system. *J Dairy Res.* (2007) 74:40–6. doi: 10.1017/S0022029906002184
119. Greenough PR, Bergsten C, Brizzi A, Mülling CKW. *Bovine Laminitis and Lameness - A Hands on Approach*. Philadelphia, PA: Saunders Elsevier (2007).
120. Wilshire JA, Bell NJ. An economic review of cattle lameness. *Cattle Pract*. (2009) 17:136–41.
121. Shearer JK, Stock ML, Van Amstel SR, Coetzee JF. Assessment and management of pain associated with lameness in cattle. *Vet Clin North Am Food Anim Pract.* (2013) 29:135–56. doi: 10.1016/j.cvfa.2012.11.012
122. Warnick LD, Janssen D, Guard CL, Grohn YT. The effect of lameness on milk production in dairy cows. *J Dairy Sci.* (2001) 84:1988–97. doi: 10.3168/jds.S0022-0302(01)74642-5
123. Green LE, Hedges VJ, Schukken YH, Blowey RW, Packington AJ. The impact of clinical lameness on the milk yield of dairy cows. *J Dairy Sci.* (2002) 85:2250–6. doi: 10.3168/jds.S0022-0302(02)74304-X
124. Amory JR, Barker ZE, Wright JL, Mason SA, Blowey RW, Green L. Associations between sole ulcer, white line disease and digital dermatitis and the milk yield of 1824 dairy cows on 30 dairy cow farms in England and Wales from February 2003-November 2004. *Prev Vet Med.* (2008) 83:381–91. doi: 10.1016/j.prevetmed.2007.09.007
125. Hernandez J, Shearer JK, Webb DW. Effect of lameness on the calving-to-conception interval in dairy cows. *J Am Vet Med Assoc.* (2001) 218:1611–4. doi: 10.2460/javma.2001.218.1611
126. Garbarino EJ, Hernandez JA, Shearer JK, Risco CA, Thatcher WW. Effect of lameness on ovarian activity in postpartum Holstein cows. *J Dairy Sci.* (2004) 87:4123–31. doi: 10.3168/jds.S0022-0302(04)73555-9
127. Huxley JN, Burke J, Roderick S, Main DC, Whay HR. Animal welfare assessment bench marking as a tool for health and welfare planning in organic dairy herds. *Vet Rec.* (2004) 155:237–9. doi: 10.1136/vr.155.8.237
128. Enting H, Kooij D, Dijkhuizen AA, Huirne RBM, Noordhuizen-Stassen EN. Economic losses due to clinical lameness in dairy cattle. *Livest Prod Sci.* (1997) 49:259–67.
129. Booth CJ, Warnick LD, Grohn YT, Maizon DO, Guard CL, Janssen D. Effect of lameness on culling in dairy cows. *J Dairy Sci.* (2004) 87:4115–22. doi: 10.3168/jds.S0022-0302(04)73554-7
130. Vermunt JJ, Leach DH. A scanning electron microscopic study of the vascular system of the bovine hind limb claw. *New Zeal Vet J.* (1992) 40:146–54.
131. Becker J, Steiner A, Kohler S, Koller-Bähler A, Wüthrich M, Reist MI. Lameness and foot lesions in Swiss dairy cows: I. Prevelance. *Schweiz Arch Tierheilkd*. (2014) 156:71–8. doi: 10.1024/0036-7281/a000553
132. Nocek JE. Bovine acidosis: implications on laminitis. *J Dairy Sci.* (1997) 80:1005–28.
133. Vermunt JJ, Greenough PR. Predisposing factors of laminitis in cattle. *Br Vet J.* (1994) 150:151–64.
134. Clarkson MJ, Downham DY, Faull WB, Hughes JW, Manson FJ, Merritt JB, et al. Incidence and prevalence of lameness in dairy cattle. *Vet Rec.* (1996) 138:563–7.
135. Vermunt JJ, Greenough PR. Sole haemorrhages in dairy heifers managed under different underfoot and environmental conditions. *Br Vet J.* (1996) 152:57–73.
136. Ossent P, Lischer C. Bovine lamninitis: the lesions and their pathogenesis. *In Pract.* (1998) 25:415–27.
137. Jeffcott LB, Field JR, McLean JG, O'Dea K. Glucose tolerance and insulin sensitivity in ponies and Standard bred horses. *Equine Vet J.* (1986) 18:97–101.
138. Treiber KH, Hess TM, Kronfeld DS, Boston RC, Geor R, Harris PA. Insulin resistance and compensation in laminitis-predisposed ponies characterized by the Minimal Model. *Pferdeheilkunde* (2005) 21:91–2.
139. Treiber KH, Kronfeld DS, Hess TM, Byrd BM, Splan RK, Staniar WB. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. *J Am Vet Med Assoc.* (2006) 228:1538–45. doi: 10.2460/javma.228.10.1538
140. De Laat MA, McGowan CM, Sillence MN, Pollitt CC. Equine laminitis: induced by 48 h hyperinsulinaemia in Standardbred horses. *Equine Vet J.* (2010) 42:129–35. doi: 10.2746/042516409X475779
141. De Laat MA, Kyaw-Tanner MT, Sillence MN, McGowan CM, Pollitt CC. Advanced glycation endproducts in horses with insulin-induced laminitis. *Vet Immunol Immunopathol.* (2012) 145:395–401. doi: 10.1016/j.vetimm.2011.12.016
142. Gearhart MA, Curtis CR, Erb HN, Smith RD, Sniffen CJ, Chase LE, et al. Relationship of changes in condition score to cow health in Holsteins. *J Dairy Sci.* (1990) 73:3132–40. doi: 10.3168/jds.S0022-0302(90)79002-9
143. Geelen MJ, Wensing T. Studies on hepatic lipidosis and coinciding health and fertility problems of high-producing dairy cows using the "Utrecht fatty liver model of dairy cows": a review. *Vet Q.* (2006) 28:90–104. doi: 10.1080/01652176.2006.9695214
144. Holtenius K, Agenas S, Delavaud C, Chilliard Y. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. *J Dairy Sci.* (2003) 86:883–91. doi: 10.3168/jds.S0022-0302(03)73671-6
145. Leach KA, Logue DN, Kempson SA, Offer JE, Ternent HE, Randall JM. Claw lesions in dairy cattle: development of sole and white

line haemorrhages during the first lactation. *Vet J.* (1997) 154:215–25. doi: 10.1016/S1090-0233(97)80024-X
146. Livesey CT, Harrington T, Johnston AM, May SA, Metcalf JA. The effect of diet and housing on the development of sole haemorrhages, white line haemorrhages and heel erosions in Holstein heifers. *Anim Sci.* (1998) 67:9–16. doi: 10.1017/S1357729800009747
147. Webster AJF. Effects of housing and two forage diets on the development of claw horn lesions in dairy cows at first calving and in first lactation. *Vet J.* (2001) 162:56–65. doi: 10.1053/tvjl.2001.0569
148. Tarlton JF, Holah DE, Evans KM, Jones S, Pearson GR, Webster AJ. Biomechanical and histopathological changes in the support structures of bovine hooves around the time of first calving. *Vet J.* (2002) 163:196–204. doi: 10.1053/tvjl.2001.0651
149. Liu H, Yu S, Zhang H, Xu J. Angiogenesis impairment in diabetes: role of methylglyoxal-induced receptor for advanced glycation end products, autophagy and vascular endothelial growth factor receptor 2. *PLoS ONE* (2012) 7:e46720. doi: 10.1371/journal.pone.0046720
150. Shinohara M, Thornalley PJ, Giardino I, Beisswenger P, Thorpe SR, Onorato J, et al. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J Clin Invest.* (1998) 101:1142–7. doi: 10.1172/JCI119885
151. Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J.* (1990) 269:111–3.
152. Moller PD, Diernaes L, Sehested J, Hyldgaard-Jensen J, Skadhauge E. Absorption and fate of L- and D-lactic acid in ruminants. *Comp Biochem Phys A* (1997) 118:387–8. doi: 10.1016/S0300-9629(96)00325-8
153. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J Dairy Sci.* (2009) 92:1060–70. doi: 10.3168/jds.2008-1389
154. Cooper RA, Anderson A. The formation and catabolism of methylglyoxal during glycolysis in Escherichia coli. *FEBS Lett.* (1970) 110:273–6. doi: 10.1016/0014-5793(70)80546-4
155. Ackerman RS, Cozzarelli NR, Epstein W. Accumulation of toxic concentrations of methylglyoxal by wild-type Escherichia coli K-12. *J Bacteriol.* (1974) 119:357–62.
156. Együd LG, Szent-Györgyi A. Cell division, SH, ketoaldehydes, and cancer. *Proc Natl Acad Sci USA.* (1966) 55:388–93. doi: 10.1073/pnas.55.2.388
157. Boosman R, Nemeth F, Gruys E. Bovine laminitis: clinical aspects, pathology and pathogenesis with reference to acute equine laminitis. *Vet Q.* (1991) 13:163–71. doi: 10.1080/01652176.1991.9694302
158. Visser MB, Pollitt CC. Lamellar leukocyte infiltration and involvement of IL-6 during oligofructose-induced equine laminitis development. *Vet Immunol Immunopathol.* (2011) 144:120–8. doi: 10.1016/j.vetimm.2011.07.016
159. Teodorowicz M, Swiatecka D, Savelkoul H, Wichers H, Kostyra E. Hydrolysates of glycated and heat-treated peanut 7S globulin (Ara h 1) modulate human gut microbial proliferation, survival and adhesion. *J Appl Microbiol.* (2014) 116:424–34. doi: 10.1111/jam.12358
160. Zhu L, He Z, Wu F, Ding R, Jiang Q, Zhang J, et al. Immunization with advanced glycation end products modified low density lipoprotein inhibits atherosclerosis progression in diabetic apoE and LDLR null mice. *Cardiovasc Diabetol.* (2014) 13:151. doi: 10.1186/s12933-014-0151-6
161. Rupa P, Nakamura S, Katayama S, Mine Y. Attenuation of allergic immune response phenotype by mannosylated egg white in orally induced allergy in BALB/c mice. *J Agric Food Chem.* (2014) 62:9479–87. doi: 10.1021/jf503109r
162. Hu H, Jiang H, Zhu L, Wu X, Han C. Accumulation of advanced glycation end products and subclinical inflammation in deep tissues of adult patients with and without diabetes. *Can J Diabetes* (2018) 1–8. doi: 10.1016/j.jcjd.2018.01.003
163. Gautieri A, Passini FS, Silván U, Guizar-Sicairos M, Carimati G, Volpi P, et al. Advanced glycation end-products: Mechanics of aged collagen from molecule to tissue. *Matrix Biol.* (2017) 59:95–108. doi: 10.1016/j.matbio.2016.09.001
164. Neviere R, Yu Y, Wang L, Tessier F, Boulanger E. Implication of advanced glycation end products (Ages) and their receptor (Rage) on myocardial contractile and mitochondrial functions. *Glycoconj J.* (2016) 33:607–17. doi: 10.1007/s10719-016-9679-x
165. Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, et al. The Pattern Recognition Receptor (RAGE) Is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. *J Exp Med.* (2003) 198:1507–15. doi: 10.1084/jem.20030800
166. Price CL, Sharp PS, North ME, Rainbow SJ, Knight SC. Advanced glycation end products modulate the maturation and function of peripheral blood dendritic cells. *Diabetes* (2004) 53:1452–8. doi: 10.2337/diabetes.53.6.1452
167. Schmidt AM, Hori O, Brett J, Yan SD, Wautier JL, Stern D. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler Thromb.* (1994) 14:1521–8.
168. Chavakis T, Bierhaus A, Nawroth PP. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microbes Infect.* (2004) 6:1219–25. doi: 10.1016/j.micinf.2004.08.004
169. Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med.* (2005) 83:876–86. doi: 10.1007/s00109-005-0688-7
170. Zill H, Bek S, Hofmann T, Huber J, Frank O, Lindenmeier M, et al. RAGE-mediated MAPK activation by food-derived AGE and non-AGE products. *Biochem Biophys Res Commun.* (2003) 300:311–5. doi: 10.1016/S0006-291X(02)02856-5
171. Sárkány Z, Ikonen TP, Ferreira-da-Silva F, Saraiva MJ, Svergun D, Damas AM. Solution Structure of the Soluble Receptor for Advanced Glycation End Products (sRAGE). *J Biol Chem.* (2011) 286:37525–34. doi: 10.1074/jbc.M111.223438
172. Sukkar MB, Ullah MA, Gan WJ, Wark PA, Chung KF, Hughes JM, et al. RAGE: a new frontier in chronic airways disease. *Br J Pharmacol.* (2012) 167:1161–76. doi: 10.1111/j.1476-5381.2012.01984.x
173. Santilli F, Vazzana N, Bucciarelli LG, Davì G. Soluble forms of RAGE in human diseases: clinical and therapeutical implications. *Curr Med Chem.* (2009) 16:940–52. doi: 10.2174/092986709787581888
174. Falcone C, Bozzini S, D'Angelo A, Matrone B, Colonna A, Benzi A, et al. Plasma levels of soluble receptor for advanced glycation end products and coronary atherosclerosis: possible correlation with clinical presentation. *Disease Markers* (2013) 35:135–40. doi: 10.1155/2013/129360
175. Seetharaman J, Kanigsberg A, Slaaby R, Leffler H, Barondes SH, Rini JM. X-ray crystal structure of the human Galectin-3 carbohydrate recognition domain at 2.1-Å resolution. *J Biol Chem.* (1998) 273:13047–52.
176. Tsai HF, Wu CS, Chen YL, Liao HJ, Chyuan IT, Hsu PN. Galectin-3 suppresses mucosal inflammation and reduces disease severity in experimental colitis. *J Mol Med.* (2016) 94:545–56. doi: 10.1007/s00109-015-1368-x
177. Díaz-Alvarez L, Ortega E. The many roles of Galectin-3, a multifaceted molecule, in innate immune responses against pathogens. *Med Inflamm.* (2017) 2017:9247574. doi: 10.1155/2017/9247574
178. Araki N, Higashi T, Mori T, Shibayama R, Kawabe Y, Kodama T, et al. macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the maillard reaction. *Eur J Biochem.* (1995) 230:408–15. doi: 10.1111/j.1432-1033.1995.0408h.x
179. Chatzigeorgiou A, Kandaraki E, Piperi C, Livadas S, Papavassiliou AG, Koutsilieris M, et al. Dietary glycotoxins affect scavenger receptor expression and the hormonal profile of female rats. *J Endocrinol.* (2013) 218:331–7. doi: 10.1530/JOE-13-0175
180. Hamasaki S, Kobori T, Yamazaki Y, Kitaura A, Niwa A, Nishinaka T, et al. Effects of scavenger receptors-1 class A stimulation on macrophage morphology and highly modified advanced glycation end product-protein phagocytosis. *Sci Rep.* (2018) 8:5901. doi: 10.1038/s41598-018-24325-y
181. Moghaddam AE, Hillson WR, Noti M, Gartlan KH, Johnson S, Thomas B, et al. Dry roasting enhances peanut-induced allergic sensitization across mucosal and cutaneous routes in mice. *J Allergy Clin Immunol.* (2014) 134:1453–6. doi: 10.1016/j.jaci.2014.07.032

182. Arena S, Salzano AM, Renzone G, D'Ambrosio C, Scaloni A. Non-enzymatic glycation and glycoxidation protein products in foods and diseases: an interconnected, complex scenario fully open to innovative proteomic studies. *Mass Spectrom Rev.* (2014) 33:49–77. doi: 10.1002/mas.21378
183. Byun K, Yoo YC, Son M, Lee J, Jeong G-B, Mok Park Y, et al. Advanced glycation end-products produced systemically and by macrophages: a common contributor to inflammation and degenerative diseases. *Pharmacol Ther.* (2017) 177:44–55. doi: 10.1016/j.pharmthera.2017.02.030
184. Ott C, Jacobs K, Hauke E, Navarrete Santos A, Grune T, Simm A. Role of advanced glycation end products in cellular signaling. *Redox Biol.* (2014) 2:411–29. doi: 10.1016/j.redox.2013.12.016
185. Tamura Y, Adachi H, Osuga J-I, Ohashi K, Yahagi N, Sekiya M, et al. FEEL-1 and FEEL-2 are endocytic receptors for advanced glycation end products. *J Biol Chem.* (2003) 278:12613–7. doi: 10.1074/jbc.M210211200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with one of the authors WH.

frontiers in Nutrition

BRIEF RESEARCH REPORT
published: 18 September 2018
doi: 10.3389/fnut.2018.00081

Abundantly Present miRNAs in Milk-Derived Extracellular Vesicles Are Conserved Between Mammals

Martijn J. C. van Herwijnen[1], Tom A. P. Driedonks[1†], Basten L. Snoek[2†], A. M. Theresa Kroon[1], Marije Kleinjan[1], Ruurd Jorritsma[3], Corné M. J. Pieterse[4], Esther N. M. Nolte-'t Hoen[1] and Marca H. M. Wauben[1]*

[1] Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [2] Theoretical Biology and Bioinformatics, Department of Biology, Science4Life, Utrecht University, Utrecht, Netherlands, [3] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [4] Plant-Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Pinyi Lu,
Biotechnology HPC Software Applications Institute (BHSAI), United States

Reviewed by:
Paola de Candia,
MultiMedica (IRCCS), Italy
Patrick Provost,
Laval University, Canada
Xin Liu,
Stanford University, United States

***Correspondence:**
Martijn J. C. van Herwijnen
M.J.C.vanHerwijnen@uu.nl

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: 31 May 2018
Accepted: 20 August 2018
Published: 18 September 2018

Citation:
Herwijnen MJCv, Driedonks TAP, Snoek BL, Kroon AMT, Kleinjan M, Jorritsma R, Pieterse CMJ, Hoen ENMN and Wauben MHM (2018) Abundantly Present miRNAs in Milk-Derived Extracellular Vesicles Are Conserved Between Mammals. Front. Nutr. 5:81. doi: 10.3389/fnut.2018.00081

Mammalian milk is not only a source of nutrition for the newborn, but also contains various components that regulate further development. For instance, milk is an abundant source of microRNAs (miRNAs), which are evolutionary conserved small non-coding RNAs that are involved in post-transcriptional regulation of target mRNA. MiRNAs present in milk can occur in extracellular vesicles (EVs), which are nanosized membrane vesicles released by many cell types as a means of intercellular communication. The membrane of EVs protects enclosed miRNAs from degradation and harbors molecules that allow specific targeting to recipient cells. Although several studies have investigated the miRNA content in milk EVs from individual species, little is known about the evolutionary conserved nature of EV-associated miRNAs among different species. In this study, we profiled the miRNA content of purified EVs from human and porcine milk. These data were compared to published studies on EVs from human, cow, porcine, and panda milk to assess the overlap in the top 20 most abundant miRNAs. Interestingly, several abundant miRNAs were shared between species (e.g., let-7 family members let-7a, let-7b, let-7f, and miR-148a). Moreover, these miRNAs have been implicated in immune-related functions and regulation of cell growth and signal transduction. The conservation of these miRNA among species, not only in their sequence homology, but also in their incorporation in milk EVs of several species, suggests that they are evolutionarily selected to regulate cell function in the newborn.

Keywords: milk, miRNA, extracellular vesicles, exosomes, immune modulation

INTRODUCTION

Mother's milk provides the newborn with more than only nutrition: it contains various complex macromolecular structures that carry signaling molecules that are delivered to the newborn. One of these signaling moieties are extracellular vesicles (EVs). EVs are cell-derived lipid bilayer-enclosed vesicles containing selectively incorporated proteins, lipids, and nucleic acids (mainly small RNAs) (1–3), which are in turn selectively delivered to recipient cells to modulate their functions (4, 5). Hence, the transfer of maternal milk EVs to the newborn allows for cross-organism communication.

EVs are generally classified into exosomes and microvesicles, which have distinct biogenesis pathways and may differ in size and cargo (1, 2). Currently available methodologies do not allow discrimination between exosomes and microvesicles as the size, surface markers, and buoyant densities of these vesicle subtypes overlap (2). Therefore, we will use the generic term EVs in this study even though previously published studies attribute their findings to certain subtypes, mostly exosomes.

EVs have been identified and characterized in human milk (6–9), as well as in milk from other species, such as cow (10, 11), buffalo (12), pig (13), wallaby (14), horse (15), camel (16), rat (17), and panda (18). Additionally, extensive miRNA profiling of milk EVs has been performed in human (8–20), cow (21), pig (13, 22), and panda (18), and several high abundant miRNAs have been identified in each of these species. These abundantly miRNA species might be involved in the specific targeting of signaling pathways in the newborn. However, the EV isolation procedures used in these studies only enabled enrichment for milk EVs but precluded isolation of pure EVs (23). In the current study, we employed an optimized protocol that we previously developed for recovery and characterization of EVs from fresh milk (7, 9) in order to assess which miRNAs were most abundant in purified EVs from human and porcine milk. Additionally, we compared the identified miRNA profiles from this study with the previously reported high abundant EV-associated miRNAs from human, bovine, porcine, and panda milk. This allowed us to compare the impact of EV isolation procedures on the analysis of miRNAs in milk EVs and to examine the conserved nature of highly abundant miRNAs present in milk EVs from different species. Furthermore, we speculate on the potential impact on the newborn's development via these conserved milk EVs-associated miRNAs.

METHODS

Milk EVs Isolation

Milk EVs were isolated as previously described (7, 9). Fresh human milk from a pool of four breast-feeding mothers 3 until 9 months after delivery was obtained after informed consent of the donors and approval by the local ethics committee. Porcine milk was obtained from two sows between 2 and 3 weeks after delivery after approval by the local ethics committee. Raw milk was centrifuged twice at 3,000 × *g* (Beckman Coulter Allegra X-12R, Fullerton, CA) and the milk supernatant was subjected to differential centrifugation at 5,000 × *g* and 10,000 × *g* in sterilized and new SW40 tubes (Beckman Coulter). The 10,000 × *g* supernatant was loaded onto a sucrose gradient (ranging from 2.0 to 0.4 M sucrose) and ultracentrifuged at 192,000 × g (in a Beckman Coulter Optima L-90K with a SW40 rotor) for 15–18 h (k-factor 144.5). EV-containing fractions (1.12–1.18 g/ml) were harvested, pooled, and centrifuged at 100,000 × g for 65 min. After centrifugation, supernatant was removed and EV pellets were aliquoted and stored at 80°C.

EV-RNA Isolation

Small RNA was isolated using the miRNeasy Micro kit according to the small RNA enrichment protocol provided by the manufacturer (Qiagen, Hilden, Germany). RNA yield and size profile were assessed using Agilent 2100 Bioanalyzer and Pico 6000 RNA chips (Agilent Technologies, Waldbronn, Germany) (see **Table 1** for comparison between studies).

Preparation of Small RNA Sequencing Libraries

Eight nanograms of milk EV-derived small RNA was treated with DNase (Turbo DNA-free kit (Life Technologies, Carlsbad, CA), pelleted using Pellet Paint (Merck, Darmstadt, Germany), and subsequently reconstituted in 6 µl milliQ water (human samples) or 12 µl milliQ water (porcine samples). For the human samples, cDNA libraries were prepared using the NebNext small RNA library prep kit for Illumina (New England Biolabs, Ipswich, MA) with the following adaptations: 3′ adapter ligation was carried out overnight at 16°C; Kapa HiFi Readymix 2× PCR mastermix (Kapa Biosystems, Wilmington, MA) was used for PCR amplification as follows: 2 min at 95°C; 17 cycles of 20 s at 98°C, 30 s at 62°C, 15 s at 70°C, and a final elongation step of 5 min at 70°C. For the porcine samples, cDNA libraries were prepared as indicated by manufacturer with the Illumina TruSeq kit (Illumina Inc., San Diego, CA) with PCR amplification up to 17 cycles. cDNA was purified using AMPure XP beads (Beckman Coulter, Brea, CA) and quantified using Agilent 2100 Bioanalyzer and DNA HiSensitivity chips. Adapter dimers were removed by TBE gel purification (146–400 nt of which 126 nt derive from adapters for human, and a size range of 150–175 bp for porcine samples).

miRNA Profiling

Data quality was checked with FastQC and reads were processed with cutadapt (v1.8) to remove low quality reads. Sequences with a minimal length of 15 bp (human) or maximum length of 25 bp (porcine) after adapter trimming were retained. The end-trimmed sequences were mapped to the miRNA hairpin sequences using bowtie (v1.1.1 for human and v1.2.2 for porcine) with default settings. miRNA sequences were retrieved from miRbase (v21) and for both human and porcine the combined number of reads per miRNA was used to find the top miRNAs. **Supplementary File 1** lists all miRNAs identified in human and porcine milk EV, together with sequencing depth and mapping rates. Sequencing data were deposited in NCBI's Gene Expression Omnibus (GEO) under accession number GSE118409.

Literature Search Previously Published Milk EVs miRNA Analysis

To collect published data on miRNA profiling of milk EVs from different species, studies listed in PubMed up to March 2018 were selected based on the keywords "milk" and "microRNA(s)," "miRNA(s)," "extracellular vesicle(s)," "exosome(s)," or "microvesicles." Additionally, the bibliographies from the retrieved articles were searched to find additional sources. Subsequently, the experimental procedures in these papers were evaluated. Only those papers in which

TABLE 1 | Overview of experimental details for isolation and characterization of milk EVs miRNAs in this study and previously published studies.

	This study	Zhou (8)	Simpson (20)	Liao (19)	Izumi (21)	This study	Gu (13)	Chen (22)	Ma (18)
Species	Human	Human	Human	Human	Bovine	Porcine	Porcine	Porcine	Panda
Donors	Pool of 4 individuals	4 Individuals	54 Individuals	12 Individuals	3 Individuals	2 Individuals	3 Individuals	NR	3 Individuals
Time post-partum	3–9 months	60 days	3 months	1–8 months	NR	3–4 weeks	0–28 days	1–5 days	0–30 days
Storage of whole milk at −80°C	No	Yes	Yes	Yes	Yes	No	No	Yes	No
EV isolation procedure	Differential centrifugation; density gradient	Differential centrifugation; Exoquick	Differential centrifugation; Exoquick	Exoquick	Differential centrifugation; ultra-centrifugation	Differential centrifugation; density gradient	Exoquick	Differential centrifugation; ultra-centrifugation	Differential centrifugation; Exoquick
miRNA extraction kit	miRNeasy	TRIzol-LS	miRNeasy	TRIzol	miRNeasy	miRNeasy	TRIzol-LS	TRIzol	TRIzol-LS
Library preparation kit	New England Biolabs, NebNext	NR	Epicentre Biotechnologies, ScriptMiner	Illumina TruSeq	Not applicable	Illumina TruSeq	NR	NR	NR
miRNA profiling method	HiSeq 2000 (Illumina)	Genome Analyzer II (Illumina)	HiSeq 2000 (Illumina)	HiSeq 2500 (Illumina)	Microarray (670 bovine miRNA)	HiSeq 2000 (Illumina)	Genome Analyzer II (Illumina)	Solexa sequencing	Genome Analyzer II (Illumina)

To be selected, a study should apply a method for the enrichment of EVs from the milk sample in combination with miRNA profiling by microarray or small RNA sequencing. The selected papers focused on human, bovine, porcine or panda milk. Variations between sample numbers and time of sample collection are indicated, as well as differences in milk storage, EVs/miRNA isolation procedures, and miRNA profiling methods. NR, not reported.

extensive miRNA profiling was performed either by deep sequencing or microarray on milk samples enriched (by differential centrifugation and/or precipitation) for milk EVs were selected for further comparison. In total seven studies were selected to compare the most abundantly present miRNAs (see **Table 1** for comparison of selected studies).

In silico Analysis

Tarbase (24) was used to screen for validated targets of identified miRNAs and TargetScan (25) was used to assess conservation of these target sites. Funrich (26) was used for GO analysis on the identified targets.

RESULTS

miRNA Profiling of Purified EVs From Human and Porcine Milk

We show for the first time the miRNA profile of purified milk EVs isolated after differential centrifugation followed by density gradient separation. We identified 309 mature miRNAs in human milk EVs and 218 mature miRNAs in porcine milk EVs (**Supplementary File 1**), These numbers are in range with the miRNAs that were previously identified in milk EV enriched samples of human [$n = 602$ (8), $n = 125$ (20) and $n = 610$ (19); average of 446 ± 278 miRNAs] and porcine [$n = 234$ (13) and $n = 491$ (22); average of 363 ± 182 miRNAs]. Hence, the number of identified miRNAs seems not to differ greatly between purified EVs and EV-enriched samples or the RNA extraction and profiling methods used.

Milk EVs From Different Species Share Abundantly Present miRNAs

In order to identify similarities in the miRNA composition of milk EVs from different species, we compared the top 20 most abundant miRNAs detected in purified milk EVs to the reported top 20 most abundant miRNAs in the selected publications. We observed substantial overlap in the top-ranked miRNAs observed in the different studies, with 19 miRNAs being abundantly detected in at least four out of nine studies (**Table 2**). Interestingly, four miRNAs were identified in high abundance in all four species examined. These included miRNA let-7 family members let-7a, let-7b, and let-7f, as well as miR-148a. These miRNAs were fully conserved at the sequence level in all species (**Supplementary File 1**). In addition to the similarities observed in miRNA content of EV, we also identified miRNAs that were abundantly present in milk EVs from all but one species. For example, miR-20a, miR-26a, and miR-141 were not present in the top 50 most abundant miRNAs in any of the three porcine studies, while these miRNAs are in the porcine genome. Additionally, let-7c was not abundantly present in any of the four human studies (see **Supplementary File 1** for the full **Table 2**). Furthermore, we also observed differences in miRNA profiles reported in studies investigating milk EVs from the same species. Many factors may underlie these differences, including pre-analytic variables, differences in milk EVs and RNA isolation protocols,

TABLE 2 | Overlap in top 20 most abundant miRNAs detected in milk EVs from human, cow, pig, and panda.

Top 20 ranked	This study	Zhou et al. (8)	Simpson et al. (20)	Liao et al. (19)	Izumi et al. (21)	This study	Gu et al. (13)	Chen et al. (22)	Ma et al. (18)	Times identified in top 20
miRNA	**Human**	**Human**	**Human**	**Human**	**Bovine**	**Porcine**	**Porcine**	**Porcine**	**Panda**	
let-7a-5p	5	6	6	17	8	1	10	8	5	9
miR-148a-3p	2	1	1	2	14		1	•	2	7
miR-30a-5p	10	NR	13	11	•	2	2	13	8	7
let-7f-5p	9	3	7	•	17	6	NR	9	13	7
miR-30d-5p	1	NR	3	4	•	5	5	•	17	6
let-7b-5p	8	NR	4	15	4		NR	•	1	5
miR-21-5p	7	NR	10	•		4	12	•	15	5
miR-22-3p	18	NR	2	1		•	NR	17	16	5
miR-320a-3p	17	NR	•	20	15	10	NR	3	•	5
miR-191-5p	•	NR	•	13		3	9	7	•	4
miR-200a-3p	3	9	5	16	•		NR		•	4
miR-181a-5p	•	NR	•	3		20	NR	4	10	4
miR-92a-3p	•	NR	•	8		13	NR	14	3	4
miR-182-5p	•	8		10		19	4			4
miR-141-3p		7	20	5	10		NR		•	4
let-7g-5p	11	NR	•	14	•	9	NR	•	9	4
let-7c		NR			13	7	NR	10	19	4
miR-375-3p	14	NR	17	9	•		13			4
miR-26a-5p	•	NR	18	6	20		NR		20	4
miR-200c-3p	4	NR	•	•	9	8	NR		•	3

*The top 20 most abundant miRNA (this study) were compared to the top 20 miRNA reported in the indicated publications on milk EVs (see **Supplementary File 1** for all data; Table shows the 20 most abundant miRNA ranked according to their presence in the top 20 of the selected studies). If a miRNA was present in the top 20, their ranking is stated and if their position is 21–50, this is depicted with •. NR, not reported (these two studies only reported the top 10 or the top 13 most abundant miRNA). Red shading indicates a high ranking (position 1–10), while blue indicates a low ranking (position 11-20). Those miRNA that were identified within the top 20 of all species (in at least one human or porcine study, and in the bovine and panda study) are depicted in bold.*

time in lactation period, and inter-individual differences in milk composition.

Conserved Milk EV-Associated miRNAs Have Immune-Related Functions

Milk EVs have been indicated to modulate immune cells (6) and promote epithelial cell growth (27). Importantly, milk-derived miRNAs have been shown to survive harsh conditions including RNase digestion (8, 13, 14, 18), low pH (13, 14, 18), and *in vitro* gastro-intestinal digestion (19, 28). This indicates that milk EVs resist gastro-intestinal conditions and therefore presumably remain intact in the newborn gastro-intestinal tract. Even though no strong direct *in vivo* evidence exists, milk EVs can be taken up *in vitro* by intestinal epithelial cells (19, 29, 30), vascular epithelial cells (31), and macrophages (11, 21, 32). This suggests that EV cargo can reach the cells at the mucosa of the newborn.

In order to link the conserved milk EV miRNAs to relevant physiological processes, we identified 24 validated target (**Supplementary File 1**) and used GO analysis to determine that these targets are involved in "cell growth and/or maintenance," "cell communication" and "signal transduction" (**Supplementary File 1**). In fact, let-7a/b/f-5p and miR-148-3p have been shown to regulate signal transduction by downregulating the transcription factor NF-κB resulting in a dampened immune response (33–35). Collectively, these data suggest that milk EVs harbor evolutionary miRNAs with immunomodulatory functions that can regulate the newborns development.

DISCUSSION

In this study, we isolated EVs from human and porcine milk and determined the presence and abundance of miRNAs. The top 20 of most abundantly present miRNAs from the purified human or porcine milk EVs were compared to previously reported miRNAs that were profiled from EV-enriched samples from human, bovine, porcine, and panda milk. Four specific miRNAs appeared to be highly abundant and conserved not only in their sequence, but also in their presence in milk of several species.

At first glance the number of isolated miRNA species does not seem to differ greatly between purified and EV-enriched milk samples and the four conserved miRNAs were identified irrespective of further EV purification and disparate RNA isolation and profiling methods. However, we also observed clear differences along the line reported earlier by van Deun et al. (36). For instance, miR-100-5p was only detected in

high abundance in the purified human milk EVs from this study and was not present in the top 50 of other human studies. In contrast, miR-30b-5p was not abundantly present in purified human milk EV, while it was detected in the three other human studies. For porcine milk EVs there were many discrepancies between the identification of miRNAs between purified EVs compared to the other isolation methods. This could also be caused by a divergence in RNA isolation, library preparations, and sequencing method, which was different for this study compared to Gu et al. and Chen et al. A first step would be to standardize milk collection, milk storage, milk EV isolation, miRNA extraction, miRNA profiling, and data analysis. Finally, adequate reporting of experimental details should be a priority in the EV field to increase rigor and reproducibility (37).

In conclusion, the evolutionary conserved character of a selected set of miRNAs in milk EVs is remarkable. Their presence in EVs suggests a possible conserved role in maternal to newborn cross-organism communication. By targeting a variety of genes, including those that are involved in the regulation of the newborn's epithelial barrier and immune system these maternal milk EV-associated miRNAs might contribute to the guided further development of the newborn.

ETHICS STATEMENT

For human subjects: This study was carried out in accordance with the recommendations of Medical Research Involving Human Subjects Act (WMO), Medical Research Ethics Committee UMC Utrecht. The protocol was approved by the Medical Research Ethics Committee UMC Utrecht. All subjects gave written informed consent in accordance with the Declaration of Helsinki. For animal subjects: This study was carried out in accordance with the recommendations of guideline 2010/63/EU, dierexperimentencommissie (DEC) Utrecht, in which no approval for milk sampling under the described conditions was required.

AUTHOR CONTRIBUTIONS

MH, RJ, CP, EH, and MW conceived and designed the study. MH, TD, BS, AK, and MK performed experiments and analyzed the data. MH, TD, BS, AK, EH, and MW wrote and revised the manuscript. All authors contributed to editing the paper.

FUNDING

This work was supported by a Future Food Utrecht seed fund (to MW, CP, RJ), Netherlands Organization for Scientific Research (NWO Enabling Technologies project nr 435002022) (to MW, EH), the European Research Council under the 441 European Union"s Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement number 442 337581 (to EH), and an ERC Advanced Grant 269072 (to CP).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2018.00081/full#supplementary-material

REFERENCES

1. van der Grein SG, Nolte-'t Hoen EN. "Small talk" in the innate immune system via RNA-containing extracellular vesicles. *Front Immunol.* (2014) 5:542. doi: 10.3389/fimmu.2014.00542
2. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol.* (2018) 19:213–28. doi: 10.1038/nrm.2017.125
3. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC Genomics* (2012) 13:357. doi: 10.1186/1471-2164-13-357
4. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun.* (2011) 2:282. doi: 10.1038/ncomms1285
5. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* (2012) 119:756–66. doi: 10.1182/blood-2011-02-338004
6. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. *J Immunol.* (2007) 179:1969–78. doi: 10.4049/jimmunol.179.3.1969
7. Zonneveld MI, Brisson AR, van Herwijnen MJ, Tan S, van de Lest CH, Redegeld FA, et al. Recovery of extracellular vesicles from human breast milk is influenced by sample collection and vesicle isolation procedures. *J Extracell Vesicles* (2014) 3:24215. doi: 10.3402/jev.v3.24215
8. Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, et al. Immune-related microRNAs are abundant in breast milk exosomes. *Int J Biol Sci.* (2012) 8:118–23. doi: 10.7150/ijbs.8.118
9. van Herwijnen MJ, Zonneveld MI, Goerdayal S, Nolte-'t Hoen EN, Garssen J, Stahl B, et al. Comprehensive proteomic analysis of human milk-derived extracellular vesicles unveils a novel functional proteome distinct from other milk components. *Mol Cell Proteomics* (2016) 15:3412–23. doi: 10.1074/mcp.M116.060426
10. Blans K, Hansen MS, Sorensen LV, Hvam ML, Howard KA, Moller A, et al. Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* (2017) 6:1294340. doi: 10.1080/20013078.2017.1294340
11. Pieters BC, Arntz OJ, Bennink MB, Broeren MG, van Caam AP, Koenders MI, et al. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-beta. *PLoS ONE* (2015) 10:e0121123. doi: 10.1371/journal.pone.0121123
12. Baddela VS, Nayan V, Rani P, Onteru SK, Singh D. Physicochemical biomolecular insights into buffalo milk-derived nanovesicles. *Appl Biochem Biotechnol.* (2016) 178:544–57. doi: 10.1007/s12010-015-1893-7
13. Gu Y, Li M, Wang T, Liang Y, Zhong Z, Wang X, et al. Lactation-related microRNA expression profiles of porcine breast milk exosomes. *PLoS ONE* (2012) 7:e43691. doi: 10.1371/journal.pone.0043691
14. Modepalli V, Kumar A, Hinds LA, Sharp JA, Nicholas KR, Lefevre C. Differential temporal expression of milk miRNA during the lactation cycle of the marsupial tammar wallaby (*Macropus eugenii*). *BMC Genomics* (2014) 15:1012. doi: 10.1186/1471-2164-15-1012
15. Sedykh SE, Purvinish LV, Monogarov AS, Burkova EE, Grigor'eva AE, Bulgakov DV, et al. Purified horse milk exosomes contain an

unpredictable small number of major proteins. *Biochim Open* (2017) 4:61-72. doi: 10.1016/j.biopen.2017.02.004
16. Yassin AM, Abdel Hamid MI, Farid OA, Amer H, Warda M. Dromedary milk exosomes as mammary transcriptome nano-vehicle: their isolation, vesicular and phospholipidomic characterizations. *J Adv Res*. (2016) 7:749–56. doi: 10.1016/j.jare.2015.10.003
17. Hock A, Miyake H, Li B, Lee C, Ermini L, Koike Y, et al. Breast milk-derived exosomes promote intestinal epithelial cell growth. *J Pediatr Surg*. (2017) 52:755–59. doi: 10.1016/j.jpedsurg.2017.01.032
18. Ma J, Wang C, Long K, Zhang H, Zhang J, Jin L, et al. Exosomal microRNAs in giant panda (*Ailuropoda melanoleuca*) breast milk: potential maternal regulators for the development of newborn cubs. *Sci Rep*. (2017) 7:3507. doi: 10.1038/s41598-017-03707-8
19. Liao Y, Du X, Li J, Lonnerdal B. Human milk exosomes and their microRNAs survive digestion *in vitro* and are taken up by human intestinal cells. *Mol Nutr Food Res*. (2017) 61:1700082. doi: 10.1002/mnfr.201700082
20. Simpson MR, Brede G, Johansen J, Johnsen R, Storro O, Saetrom P, et al. Human breast milk miRNA, maternal probiotic supplementation and atopic dermatitis in offspring. *PLoS ONE* (2015) 10:e0143496. doi: 10.1371/journal.pone.0143496
21. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, et al. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. *J Dairy Sci*. (2015) 98:2920–33. doi: 10.3168/jds.2014-9076
22. Chen T, Xi QY, Ye RS, Cheng X, Qi QE, Wang SB, et al. Exploration of microRNAs in porcine milk exosomes. *BMC Genomics* (2014) 15:100. doi: 10.1186/1471-2164-15-100
23. Lotvall J, Hill AF, Hochberg F, Buzas EI, Di Vizio D, Gardiner C, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the international society for extracellular vesicles. *J Extracell Vesicles* (2014) 3:10.3402/jev.v3.26913. doi: 10.3402/jev.v3.26913
24. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res*. (2015) 43:153–9. doi: 10.1093/nar/gku1215
25. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* (2015) 4:e05005. doi: 10.7554/eLife.05005
26. Pathan M, Keerthikumar S, Chisanga D, Alessandro R, Ang CS, Askenase P, et al. A novel community driven software for functional enrichment analysis of extracellular vesicles data. *J Extracell Vesicles* (2017) 6:1321455. doi: 10.1080/20013078.2017.1321455
27. Chen T, Xie MY, Sun JJ, Ye RS, Cheng X, Sun RP, et al. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. *Sci Rep*. (2016) 6:33862. doi: 10.1038/srep33862
28. Benmoussa A, Lee CH, Laffont B, Savard P, Laugier J, Boilard E, et al. Commercial dairy cow milk microRNAs resist digestion under simulated gastrointestinal tract conditions. *J Nutr*. (2016) 146:2206–15. doi: 10.3945/jn.116.237651
29. Wolf T, Baier SR, Zempleni J. The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma caco-2 cells and rat small intestinal IEC-6 cells. *J Nutr*. (2015) 145:2201–6. doi: 10.3945/jn.115.218586
30. Golan-Gerstl R, Elbaum Shiff Y, Moshayoff V, Schecter D, Leshkowitz D, Reif S. Characterization and biological function of milk-derived miRNAs. *Mol Nutr Food Res*. (2017) 61:1700009. doi: 10.1002/mnfr.201700009
31. Kusuma RJ, Manca S, Friemel T, Sukreet S, Nguyen C, Zempleni J. Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis. *Am J Physiol Cell Physiol*. (2016) 310:C800–7. doi: 10.1152/ajpcell.00169.2015
32. Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med*. (2011) 9:9. doi: 10.1186/1479-5876-9-9
33. Patel V, Carrion K, Hollands A, Hinton A, Gallegos T, Dyo J, Sasik R, et al. The stretch responsive microRNA miR-148a-3p is a novel repressor of IKBKB, NF-kappaB signaling, and inflammatory gene expression in human aortic valve cells. *FASEB J*. (2015) 29:1859–68. doi: 10.1096/fj.14-257808
34. Liu X, Zhan Z, Xu L, Ma F, Li D, Guo Z, et al. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha. *J Immunol*. (2010) 185:7244–51. doi: 10.4049/jimmunol.1001573
35. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* (2009) 139:693–706. doi: 10.1016/j.cell.2009.10.014
36. Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, Vandesompele J, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles* (2014) 3:10.3402/jev.v3.24858. doi: 10.3402/jev.v3.24858
37. EV-TRACK Consortium, Van Deun J, Mestdagh P, Agostinis P, Akay O, Anand S, et al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat Methods* (2017) 14:228–32. doi: 10.1038/nmeth.4185

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PC declared a past co-authorship with several of the authors EH & MW to the handling Editor.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 September 2018
doi: 10.3389/fimmu.2018.02178

Genomic Characterization of *Lactobacillus delbrueckii* TUA4408L and Evaluation of the Antiviral Activities of its Extracellular Polysaccharides in Porcine Intestinal Epithelial Cells

Paulraj Kanmani[1,2†], Leonardo Albarracin[1,3,4†], Hisakazu Kobayashi[1,2], Elvira Maria Hebert[3], Lucila Saavedra[3], Ryoya Komatsu[1,2], Brian Gatica[5], Ayako Miyazaki[6], Wakako Ikeda-Ohtsubo[1,2], Yoshihito Suda[7], Hisashi Aso[2,8], Shintaro Egusa[9], Takashi Mishima[10], Alexis Salas-Burgos[5], Hideki Takahashi[11,12], Julio Villena[1,3] and Haruki Kitazawa[1,2*]*

[1] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2] Livestock Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [3] Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [4] Scientific Computing Laboratory, Computer Science Department, Faculty of Exact Sciences and Technology, National University of Tucuman, Tucuman, Argentina, [5] Department of Pharmacology, University of Concepcion, Concepcion, Chile, [6] Viral Diseases and Epidemiology Research Division, National Institute of Animal Health, NARO, Tsukuba, Japan, [7] Department of Food, Agriculture, and Environment, Miyagi University, Sendai, Japan, [8] Cell Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [9] Research & Development Division, Marusan-Ai Co., Ltd., Okazaki, Japan, [10] Graduate School of Regional Innovation Studies, Mie University, Tsu, Japan, [11] Laboratory of Plant Pathology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [12] Plant Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

In lactic acid bacteria, the synthesis of exopolysaccharides (EPS) has been associated with some favorable technological properties as well as health-promoting benefits. Research works have shown the potential of EPS produced by lactobacilli to differentially modulate immune responses. However, most studies were performed in immune cells and few works have concentrated in the immunomodulatory activities of EPS in non-immune cells such as intestinal epithelial cells. In addition, the cellular and molecular mechanisms involved in the immunoregulatory effects of EPS have not been studied in detail. In this work, we have performed a genomic characterization of *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L and evaluated the immunomodulatory and antiviral properties of its acidic (APS) and neutral (NPS) EPS in porcine intestinal epithelial (PIE) cells. Whole genome sequencing allowed the analysis of the general features of *L. delbrueckii* TUA4408L genome as well as the characterization of its EPS genes. A typical EPS gene cluster was found in the TUA4408L genome consisting in five highly conserved genes *epsA-E*, and a variable region, which includes the genes for the polymerase *wzy*, the flippase *wzx*, and seven glycosyltransferases. In addition, we demonstrated here for the first time that *L. delbrueckii* TUA4408L and its EPS are able to improve the resistance of PIE cells against rotavirus infection by reducing viral replication and

OPEN ACCESS

Edited by:
Willem Van Eden, Utrecht University, Netherlands

Reviewed by:
Christine Jansen, Utrecht University, Netherlands
Huub Savelkoul, Wageningen University & Research, Netherlands

***Correspondence:**
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp
Julio Villena
jcvillena@cerela.org.ar

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *11 April 2018*
Accepted: *03 September 2018*
Published: *24 September 2018*

Citation:
Kanmani P, Albarracin L, Kobayashi H, Hebert EM, Saavedra L, Komatsu R, Gatica B, Miyazaki A, Ikeda-Ohtsubo W, Suda Y, Aso H, Egusa S, Mishima T, Salas-Burgos A, Takahashi H, Villena J and Kitazawa H (2018) Genomic Characterization of Lactobacillus delbrueckii TUA4408L and Evaluation of the Antiviral Activities of its Extracellular Polysaccharides in Porcine Intestinal Epithelial Cells. Front. Immunol. 9:2178. doi: 10.3389/fimmu.2018.02178

regulating inflammatory response. Moreover, studies in PIE cells demonstrated that the TUA4408L strain and its EPS differentially modulate the antiviral innate immune response triggered by the activation of Toll-like receptor 3 (TLR3). *L. delbrueckii* TUA4408L and its EPS are capable of increasing the activation of interferon regulatory factor (IRF)-3 and nuclear factor κB (NF-κB) signaling pathways leading to an improved expression of the antiviral factors interferon (IFN)-β, Myxovirus resistance gene A (MxA) and RNaseL.

Keywords: porcine intestinal epithelial cells, immunobiotics, TLR3, Rotavirus, antiviral activity, genome sequence, *Lactobacillus delbrueckii* TUA4408L

INTRODUCTION

Polysaccharides are widespread in nature, and their production has been described in several species of pathogenic and commensal bacteria. Variations in the sugar-building units, glycosidic linkage, anomeric configuration, monosaccharide decoration, and molecular weight result in an enormous diversity of polysaccharides. Because of this heterogeneity, bacterial polysaccharides display diverse chemical, physical and biological properties (1, 2). Bacteria are able to synthesize cytoplasmic storage polysaccharides and exocellular polysaccharides including the tightly linked capsular polysaccharides (CPS) and the loosely associated with the cell surface exopolysaccharides (EPS) (1, 2). It was established that exocellular polysaccharides are involved in the interaction of bacteria with their environment. CPS and EPS have been shown to participate in the formation of bacterial biofilms, adhesion to abiotic and biotic surfaces as well as in the interaction with the immune system (3–5).

In lactic acid bacteria (LAB), the synthesis of EPS has long been associated with some favorable technological properties, especially in food production where they act as viscosifying, stabilizing, emulsifying, or gelling agents. In addition, functional and health-promoting benefits have been attributed to the EPS produced by some LAB strains (2, 5). In this regard, it was reported that surface EPS produced by lactobacilli are able to modulate the immune system. Studies by Yasuda et al. (6) demonstrated that high molecular mass polysaccharides from *Lactobacillus casei* strain Shirota have anti-inflammatory effects while mutant types of this bacterium lacking EPS are potent inducers of interleukin (IL)-12, tumor necrosis factor (TNF)-α, and IL-6 in macrophages. It was also shown that EPS from *L. rhamnosus* RW-9595M exerted immunosuppressive properties in macrophages by inducing high levels of IL-10 (7). In addition to the anti-inflammatory activities, some research works have demonstrated that EPS from lactobacilli are also capable of stimulating the immune system. It was shown that EPS produced by *L. paracasei* DG is able to enhance the expression of TNF-α, IL-6, IL-8, and macrophage inflammatory protein 3α (MIP-3α) in the human monocytic cell line THP-1 (8). EPS derived from *L. rhamnosus* KL37 was also capable of improving TNF-α, IL-6, and IL-12 in macrophages in a mitogen-activated protein kinases (MAPK)-dependent manner (9).

Research works clearly show the potential of EPS produced by lactobacilli to favorably modulate the immune system. However, there are several points that have not yet been studied in depth in relation to the application of immunomodulatory EPS from lactobacilli: (a) most studies were performed in immune cells and few works have concentrated in the immunomodulatory activities of EPS in non-immune cells such as intestinal epithelial cells; (b) the cellular and molecular mechanisms involved in the immunoregulatory effects have not been studied in detail; (c) most research works evaluated the immunological effects of EPS produced by *L. casei* and *L. rhamnosus* while other species of *Lactobacillus* that are also capable of producing important quantities of EPS such as *L. delbrueckii* has not been investigated, and (d) very few studies have demonstrated that EPS from lactobacilli can exert a real beneficial effect through the modulation of the immune system such as increasing the resistance to bacterial or viral infections.

We have conducted research aimed at deepening in the knowledge of the points mentioned above. In previous studies, we evaluated the immunomodulatory properties of several lactobacilli strains according to their capacity to differentially modulate the immune response of porcine intestinal epithelial (PIE) cells triggered by the activation of Toll-like receptor 4 (TLR4). Our previous studies showed that the PIE cell line preserves all the immunological characteristics of primary epithelial cells and therefore, this cell line is a useful *in vitro* tool to evaluate immune responses (10). In addition, this cell line has been used by our group to evaluate immunomodulatory microorganisms directed to pigs or humans taking into consideration the anatomical, physiological, and immunological similarities of the gastrointestinal tract of both hosts. Among the strains evaluated in PIE cells, *L. delbrueckii* subsp. *delbrueckii* TUA4408L, an strain isolated from a Japanese traditional pickle fermented food, was able to reduce the expression of proinflammatory cytokines after TLR4 activation. This immunomodulatory activity was related to its ability to diminish the activation of nuclear factor κB (NF-κB) and MAPK pathways in a TLR2-dependent manner (11). Moreover, the EPS of the TUA4408L strain was fractionated into acidic (APS) and neutral (NPS) fractions and, it was demonstrated that both APS and NPS differentially regulated the expression of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in PIE cells after the stimulation with the TLR4 agonist. Both APS and NPS reduced the activation of NF-κB, ERK-MAPK, and p38-MAPK pathways while NPS also decreased the activation of JNK-MAPK pathway (11).

Recently, substantial progress has been achieved in genomic sequencing of LAB and now several collections of lactobacilli

genomes are available. Until the writing of this manuscript was completed, fifty complete and draft genomes belonging to the *L. delbrueckii* species were published in the NCBI database, including *L. delbrueckii* subsp. *bulgaricus*, subsp. *lactis*, subsp. *delbruekii*, subsp. *jakobsenii*, subsp. *indicus*, and subsp. *sunkii*. Among them, two complete and two draft genomes are available for different strains of *L. delbrueckii* subsp. *delbruekii*. Therefore, few genomic analyzes have been performed with this species of LAB.

In this work, we further advanced in the characterization of the immunomodulatory properties of *L. delbrueckii* TUA4408L and its EPSs. Whole genome sequencing allowed the analysis of the general features of *L. delbrueckii* TUA4408L genome as well as the characterization of its EPS gene cluster. In addition, studies in PIE cells demonstrated that the TUA4408L strain and its EPSs are able to modulate the antiviral innate immune response triggered by TLR3 activation. Moreover, we demonstrated here for the first time that *L. delbrueckii* TUA4408L and its EPSs are capable of improving the resistance of intestinal epithelial cells against rotavirus infection by reducing viral replication and differentially modulating antiviral response.

MATERIALS AND METHODS

Microorganisms

Lactobacillus delbrueckii subsp. *delbrueckii* TUA4408L was isolated from sunki-zuke, a japanease traditional pickle fermented in salt-free conditions. The TUA4408L strain was maintained in deMan-Rogosa-Sharp (MRS) medium at 4°C for further usage. For experiments, TUA4408L strain was propagated in soy milk at 43°C for 16 h, washed with PBS and diluted in Dulbecco's modified Eagle's medium (DMEM, Invitrogen corporation, Carlsbad, CA). The EPSs from *L. delbrueckii* TUA4408L were extracted and fractionated by the method of Kitazawa et al. (12). The acid (APS) and neutral (NPS) EPSs were fractionated by ion-exchange chromatography as described previously (11).

Rotavirus strain UK was used in challenge experiments considering its ability to efficiently replicate in PIE cells, as described previously (13). Rotavirus UK was treated with 10 μg/ml of trypsin (Sigma, Type I) at 37°C for 30 min, and then inoculated onto confluent MA104 cells. After 1 h of absorption, the inoculum was removed and the cells were incubated with serum-free MEM (1 μg/ml of trypsin) at 37°C. After the cytopathic effect reached more than 80% of cells, three rounds of freezing and thawing were performed to harvest the culture supernatant. The virus stock was stored at −80°C for further experiments.

Bioinformatic Analysis

L. delbrueckii subsp. *delbrueckii* TUA4408L genome was sequenced with the PacBio sequencing platform and genome assembly was performed using HGAP 3.0 (14), with default options and the Minimum Seed Read Length was adjusted to 2,000. *L. delbrueckii* TUA4408L genome annotation was carried out using NCBI Prokaryotic Genome Annotation Pipeline (15). Further annotation was obtained by using the SEED-based automated annotation system provided by the RAST server (16). Genome sequencing project was deposited in GenBank under accession number CP021136.

Circular genome maps were generated using the CGView Server (17) based on the information generated by the genome annotation. Bioinformatics analyses of the TUA4408L genome included the use of Antibiotic Resistance Genes Database (ARDB) (18), and Comprehensive Antibiotic Resistance Database (CARD) (19) to evaluate antibiotic resistance genes, PHAge Search Tool (PHAST) and IslandViewer software were used to identify prophages and genomics islands, respectively (20, 21). Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas arrays were identified in bacterial genome using CRISPRfinder (22). The presence and type of bacteriocin genes was evaluated using BAGEL4 software and BLASTx algorithm.

Two phylogenetic trees were constructed based on multilocus sequence typing (MLST), and the 16S rRNA gene, respectively. Seven different housekeeping genes of *L. delbrueckii* were used to construct the MLST phylogenetic tree: *fusA*, *gyrB*, *hsp60*, *ileS*, *pyrG*, *recA*, and *recG* (23). The 16S rRNA gene sequences and the corresponding nucleic acid sequences from the seven genes for MLST were respectively aligned with those of the most closely related species using the multiple alignment program MUltiple Sequence Comparison by Log- Expectation (MUSCLE) (24), a bioinformatic tool included in MEGA7 (25).

For comparative genomic analysis, the complete genomes of *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L, KCTC 13731, DSN 20074, NBRC 3202, and KACC 13439 strains were used. Nucleotide sequences were obtained from GenBank and genomes were reannotated using Prokka (15). Roary 3.11.2, is a high-speed stand-alone pan genome pipeline, which takes annotated assemblies in GFF3 format produced by Prokka (26) and calculates the pan genome was used.

Cell Culture

The PIE cell line was originally established at Tohoku University from intestinal epithelia of unsuckled neonatal swine (10). PIE cells were maintained in DMEM medium supplemented with 10 % fetal calf serum (FCS), penicillin (100 mg/ml), and streptomycin (100 U/ml) at 37°C in a humidified atmosphere of 5% CO_2. PIE cells grow rapidly without any transformation or immortalization. Their proliferative capability diminishes after 50 passages in culture. Therefore, PIE cells in this study had between 20th and 40th passages. The cultures were passaged routinely after reaching confluence of 80–90%.

Analysis of Antiviral Immunity in PIE Cells

PIE cells were seeded at 3.0×10^4 cells in 12 well type I collagen coated plates and incubated at 37°C, 5% CO_2. After 3 days of culturing period, 1 ml of DMEM containing either *L. delbrueckii* (5×10^7 cells/ml), APS or NPS (100 ug/ml) was added. Cells were incubated at 37°C, 5% CO_2 for 48 h. PIE cells were washed three times with fresh medium to eliminate lactobacilli or EPSs and subsequently stimulated with 10 ug/ml of poly(I:C) (Sigma Aldrich, USA) for 12 h. The expressions of interferon (IFN)-β, IL-6, IL-8, MCP-1, TNF-α, RNaseL, Myxovirus resistance gene A

(MxA), retinoic acid-inducible gene I receptor (RIG-I), and TLR3 were quantified by qPCR as described below.

Analysis of TLR Negative Regulators in PIE Cells

In order to analyze the expression of negative regulators of TLR signaling, PIE cells were seeded at 3.0×10^4 cells/well in 12 well type I collagen coated plates and cultured at 37°C, 5% CO_2 for 3 days. Then, 1 ml of DMEM containing either *L. delbrueckii* (5×10^7 cells/ml), APS or NPS (100 ug/ml) was added. After 48 h of stimulation, PIE cell were stimulated with poly(I:C) as described above, for 3, 6, and 12 h. The expression of TLR negative regulators single immunoglobulin IL-1-related receptor (SIGIRR), Toll interacting protein (Tollip), zinc finger protein A20 (A20), B-cell lymphoma 3-encoded protein (Bcl-3), mitogen-activated protein kinase-1 (MKP-1), and interleukin-1 receptor-associated kinase M (IRAK-M) were quantified by qPCR as described below.

RNA Extraction and qPCR

The total RNA was isolated by using TRIzol reagent (Invitrogen). The purity and quantity of RNA was analyzed by Nano drop spectrophotometer ND-1000 UV-Vis (NanoDrop Technologies, USA). The quantified RNA (500 ng) was used to synthesize cDNA by Thermal cycler (BIO-RAD, USA). The reaction mixtures (10 μl) were prepared using Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer instructions. The qPCR was performed in a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) with platinum SYBR green (qPCR supermix uracil-DNA glycosylase with 6-carboxyl-X-rhodamine, Invitrogen). The total volume of reaction mixture was 10 μl, which contained 2.5 μl of cDNA, and 7.5 μl of master mix that included RT enzyme, SYBR green, forward and reverse primers (1 pmol/μl). The reaction cycles were performed first at 50°C for 5 min; followed by 95°C for 5 min; then 40 cycles at 95°C for 15 s, at 60°C for 30 s and at 72°C for 30 s. According to the minimum information for publication of qPCR experiments guidelines, β-actin was used as a housekeeping gene because of its high stability across porcine various tissues (27, 28). Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples. Primers were described previously (11, 13).

Western Blotting Analysis

PIE cells were seeded (1.8×10^5 cells/dish) in 60 mm dishes and incubated at 37°C, 5% CO_2 for 3 days. The confluent PIE cells were stimulated with lactobacilli or EPSs as described above. After stimulation, PIE cells were washed three times with fresh DMEM medium, treated with 10 ug/ml of poly(I:C) and studied in different time intervals (0, 30, 60, 120, 180, and 240 min). Then, PIE cells were washed three times with PBS and the harvested cells were lysed by adding 200 ul of CellLytic M cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA), containing protease inhibitors (Complete mini, PhosSTOP, Roche, Mannheim, Germany). Afterwards, the lysed cells were transferred to fresh Eppendorf tubes (1.5 ml), kept on ice for 5–10 min and sonicated three times at 50% for 3 sec and, centrifuged at 150 × 100 rpm for 5 min. Then, the concentration of protein in the resulting supernatants was estimated by using bicinchoninic acid (BCA) assay kit (Thermo Scientific, Pierce, Rockford, IL).

For western blot analysis, the protein samples (8 ug/sample) were loaded on 10% SDS-polyacrylamide gels and the electrophoretically separated proteins were transferred to a nitrocellulose membrane (Trans-Blot TurboTM, BIO-RAD). The membranes were cut and incubated with blocking buffer for 1–2 h before incubating with primary and secondary antibodies. TNF receptor-associated factor (TRAF3), Interferon regulatory factor 3 (IRF-3), p38 and the nuclear factor kappa B inhibitor alpha (IkBα) were evaluated using TRAF3 antibody (Cat. #4729), Phospho-IRF3 (Ser396) (4D4G, Cat. #4947) rabbit antibody, Phospho-p38 mitogen-activated protein kinase (Thr180/Tyr182) antibody (p-p38, Cat. #9211); and I kappaB-alpha antibody (IkBa, Cat. #9242) from Cell Signaling Technology (Beverly, MA, USA) at 1,000 times dilution of their original concentration, overnight at room temperature. Then, membranes were washed with TBS-T buffer and incubated with anti-rabbit IgG, AP-linked antibody (Cat. #7054) for 1–2 h at room temperature. After washing with TBS-T buffer, the membranes were spread with 200 ul of ECF substrate (GE Healthcare Japan Co., Tokyo, Japan) to detect optical protein bands and photographed by blot analyzer (Quantity One W409). The exhibited proteins bands were estimated from the peak area of densitogram by using Image J software (National Institute of Health, Bethesda, MD, USA). To detect each total protein, the same membranes were incubated in stripping solution for 10 min (Western Blot Re-Probe Kit, #JZ-008, Jacksun Easy Biotech, Inc., New York, USA), blocked (1–2 h), and subsequently incubated with IRF3 (D83B9, Cat. #4302) antibody, p38 MAPK antibody (p38, Cat. #9212), and β-actin (13E5, Cat. #4970) rabbit antibody, from Cell Signaling Technology.

Role of TLRs in the Immunomodulatory Activities of *L. delbrueckii* TUA4408L

The role of TLR2 and TLR4 in the immunomodulatory activities of *L. delbrueckii* TUA4408L and its EPSs were analyzed by using blocking experiments. Unlabeled anti-porcine TLR2 and TLR4-rabbit IgG antibodies (Biolegend, San Diego, CA) were used to block TLRs expression in PIE cells as described previously (11). Briefly, PIE cells were cultured (3.0×10^4 cells/ml) in 12 well type I collagen coated plates at 37°C, 5% CO_2. After 3 days, the confluent cells were incubated with unlabeled anti-porcine TLR2 or TLR4 IgG antibodies (200 ng/ml) for 12 h. After treatment with blocking antibodies, PIE cells were treated with *L. delbrueckii* TUA4408L or its EPSs as described above. Finally, PIE cells were washed three times with fresh medium and subsequently stimulated with poly(I:C) (10 ug/ml) for 12 h at 37°C, 5% CO_2. The expression of IFN-β, IL-6, IL-8, MCP-1, and TNF-α were examined by qPCR as described before.

Rotavirus Infection

PIE cells were plated (5.0×10^3) in 96 well type I collagen coated microplate (SUMILON, Tokyo, Japan) and incubated at 37°C, 5 % CO_2. One ml of DMEM containing either

L. delbrueckii (5×10^7 cells/ml), APS or NPS (100 ug/ml) was added. After 48 h incubation, the cells were treated with trypsin-activated rotavirus UK, and incubated at 37°C, 5% CO_2. At hour 16 post-inoculation, PIE cells were fixed after removal of the inoculums and the infected virus titer were analyzed by immunofluorescence staining as described previously (13). Infection rate of UK rotavirus in PIE cells is 4.6 log10 FFU/0.1 ml (13). In addition, the expression of IFN-β, MxA, RIG-I, TLR3, IL-6, IL-8, and MCP-1 were quantified by qPCR.

Statistical Analysis

Statistical analyses were performed using the GLM and REG procedures available in the SAS computer program (SAS, 1994). Comparisons between mean values were carried out using one-way analysis of variance and Fisher's least-significant-difference (LSD) test. For these analyses, P values of < 0.05 were considered significant.

RESULTS

General Features of *L. delbrueckii* TUA4408L Genome

The genome of *L. delbrueckii* TUA4408L was assembled into a single circular chromosome composed of 2,012,440 bp with 49.9% G+C content (**Figure 1A**; **Table 1**). Genome sequencing also revealed that TUA4408L strain is a plasmid-free bacterium. A total of 2,029 genes (1,755 protein-coding genes), 27 rRNA (including 5S, 16S, and 23S genes), 95 tRNA, 3 ncRNA, and 222 pseudo genes were found in the circular chromosome of TUA4408L strain (**Table 1**).

By using the PHage Search Tool (PHAST), *L. delbrueckii* TUA4408L genome was predicted to have two incomplete prophage regions, located at positions 136643–147495 (10.8 Kbp) and 474436–498733 (24.2 Kbp). In addition, genomics islands were found in different blocks of TUA4408L genome (**Figure 1B**). The *in silico* analysis of the distribution, the presence and type of bacteriocin genes was evaluated in *L. delbrueckii* TUA4408L using BAGEL4 software and BLASTx algorithm. Analysis of genome sequence of the TUA4408L strain revealed the presence of two ORF encoding enterolysin A, a cell wall-degrading bacteriocin widely distributed among *Enterococcus strains* (29, 30). This gene is also detected among *L. delbrueckii* genomes with high percentage of identity (data not shown). Antibiotic resistance genes were not found in the TUA4408L strain by using ARDB and CARD. The genome of *L. delbrueckii* TUA4408L contains CRISPR loci and Cas proteins that provide sequence-specific protection against foreign DNA (31). In the TUA4408L genome we found a type IC CRISPR–Cas system constituted by *cas3*, *cas5*, *cas8c*, *cas7*, *cas4*, *cas1*, *cas2*, and a CRISPR array with 33 short direct repeats and 18 spacers.

RAST analysis indicated that *L. delbrueckii* TUA4408L has a remarkable potential to metabolize sugars since its genome

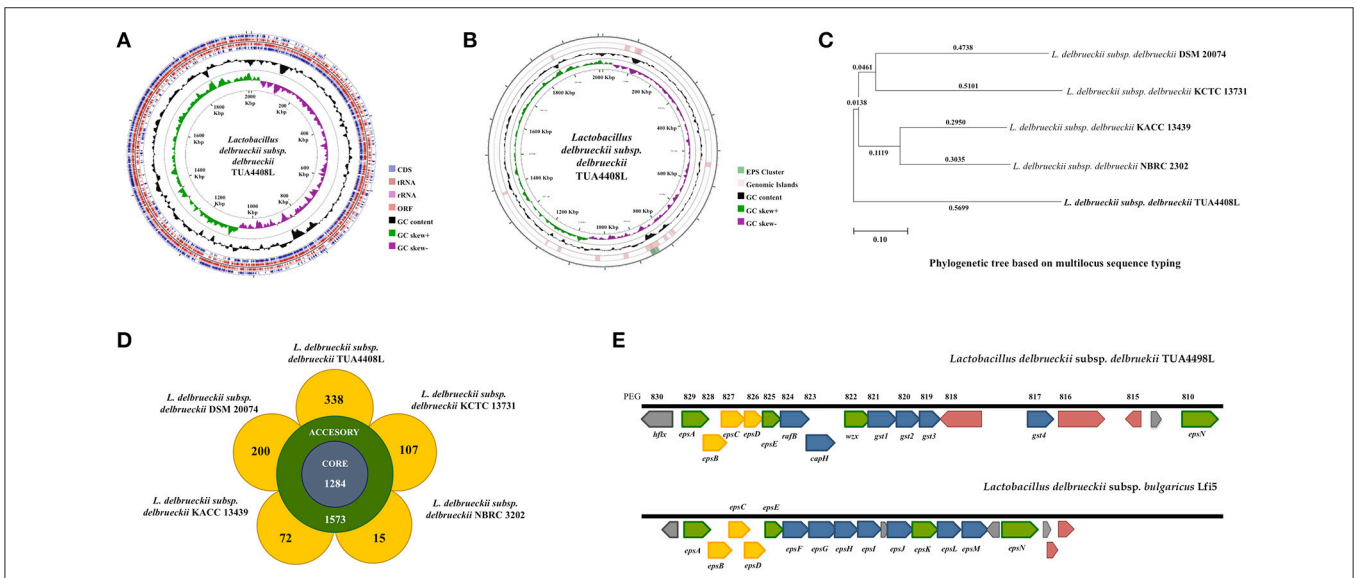


FIGURE 1 | Genomic analysis of the exopolysaccharide (EPS) producing strain *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L. **(A)** Genome was sequenced with the PacBio sequencing platform, assembled with HGAP 3.0, and annotated with NCBI Prokaryotic Genome Annotation Pipeline. Circular genome map of the TUA4408 strain was generated with the CGView Server. Genome sequencing project was deposited in GenBank under accession number CP021136. **(B)** EPS gene cluster and genomic islands are highlighted in the circular genome map of the TUA4408 strain was generated with the CGView Server. **(C)** Phylogenetic tree of *Lactobacillus delbrueckii* subsp. *delbrueckii* constructed based on multilocus sequence typing (MLST) genes. Seven different housekeeping genes of *L. delbrueckii* were used to construct the MLST phylogenetic tree including *fusA*, *gyrB*, *hsp60*, *ileS*, *pyrG*, *recA*, and *recG*. **(D)** Pangenome analysis of *L. delbrueckii* subsp. *delbrueckii*. The complete genomes of TUA4408L, KCTC 13731, DSN 20074, NBRC 3202, and KACC 13439 strains were used. Nucleotide sequences were obtained from GenBank and genomes were reannotated using Prokka. Roary 3.11.2 pangenome pipeline was used for analysis. **(E)** Analysis of the EPS gene cluster of TUA4408L strain.Comparison of EPS gene cluster among *L. delbrueckii* subsp. *delbrueckii* TUA4408L and *L. delbrueckii* subsp. *bulgaricus* Lfi5. The predicted functions of genes are indicated by colors as follow: assembly (green), modulation (yellow), and glycosyltransferase (blue).

TABLE 1 | Comparison of the general genome features of sequenced *Lactobacillus delbrueckii* subsp. *delbrueckii*.

Attribute	*Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L	*Lactobacillus delbrueckii* subsp. *delbrueckii* KCTC 13731	*Lactobacillus delbrueckii* subsp. *delbrueckii* DSM 20074	*Lactobacillus delbrueckii* subsp. *delbrueckii* ACC 13439	*Lactobacillus delbrueckii* subsp. *delbrueckii* NBRC 3202
GenBank accession	CP021136	CP018216	CP018615	LHPL00000000	BEWJ00000000
Genome size (bp)	2,012,440	1,910,506	1,953,716	1,766,190	1,787,093
DNA GC content (%)	49.9	50.0	49.6	50.1	50.3
Level	Complete genome	Complete genome	Complete genome	Conting	Conting
Plasmid	Not reported	Not reported	Not reported	Not reported	Not reported
rRNA genes (5S, 16S, 23S)	27 (9, 9, 9)	24 (8, 8, 8)	27 (9, 9, 9)	4 (0, 3, 1)	3 (1, 1, 1)
tRNA genes	95	84	95	65	50
Other ncRNA genes	3	3	3	Not reported	3
Proteins	1,755	1,617	1,587	1,521	1,599
Predicted genes	2,029	1,921	1,984	1,804	1,816
Pseudo genes	149	193	272	214	161
CRISPR Arrays	1	1	Not reported	Not reported	1

contains several genes related to chitin, N-acetylglucosamine, sucrose, maltose, maltodextrin, mannitol, and D-ribose utilization. In addition, genes for trehalose, lactose and galactose uptake and utilization, as well as trehalose biosynthesis were found in the genome.

We also compared the genome of the TUA4408L strain with the available genomes of other *L. delbrueckii* subsp. *delbrueckii* strains including DSM 20074, NBRC 2302, KCTC 13731, and KACC 13439 (**Figures 1C,D**). Phylogenetic tree constructed on multilocus sequence typing (MLST) showed that *L. delbrueckii* TUA4408L clustered separated from the other sequenced strains (**Figure 1C**). In order to obtain better insight into the specific features of *L. delbrueckii* TUA4408L, we performed a pangenome analysis with the TUA4408L genome and the four genomes mentioned before. We found that 1,284 genes belong to the coregenome sheared by the five strains evaluated while 1,573 genes belong to the accessory pangenome (**Figure 1D**). A relative low number of unique genes were found for NBR 2303 (15 genes), and KACC 13439 (72 genes) strains, probably because they are not completely sequenced. *L. delbrueckii* KCCT 13731 and DSM 20074 strains had 107 and 200 unique genes, respectively (**Figure 1D**). Interestingly, *L. delbrueckii* TUA4408L had 338 unique genes including 136 proteins with known functions and 202 hypothetical proteins.

Among the unique genes present in the *L. delbrueckii* TUA4408L genome, we found glycosyltransferases involved in EPS biosynthesis (discussed below). In addition, several genes involved sugar metabolism were found in the TUA4408L strain but not in the other *L. delbrueckii* subsp. *delbrueckii* strains analyzed (**Supplemental Table 1**). The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), a major carbohydrate active transport system, catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane. Several PTS genes were found in the group of unique genes from the TUA4408L strain including those involved in the transport of glucose (*crr3*), lactose (*lacF*), mannose (*manX, manZ*), oligoglucomannans such as cellobiose (*celA*, *celD, bglK*) and mannobiose (*gmuC*), lichenan (*licA*, *licC*), maltose (*malP*), the chitin disaccharide N,N'-diacetylchitobiose (*chbB*), and L-sorbose (*sorA, sorB*) (**Supplemental Table 1**). Transporters for trehalose (*sugC*), ribose (*rbsB*), and L-arabinose (*araQ*), genes involved in the utilization of different sugars including *bglA* that plays a major role in the utilization of arbutin or salicin, and *levS* that participates in starch and sucrose metabolism, as well as genes involved in the biosynthesis of glycogen (*glgA*, *glgB*, *glgC*, and *glgD*), galactofuranose glycoconjugates (*glf*), and sucrose (*inuJ*) were also found in the group of unique genes of TUA4408L.

Characterization of EPS Gene Cluster of *L. delbrueckii* TUA4408L

As expected, all the genes necessary for EPS production were found in the genome of *L. delbrueckii* TUA4408L (**Figure 1E**). In LAB, it has been described that a typical EPS gene cluster consists of five highly conserved genes *epsA*, *epsB*, *epsC*, *epsD,* and *epsE*, and a variable region, which includes the genes for the polymerase *wzy*, the flippase *wzx*, and a variable number of glycosyltransferases and other polymer-modifying enzymes [reviewed in (2)]. This type of EPS gene cluster structure has been described for *L. delbrueckii* subsp. *bulgaricus* Lfi5 (32). An 18Kpb DNA region containing 14 genes, designated *epsA* to *epsN*, was isolated by genomic DNA library screening and inverted PCR for the Lfi5 strain (**Figure 1E**). Similarly, *epsA* to *epsE* genes are present in the genome of *L. delbrueckii* TUA4408L (**Figure 1E**), which show high percentages of identity with those described in the Lfi5 strain (**Supplemental Table 2**). Therefore, putative functions for these EPS gene products in *L. delbrueckii* TUA4408L could be assigned by sequence similarities: *epsA* would be a positive regulator of the *eps* operon, the *epsBCD* would be a phosphoregulatory system, while *epsE* would be the phosphoglucosyltransferase initiating the biosynthesis

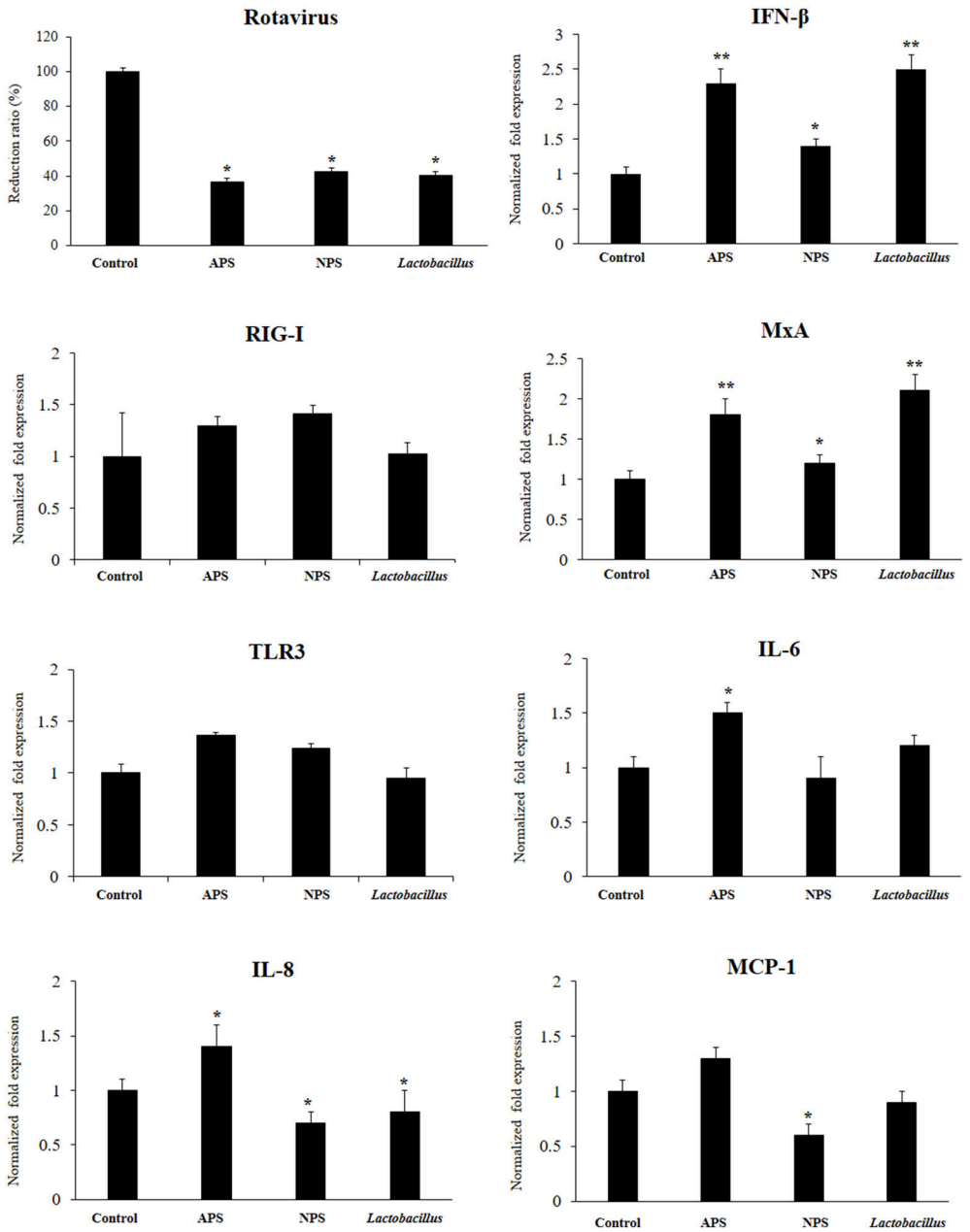


FIGURE 2 | Antiviral activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were treated with lactobacilli, APS, NPS, or medium for 48 h and then infected with rotavirus. The virus titers were analyzed by immunofluorescence staining 16 h after challenge. The expression of interferon β (IFN-β), MxA, Toll-like receptor (TLR)-3, retinoic acid-inducible gene I receptor (RIG-I), interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1) was evaluated 16 h after rotavirus challenge. The results are expressed as mean ± SD and represent data from three independent experiments ($n = 3$ in each experiment). Asterisks indicate significant differences when compared to rotavirus-challenged control PIE cells (*$P < 0.05$ and **$P < 0.01$) .

of EPS. The polymerase *wzy* (*epsK*) and the flippase *wzx* (*epsN*) are also found in the EPS cluster of *L. delbrueckii* TUA4408L (**Figure 1E**). In addition, seven genes coding for glycosyltransferases are present in the TUA4408L genome including members of the glycosyltransferases superfamilies GTB, glyco-transf-2-3, Caps-Shynth and Core-2/I-branching enzyme (**Supplemental Table 3**). Most of the genes in the EPS cluster of *L. delbrueckii* TUA4408L are tightly coupled (**Figure 1E**); significant intergenic gaps only exist between *gst3* and *gst4* (3,989 bp) and between *gst4* and *epsN* (5,754 bp). However, predictable transcription terminators or consensus promoter sequences were not found in these gaps.

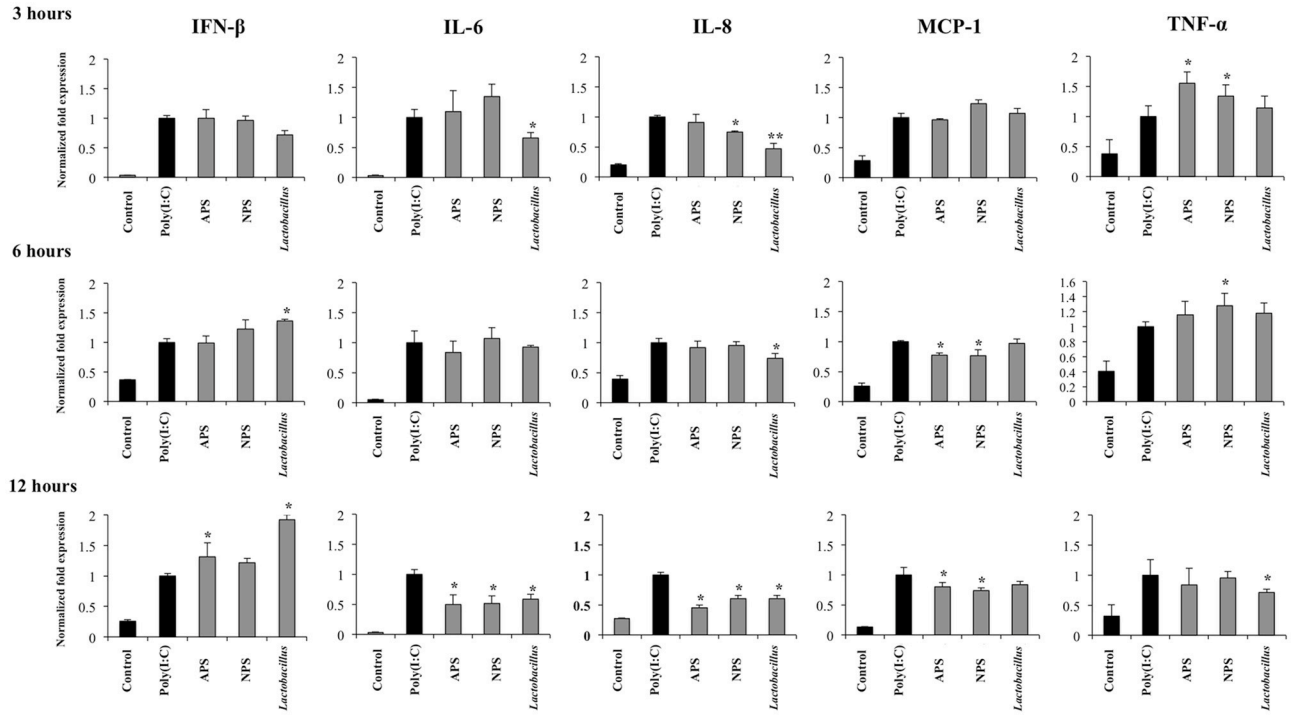


FIGURE 3 | Immunomodulatory activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were treated with lactobacilli, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of interferon β (IFN-β), interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF-α) was evaluated at 3, 6, and 12 h after poly(I:C) challenge. The results are expressed as mean ± SD and represent data from three independent experiments (n=3 in each experiment). Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$, **$P < 0.01$).

L. delbrueckii TUA4408L and its EPSs Improve Resistance of PIE Cells to Rotavirus Infection and Differentially Modulate Antiviral Immune Response

We aimed to evaluate the capacity of EPSs from *L. delbrueckii* TUA4408L to improve the resistance against rotavirus infection in PIE cells that are susceptible to the infection with this pathogen (27). PIE cells were stimulated with *L. delbrueckii*, APS or NPS for 48 h and then challenged with rotavirus strain UK. As we described previously, rotavirus UK efficiently replicated in PIE cells (**Figure 2**). Of interest, the three treatments *L. delbrueckii*, APS and NPS were able to significantly reduce rotavirus replication in PIE cells (**Figure 2**).

The innate antiviral immune response triggered by rotavirus infection was also evaluated. The challenge of PIE cells with rotavirus significantly increased the expression of IFN-β, MxA, IL-6, IL-8, and MCP-1 when compared to untreated control cells, in line with our previous findings (27). Treatment of PIE cells with *L. delbrueckii* TUA4408L, NPS or APS significantly increased the expression of IFN-β and MxA when compared to controls (**Figure 2**). However, APS and *L. delbrueckii* were more efficient to improve the expression of both antiviral factors than NPS. No significant differences were observed between control and treated PIE cells when the expression levels of RIG-I or TLR3 were evaluated after rotavirus infection (**Figure 2**). When the inflammatory cytokines and chemokines were analyzed, it was observed that each treatment induced a characteristic change in IL-6, IL-8, and MCP-1 expression in PIE cells after rotavirus infection (**Figure 2**). APS significantly enhanced the expression of IL-6 and IL-8, while NPS reduced the expression of IL-8 and MCP-1 in infected PIE cells when compared to untreated controls. In addition, *L. delbrueckii* TUA4408L diminished IL-8 expression in in PIE cells afterrotavirus infection (**Figure 2**).

L. delbrueckii TUA4408L and its EPSs Differentially Modulate the TLR3-Triggered Cytokine Response in PIE Cells

Considering the ability of EPSs from *L. delbrueckii* TUA4408L to improve innate immune response against rotavirus, we next evaluated their influence in the innate antiviral immune response triggered by TLR3, which is known to be the main pattern recognition receptor (PRR) activated by this pathogen (33). For this purpose, PIE cells were stimulated with *L. delbrueckii*, APS, or NPS for 48 h and then challenged with the TLR3 ligand poly(I:C). The expression of IFN-β, IL-6, IL-8, MCP-1, and TNF-α were determined at different time points after poly(I:C) stimulation as shown in **Figure 3**. Poly(I:C) significantly increased the expression of all the inflammatory

factors evaluated when compared to untreated control cells, in line with our previous findings (34, 35). Treatment of PIE cells with *L. delbrueckii* TUA4408L significantly increased the expression of IFN-β at hours 6 and 12 post-poly(I:C) stimulation. APS also increased the expression of IFN-β on hour 12 while NPS did not induce changes in the expression of this type I IFN (**Figure 3**).

L. delbrueckii TUA4408L significantly reduced the expression of IL-6 and IL-8 early at hour 3 after poly(I:C) stimulation, and TNF-α at hour 12 (**Figure 3**). No effect on MCP-1 expression was observed in lactobacilli-treated cells. APS and NPS treatments significantly reduced the expression of IL-6, IL-8, and MCP-1 at hour 12 after poly(I:C) stimulation, while the both treatments enhanced the levels of TNF-α mRNA at hour 3 (**Figure 3**).

Taking into consideration the ability of *L. delbrueckii* and its EPSs to differentially modulate the expression of IFN-β in PIE cells after TLR3 activation, we next evaluated the expression of factors involved in antiviral defenses that are induced by this type I IFN including RNaseL, MxA, RIG-I, and TLR3 (**Figure 4**). The three treatments improved the expression of TLR3 (**Figure 4**) and MxA (data not shown) in poly(I:C)-treated PIE cells while no changes were observed in RIG-I (**Figure 4**) and RNaseL (data not shown) mRNA levels at hour 12 after poly(I:C) stimulation. When MxA and RNaseL were evaluated at hour 24 after poly(I:C) challenge, we found that treatments improved the expression of MxA while *L. delbrueckii* TUA4408L and APS increased RNaseL (**Figure 4**).

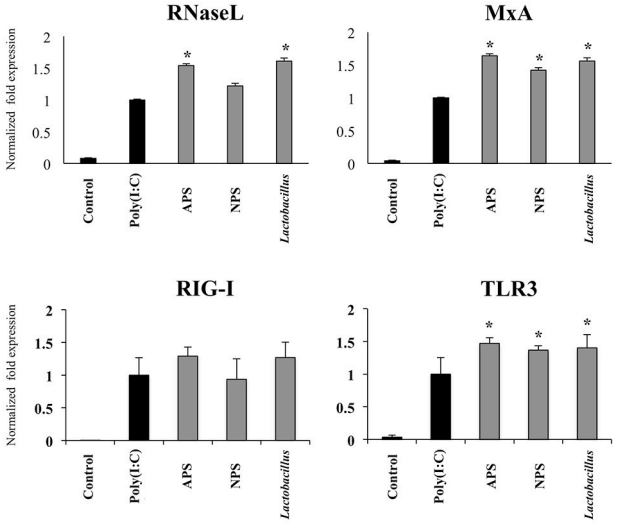


FIGURE 4 | Immunomodulatory activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were treated with lactobacilli, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of the pattern recognition receptors TLR3 and RIG-I was evaluated 12 h after poly(I:C) challenge and the antiviral factors RNAseL and MxA 24 h after stimulation. The results are expressed as mean ± SD and represent data from three independent experiments ($n = 3$ in each experiment). Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$).

L. delbrueckii TUA4408L and its EPSs Differentially Modulate TLR3 Signaling Pathway in PIE Cells

The TLR3 signaling pathway in PIE cells was studied by evaluating TRAF3, p-IRF3/IRF3, p-p38/p38, and IκBα by western blot analysis for 120 min after poly(I:C) stimulation (**Figure 5**).

Challenge of PIE cells with poly(I:C) significantly reduced the levels of the IκBα between 10 and 60 min, indicating the activation of the NF-κB pathway (**Figure 5**). IκBα levels were also reduced between minutes 10 and 30 in PIE cells prestimulated with *L. delbrueckii* TUA4408L or NPS while APS-treated cells showed no reduction in IκBα following poly(I:C) challenge. In addition, the three treatments significantly increased IκBα in PIE cells at minute 120 when compared with control cells (**Figure 5**). No significant changes were observed in the p-p38/p38 ratio in PIE cells after poly(I:C) challenge with the exception of NPS-treated cells that showed increased p-p38/p38 ratios at minutes 30 and 120 (**Figure 5**).

Poly(I:C) stimulation reduced TRAF3 at minute 30 (**Figure 5**). Similarly, diminished levels of TRAF3 were observed in APS–treated PIE at minute 30. NPS significantly increased TRAF3 at minutes 10, 30, and 120 while no changes were observed in TRAF3 levels in *L. delbrueckii*–treated PIE (**Figure 5**). In addition, enhanced p-IRF3/IRF3 ratio was observed in PIE cells 120 min after poly(I:C) challenge. However, in *L. delbrueckii*– and NPS–treated PIE cells, the p-IRF3/IRF3 ratio was significantly increased earlier at minute 10 while this increment was observed for APS treatment at minute 30 (**Figure 5**).

L. delbrueckii TUA4408L and its EPSs Modulate Negative Regulators of TLR Signaling in PIE Cells

Several intracellular and transmembrane proteins are known to modulate the activation of TLR3 acting as negative regulators. Considering the effects of *L. delbrueckii* TUA4408L and its EPSs on TLR3 signaling described above, we next aimed to evaluate whether these treatments were able to induce changes in the expression of several negative regulators of TLR3 in PIE cells after poly(I:C) challenge. The expression of the negative regulators: single immunoglobulin IL-1 related receptor (SIGIRR), toll interacting protein (Tollip), A20, B-cell lymphoma 3- encoded protein (Bcl-3), interleukin-1 receptor associated kinase M (IRAKM-1), and mitogen activated protein kinase phosphate 1 (MKP-1) were evaluated as shown in **Figure 6**.

Activation of TLR3 in PIE cells induced an early and strong up-regulation of A20 and Bcl-3 that was sustained during the studied period. In addition, increased expression of SIGIRR, Tollip, MKP-1, and IRAK-M was observed at hour 3 after poly(I:C) challenge. However, the expression of these four negative regulators returned to basal levels in hours 6 and 12 (**Figure 6**).

The three treatments *L. delbrueckii*, APS and NPS significantly reduced the expression of SIGIRR and A20 in poly(I:C)-challenged PIE cells early in hour 3 (**Figure 6**). Moreover,

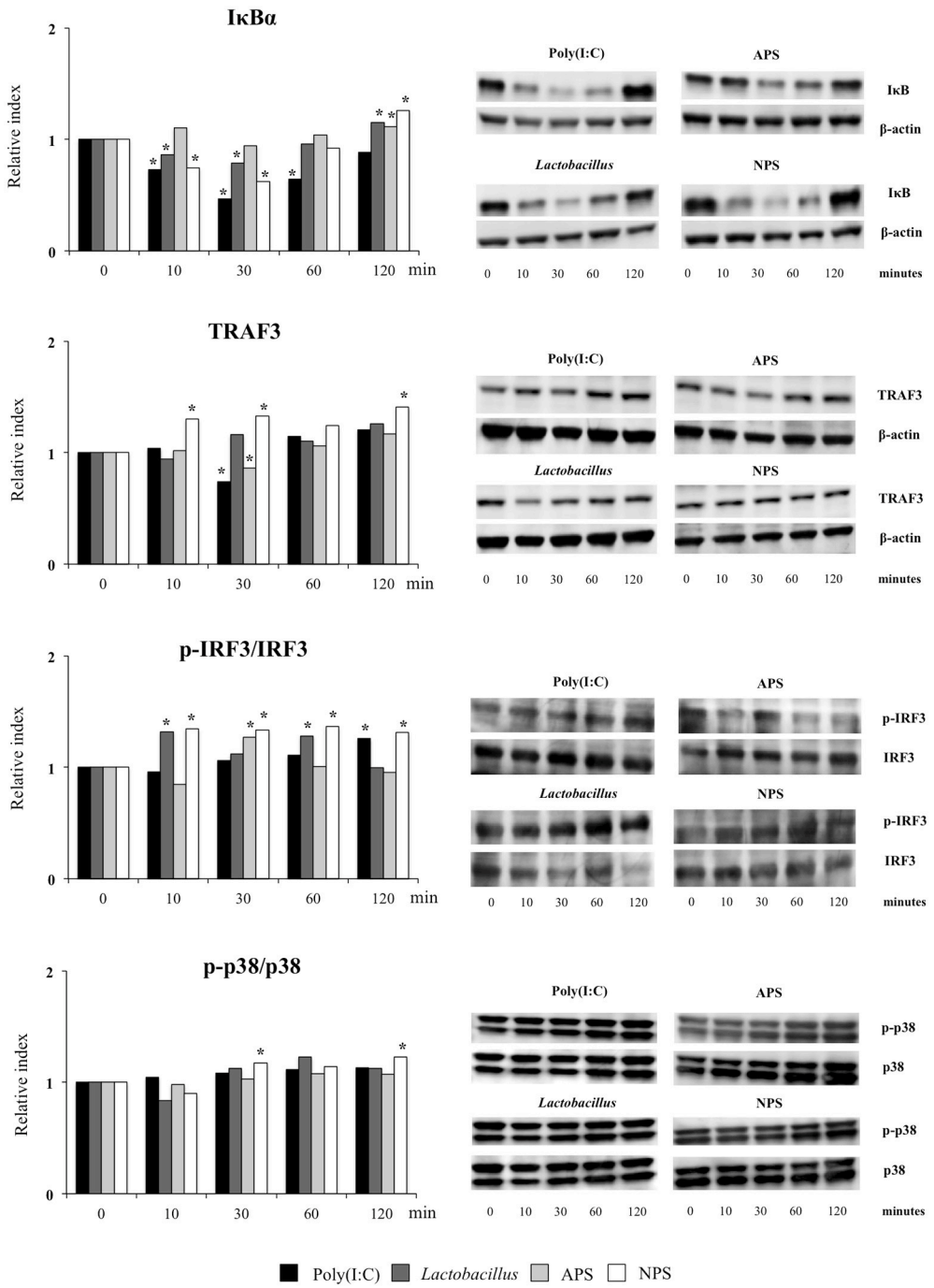


FIGURE 5 | Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L on signaling pathways in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were treated with lactobacilli, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The proteins from lysed cells were extracted at the indicated time points and separated by SDS-PAGE. Western-blot was performed to quantify TRAF3, p-IRF3/IRF3/, p-p38/p38, and IκBα levels. Intensities of proteins bands were calculated from peak area of densitogram by using image J software. Three independent experiments, one representative experiment is shown. Asterisks indicate significant differences when compared to time 0 for each group (*$P < 0.05$).

the expression of A20 remained reduced when compared to controls until hour 6 in *L. delbrueckii*- and NPS-treated PIE cells. Interestingly, A20 expression was significantly higher in *L. delbrueckii* TUA4408L-treated PIE cells at hour 12 when compared to the other experimental groups (**Figure 6**).

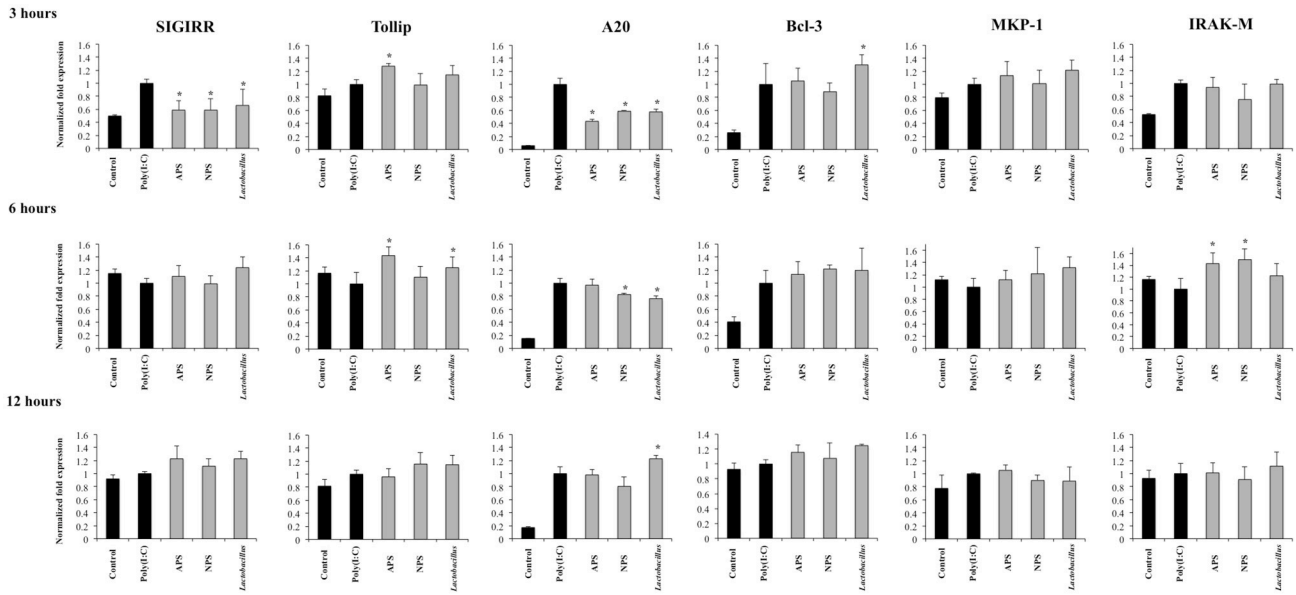


FIGURE 6 | Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L on the expression of negative regulators of the Toll-like receptor (TLR) signaling in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were treated with lactobacilli, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of single immunoglobulin IL-1-related receptor (SIGIRR), Toll interacting protein (Tollip), zinc finger protein A20 (A20), B-cell lymphoma 3-encoded protein (Bcl-3), mitogen-activated protein kinase-1 (MKP-1), and interleukin-1 receptor-associated kinase M (IRAK-M) was evaluated at 3, 6, and 12 h after poly(I:C) challenge. The results are expressed as mean ± SD and represent data from three independent experiments ($n = 3$ in each experiment). Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$).

APS treatment was able to enhance the expression of Tollip and IRAK-M between hours 3 and 6 after poly(I:C) challenge while NPS increased the expression of IRAK-M at hour 6. In addition, *L. delbrueckii* TUA4408L-treated PIE cells showed an improved expression of Tollip and Bcl-3 at hours 6 and 3 after poly(I:C) challenge, respectively (**Figure 6**).

Role of TLR2 and TLR4 on the Immunomodulatory Activities of *L. delbrueckii* TUA4408L and its EPSs

Previously, we demonstrated that the ability of *L. delbrueckii* TUA4408L and its EPSs to differentially modulate the immune response triggered by TLR4 activation is partially dependent on TLR2 and TLR4 (11). Then, we next aimed to find out whether the immunoregulatory effects observed in the context of TLR3 activation were also dependent on both TLRs. For this purpose, anti-TLR2 or anti-TLR4 blocking antibodies were used before the stimulation with *L. delbrueckii*, APS, or NPS, and the subsequent challenge with poly(I:C).

The treatment of PIE cells with anti-TLR2 antibodies abolished the ability of *L. delbrueckii* to improve IFN-β and reduce the expression of IL-6, IL-8, and TNF-α after TLR3 activation, while anti-TLR4 antibodies did not induce significant changes in the immunomodulatory activity of the TUA4408L strain (**Figure 7**). Similarly, the treatment of PIE cells with anti-TLR2 antibodies abolished the ability of NPS to reduce the expression of IL-6, IL-8, and MCP-1 after TLR3 activation, while anti-TLR4 antibodies had no effect. On the contrary, anti-TLR2 antibodies did not induce modifications in the expression of inflammatory factors in APS-treated PIE cells. However, anti-TLR4 antibodies abolished the ability of APS to improve IFN-β and reduce inflammatory cytokines after TLR3 activation (**Figure 7**).

DISCUSSION

In this work, we reported and analyzed, for the first time, the complete genome of an industrial *L. delbrueckii* subsp. *delbrueckii* strain that has remarkable immunomodulatory properties (11). Genomic analysis revealed that the TUA4408L strain has an interesting metabolic potential relating to sugar assimilation and synthesis. This finding is in line with our results demonstrating the ability of the TUA44008L strain to growth and ferment several substrates such as pickles or soymilk, and to growth in several simple mediums containing only one source of sugars (unpublished data). In addition, we reported here the identification and genomic characterization of a chromosomally located EPS cluster from *L. delbrueckii* TUA4408L. The predicted gene products in the EPS cluster are homologous to proteins involved in the biosynthesis of other bacterial polysaccharides and the genetic organization was found to be similar to the EPS cluster from other species of lactobacilli (2, 32). In LAB, a typical EPS gene cluster consists of five highly conserved genes *epsA-epsE*, the polymerase *wzy*, the flippase *wzx*, and

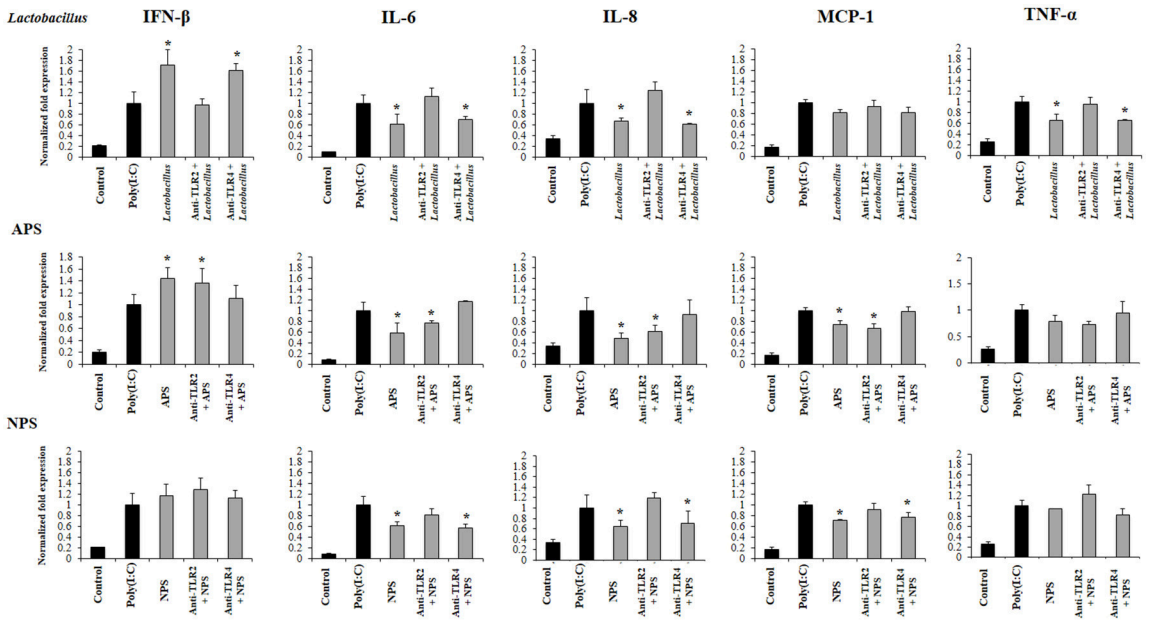


FIGURE 7 | Role of Toll-like receptor (TLR)-2 and TLR4 in the immunomodulatory activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral (NSP) and acidic (APS) EPS by standard chromatographic methods. PIE cells were incubated with anti-TLR2 or anti-TLR4 antibodies, treated with lactobacilli, APS or NPS for 48 h and, then stimulated with the TLR3 agonist poly(I:C). The expression of interferon β (IFN-β), interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF-α) was evaluated 12 h after poly(I:C) challenge. The results are expressed as mean ± SD and represent data from three independent experiments ($n = 3$ in each experiment). Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells non treated with blocking antibodies (*$P < 0.05$).

a variable number of glycosyltransferases and other polymer-modifying enzymes (2). This EPS gene cluster structure has been described and studied in *L. delbrueckii* subsp. *bulgaricus* Lfi5 (**Figure 8A**) (32). In *L. delbrueckii* TUA4408L genome, we found the genes encoding for the polysaccharide assembly machinery including those involved in the initiation of EPS biosynthesis (*epsE*), export (*wzx/epsN*), attachment (*epsA*), polymerization (*wzy/epsK*), as well as the phosphoregulatory module *epsBCD* (2). In addition, a variable region containing genes for seven putative glycosiltransferases was found in the TUA4408L EPS cluster (**Figure 8A**). It was also described that EPS gene clusters are highly diverse and their nucleotide sequences are among the most variable sequences in LAB genomes. In fact, insertion sequence elements flanking or within the operon are consistently present in the architecture of EPS gene clusters (36). We also have found this characteristic in the EPS cluster from *L. delbrueckii* TUA4408L.

A region containing seven genes for glycosyltransferases was found in *L. bulgaricus* Lfi5 EPS cluster (32), and the work suggested that all these putative glycosyltransferases were involved in the sequential biosynthesis of the EPS repeating unit. In fact, their number (seven) corresponded to the number of sugar residues present in the EPS repeating unit from Lfi5 strain (32). Preliminarily chemical evaluation of the EPS of *L. delbrueckii* TUA4408L indicated that the monosaccharaides glucose, mannose, galactose, arabinose and galacturonic acid are present in the EPS (unpublished data). Moreover, a branching glucose would be also present in the structure of the EPS repeating unit in the TUA4408L strain. Taking into consideration that glycosyltransferases constitute a large family of diverse proteins that have a wide range of enzymatic activities and functions, the prediction of the function of a putative glycosyltransferase by analyzing sequence homology is problematic because there are many examples of closely related sequences having different catalytic activity (37). Therefore, more detailed studies are necessary in order to characterize the glycosyltransferases *epsF* to *epsJ*, and *epsL* present in the *L. delbrueckii* TUA4408L EPS cluster as well as its chemical structure.

In addition to its genomic characterization, we have further advanced here in the study of the immunomodulatory properties of *L. delbrueckii* TUA4408L and its EPS. We have demonstrated that *L. delbrueckii* TUA4408L and its EPS are capable to regulate the innate immune response induced by the activation of TLR3 and improve the resistance of PIE cells against rotavirus infection.

Rotavirus is a naked double-stranded RNA (dsRNA) virus that infects mature intestinal epithelial cells. These cells sense rotavirus dsRNA through PRRs such as TLR3 and activate cellular signaling cascades to react to viral infection (33, 38). As a result of TLR3 activation, IFNs, IFN-regulated factors, cytokines, and chemokines are produced by infected cells in order to induce the recruitment and activation of immune cells and establish an antiviral state for virus clearance (33). Among the antiviral factors produced by infected cells, IFN-β is a key cytokine

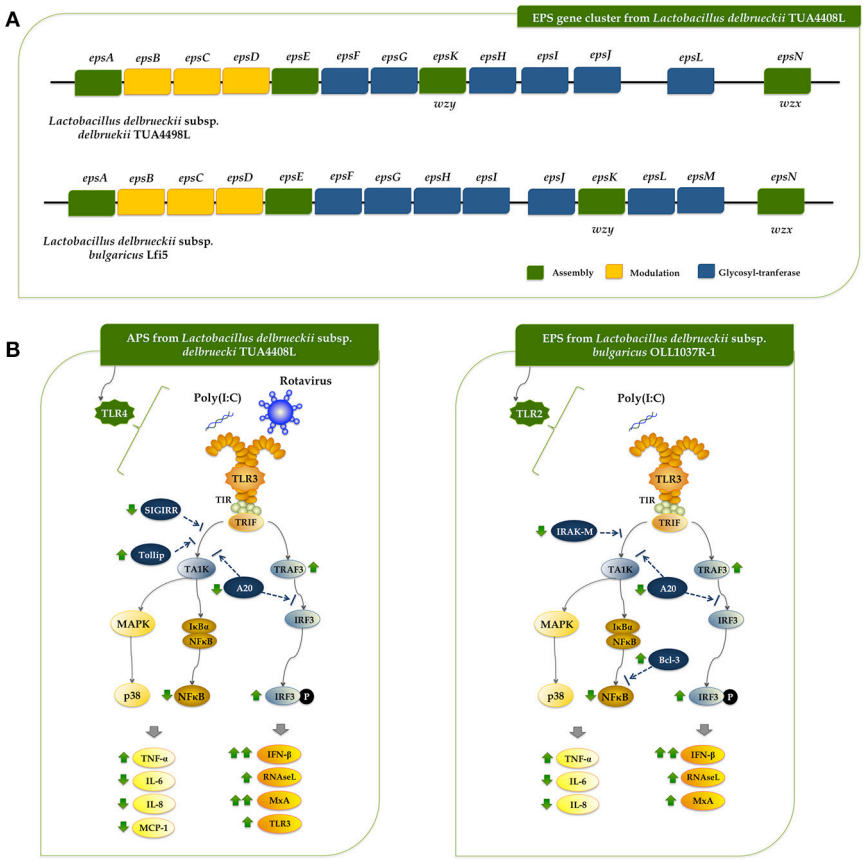


FIGURE 8 | (A) Schematic genetic organization of the exopolysaccharides (EPS) gene cluster of *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L compared with the EPS gene cluster of *Lactobacillus delbrueckii* subsp. *bulgaricus* Lfi5. Gene functional grouping is marked with different colors. The nomenclature currently used for designating the genes in *eps* clusters encoding the Wzy-dependent pathway differs among organisms. For a generic LAB *eps* gene cluster, it was proposed to designate the five first conserved genes *epsABCDE*, the polymerase *wzy/epsK* and the flippase *wzx/epsN*. This nomenclature was used to designate those genes in the TUA4408L EPS cluster. In addition, the seven glycosiltransferases present in the TUA4408L EPS cluster were designated as *epsF* to *epsJ*, and *epsL* according to their location in the cluster. It should be noted that these genes are different from those with the same designation in the *L. bulgaricus* Lfi5 EPS cluster. **(B)** Proposed mechanisms for the antiviral activity of *L. delbrueckii* subsp. *delbrueckii* TUA4408L and its acidic extracellular polysaccharide (APS), and the EPS of *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 in porcine intestinal epithelial (PIE) cells after stimulation with the Toll-like receptor (TLR)-3 agonist poly(I:C) or infection with rotavirus.

involved in the protection against rotavirus infection (39, 40), and therefore the improvement of this antiviral cytokine by intestinal epithelial cells have been used as a biomarker in the searching of beneficial microbes able to protect against rotavirus infection (reviewed in (33). In this regard, we have evaluated the suitability of PIE cells to be used as an efficient *in vitro* system for the search of beneficial microbes with antiviral capacities for humans and pigs. Our studies have shown that PIE cells have functional TLR3 which signal via IRF3 and NF-κB, inducing the up-regulation of IFN-β and the antiviral effectors MxA and RNaseL that are able to inhibiting viral replication and proliferation (41, 42). Furthermore, by studying IRF3 and NF-κB signaling pathway we have been able to find and characterize beneficial microbes with remarkable antiviral capacities including *L. casei* MEP221106 (43), *B. infantis* MCC12, *B. breve* MCC1274 (13), *L. rhamnosus* CRL1505, and *L. plantarum* CRL1506 (34, 35). In this work, by evaluating the innate antiviral immune response triggered by TLR3 activation and rotavirus infection in PIE cells we demonstrated that *L. delbrueckii* TUA4408L and its EPS are able to modulate IRF3 and NF-κB signaling pathways, improve IFN-β, MxA, and RNaseL expression, and significantly reduce rotavirus replication (**Figure 8B**).

In agreement with our previous studies (13, 34, 35, 43), we found that *L. delbrueckii* TUA4408L and its EPS differentially modulated the expression of several negative regulators of the TLR signaling pathway. In particular, a significant reduction in the expression of the zinc-finger protein A20 was observed in PIE cells treated with the APS, NPS or the TUA4408L strain. It was established that A20 is able to suppress IRF3 activation conducting to the reduction of the IFN-mediated immune response (44). Then, the reduction of A20 expression in EPS- or TUA4408L-treated PIE cells would be related the improved IRF3 activation and IFN-β, MxA, and RNaseL expression.

In addition to their ability to enhance antiviral effectors, microbes that improve protection against rotavirus infections have also the capacity to differentially modulate the expression of proinflammatory cytokines and chemokines (13, 34, 35, 43). The efficient regulation of inflammatory response induced viral attack is essential to achieve full protection against infection since unregulated inflammatory responses have been linked to cellular damage and severe mucosal injury in the gut during the course of rotavirus infection (33, 45). *L. delbrueckii* TUA4408L and its EPS have this characteristic since IL-6, IL-8, MCP-1, and TNF-α in PIE cells after TLR3 activation or rotavirus challenge were differentially expressed when compared to untreated PIE cells. The effect of the TUA4408L strain and its EPS on proinflammatory factors expression was probably coupled to their ability to differentially modulate the expression of the TLR negative regulators SIGIRR, Tollip, IRAK-M, and Bcl-3 (**Figure 8B**).

Besides epithelial cells, we have previously used porcine intestinal antigen presenting cells primary cultures to evaluate the effect of immunomodulatory probiotic strains (46) and such studies can be expanded to the analysis of the immunomodulatory capacities of *L. delbrueckii* subsp. *delbrueckii* and their EPS in antigen presenting cells in the future.

We were also interested in investigating whether TLR2 and TLR4 were involved in the antiviral activities of *L. delbrueckii* TUA4408L and its EPS since our previous studies evaluating their ability to influence immune responses in PIE cells triggered by TLR4 activation demonstrated that both PRRs were involved in their immunomodulatory effects (11). Our experiments using blocking antibodies, knockdown PIE cells, and calcium mobilization, demonstrated that the immunoregulatory capacities of *L. delbrueckii* TUA4408L EPS was dependent on TLR2 while APS exerted its immunomodulatory effect through TLR4 (11). In this work, we obtained similar results since the ability of the TUA4408L strain and its APS to modulate TLR3-triggered antiviral immune response in PIE cells was dependent on TLR2 and TLR4, respectively. It would be interesting to evaluate *in vivo* the role of TLR2 and TLR4 in the immunomodulatory effects of TUA4408L strain and its APS and also the role of TLR9 as this importantly relates to the production of immunosuppressive IL-10.

To the best of our knowledge, few studies have demonstrated the beneficial effects of EPS produced by lactobacilli in the improvement of innate antiviral immune response in general and in epithelial cells in particular. In a recent study, we evaluated the immunomodulatory activities of *L. delbrueckii* subsp. *bulgaricus* OLL1037R-1 and demonstrated that its EPS was able to induce a significant increase in the expression of IFN-α, IFN-β, MxA, and RNaseL in PIE cells after the stimulation of TLR3, in a TLR2 dependent manner (47). Interestingly, although EPS improved IFN-β, MxA, and RNaseL expression in PIE cells, this effect was not similar with purified APS or NPS from the OLL1073R-1 strain. In fact, our results indicated that the complete EPS molecule was necessary for obtaining the highest immunomodulatory/antiviral activity in PIE cells (47). On the other hand, the results presented here indicate that the beneficial effect induced by the TUA4408L strain can be reproduced completely by APS treatment, indicating that this EPS fraction is involved in the modulation of antiviral immunity. Then, our research works clearly demonstrated that the immunomodulatory properties of the EPS produced by lactobacilli are strain specific and therefore, the EPS of each potential probiotic strain should be studied in depth since it is not possible to make extrapolations even with strains of the same species. One question that remains open is how the APS from TUA4408L and the EPS from OLL1037R-1 acting through different receptors, TLR4 and TLR2 respectively, are able to induce almost identical effects on innate antiviral immunity (**Figure 8B**): improvement of IFN-β, TRAF3/IRF3, MxA, and RNaseL, reduction of A20 expression and NF-κB activation, and differential modulation of inflammatory cytokines and chemokines. The answer to this question is an important topic for future research.

The functional and genomic comparative studies of the effect of different EPS, APS, and NPS from *L. delbrueckii* subsp. *delbrueckii* strains with and without antiviral capabilities could be of great importance to deepen the knowledge of the molecular mechanisms related to its beneficial effects and to find those that are more efficient in the protection against viral infections. Moreover, the study and characterization of the EPS gene clusters of immunomodulatory lactobacilli, in particular of their variable regions, could provide bacterial biomarkers that allow an efficient screening in the search for EPS-producing strains with antiviral capabilities for its application in immunomodulatory functional foods and feeds. The genomic characterization of *L. delbrueckii* subsp. *delbrueckii* TUA4408L and the evaluation of the immunomodulatory/antiviral properties of its EPS reported here is an important advance in this line of research.

AUTHOR CONTRIBUTIONS

HA, JV, and HaK designed the study and manuscript writing. PK, RK, YS, SE, and TM did the laboratory work related to antiviral immunity. HiK, and AM did the laboratory work related to rotavirus infection. LA, EH, LS, BG, and AS-B performed genomic analysis. PK, LA, HiK and JV did the statistical analysis. HA, YS, JV and HaK participated in the data analysis and integrative discussion. HT, YS, TM, and SE contributed to data analysis and interpretation. All the authors read and approved the manuscript.

FUNDING

This study was supported by a Grant-in-Aid for Scientific Research (B)(2) (24380146, 16H05019), Challenging Exploratory Research (23658216, 26660216, 16K15028), and Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects from the Japan Society for the Promotion of Science (JSPS) to HaK. PK was supported by JSPS (Postdoctoral Fellowship for Foreign Researchers, Program No. 25-03397 and 15F15401, respectively). This work was supported by JSPS Core-to-Core Program, A. Advanced Research Networks entitled Establishment of international agricultural immunology

research-core for a quantum improvement in food safety. This study was also financially supported by grants for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Science, Sports, and Technology (MEXT) of Japan (Grant numbers: 16H06429, 16K21723, and 16H06435).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02178/full#supplementary-material

REFERENCES

1. Ullrich M. *Bacterial Polysaccharides: Current Innovations and Future Trends*. Horizon, TX: Scientific Press (2009).
2. Zeidan AA, Poulsen VK, Janzen T, Buldo P, Derkx PMF, Øregaard G, et al. Polysaccharide production by lactic acid bacteria: from genes to industrial applications. *FEMS Microbiol Rev*. (2017) 41:S168–200. doi: 10.1093/femsre/fux017
3. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* (2010) 8:623–33. doi: 10.1038/nrmicro2415
4. Caggianiello G, Kleerebezem M, Spano G. Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. *Appl Microbiol Biotechnol*. (2016) 100:3877–86. doi: 10.1007/s00253-016-7471-2
5. Laiño J, Villena J, Kanmani P, Kitazawa H. Immunoregulatory effects triggered by lactic acid bacteria exopolysaccharides: new insights into molecular interactions with host cells. *Microorganisms* (2016) 4:27. doi: 10.3390/microorganisms4030027
6. Yasuda E, Serata M, Sako T. Suppressive effect on activation of macrophages by lactobacillus casei strain shirota genes determining the synthesis of cell wall-associated polysaccharides. *Appl Environ Microbiol*. (2008) 74:4746–55. doi: 10.1128/AEM.00412-08
7. Bleau C, Monges A, Rashidan K, Laverdure JP, Lacroix M, Van Calsteren MR, et al. Intermediate chains of exopolysaccharides from Lactobacillus rhamnosus RW-9595M increase IL-10 production by macrophages. *J Appl Microbiol*. (2010) 108:666–75. doi: 10.1111/j.1365-2672.2009.04450.x
8. Balzaretti S, Taverniti V, Guglielmetti S, Fiore W, Minuzzo M, Ngo HN, et al. A novel rhamnose-rich hetero-exopolysaccharide isolated from *Lactobacillus paracasei* DG activates THP-1 human monocytic cells. *Appl Environ Microbiol*. (2017) 83:e02702–16. doi: 10.1128/AEM.02702-16
9. Ciszek-Lenda M, Nowak B, Sróttek M, Gamian A, Marcinkiewicz J. Immunoregulatory potential of exopolysaccharide from *Lactobacillus rhamnosus* KL37. Effects on the production of inflammatory mediators by mouse macrophages. *Int J Exp Pathol*. (2011) 92:382–91. doi: 10.1111/j.1365-2613.2011.00788.x
10. Moue M, Tohno M, Shimazu T, Kido T, Aso H, Saito T, et al. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. *Biochim Biophys Acta Gen Subj*. (2008) 1780:134–44. doi: 10.1016/j.bbagen.2007.11.006
11. Wachi S, Kanmani P, Tomosada Y, Kobayashi H, Yuri T, Egusa S, et al. *Lactobacillus delbrueckii* TUA4408L and its extracellular polysaccharides attenuate enterotoxigenic *Escherichia coli*- induced inflammatory response in porcine intestinal epitheliocytes via Toll-like receptor-2 and 4. *Mol Nutr Food Res*. (2014) 58:2080–93. doi: 10.1002/mnfr.201400218
12. Kitazawa H, Harata T, Uemura J, Saito T, Kaneko T, Itoh T. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from Lactobacillus delbrueckii ssp. bulgaricus. *Int J Food Microbiol*. (1998) 40:169–75. doi: 10.1016/S0168-1605(98)00030-0
13. Ishizuka T, Kanmani P, Kobayashi H, Miyazaki A, Soma J, Suda Y, et al. Immunobiotic bifidobacteria strains modulate rotavirus immune response in porcine intestinal epitheliocytes via pattern recognition receptor signaling. *PLoS ONE* (2016) 11:e0152416. doi: 10.1371/journal.pone.0152416
14. Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, et al. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat Methods* (2013) 10:563–9. doi: 10.1038/nmeth.2474
15. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* (2014) 30:2068–9. doi: 10.1093/bioinformatics/btu153
16. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res*. (2014) 42:D206-14. doi: 10.1093/nar/gkt1226
17. Grant JR, Stothard P. The CGView Server: a comparative genomics tool for circular genomes. *Nucleic Acids Res*. (2008) 36:W181–4. doi: 10.1093/nar/gkn179
18. Liu B, Pop M. ARDB - Antibiotic resistance genes database. *Nucleic Acids Res*. (2009) 37: D443–7. doi: 10.1093/nar/gkn656
19. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res*. (2017) 45:D566–73. doi: 10.1093/nar/gkw1004
20. Bertelli C, Laird MR, Williams KP, Lau BY, Hoad G, Winsor GL, et al. IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Res*. (2017) 45:W30–5. doi: 10.1093/nar/gkx343
21. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: A Fast Phage Search Tool. *Nucleic Acids Res*. (2011) 39:W347–52. doi: 10.1093/nar/gkr485
22. Grissa I, Vergnaud G, Pourcel C. CRISPRcompar: a website to compare clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res*. (2008) 36: W145–8. doi: 10.1093/nar/gkn228
23. Tanigawa K, Watanabe K. Multilocus sequence typing reveals a novel subspeciation of *Lactobacillus delbrueckii*. *Microbiology* (2011) 157:727–38. doi: 10.1099/mic.0.043240-0
24. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. (2004) 32:1792–7. doi: 10.1093/nar/gkh340
25. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol*. (2016) 33:1870–4. doi: 10.1093/molbev/msw054
26. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* (2015) 31:3691–3. doi: 10.1093/bioinformatics/btv421
27. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. (2009) 55:611–22. doi: 10.1373/clinchem.2008.112797
28. Nygard AB, Jørgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol*. (2007) 8:67. doi: 10.1186/1471-2199-8-67
29. Nilsen T, Nes IF, Holo H. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Appl Environ Microbiol*. (2003) 69:2975–84. doi: 10.1128/AEM.69.5.2975-2984.2003
30. Suárez N, Bonacina J, Hebert E, Saavedra L. Genome mining and transcriptional analysis of bacteriocin genes in enterococcus faecium CRL1879. *J Data Mining Genomics Proteomics* (2015) 6:1. doi: 10.4172/2153-0602.1000180
31. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, et al. An updated evolutionary classification of CRISPR–Cas systems. *Nat Rev Microbiol*. (2015) 13:722–36. doi: 10.1038/nrmicro3569
32. Lamothe G, Jolly L, Mollet B, Stingele F. Genetic and biochemical characterization of exopolysaccharide biosynthesis by *Lactobacillus delbrueckii subsp. bulgaricus*. *Arch Microbiol*. (2002) 178:218–28. doi: 10.1007/s00203-002-0447-x
33. Villena J, Vizoso-Pinto MG, Kitazawa H. Intestinal innate antiviral immunity and immunobiotics: beneficial effects against rotavirus infection. *Front Immunol*. (2016) 7:1–10. doi: 10.3389/fimmu.2016.00563
34. Villena J, Chiba E, Vizoso-Pinto M, Tomosada Y, Takahashi T, Ishizuka T, et al. Immunobiotic *Lactobacillus rhamnosus* strains

differentially modulate antiviral immune response in porcine intestinal epithelial and antigen presenting cells. *BMC Microbiol.* (2014) 14:126. doi: 10.1186/1471-2180-14-126
35. Albarracin L, Kobayashi H, Iida H, Sato N, Nochi T, Aso H, et al. Transcriptomic analysis of the innate antiviral immune response in porcine intestinal epithelial cells: influence of immunobiotic *Lactobacilli*. *Front Immunol.* (2017) 8:57. doi: 10.3389/fimmu.2017.00057
36. Cui Y, Xu T, Qu X, Hu T, Jiang X, Zhao C. New insights into various production characteristics of *Streptococcus thermophilus* Strains. *Int J Mol Sci.* (2016) 17:1701. doi: 10.3390/ijms17101701
37. Breton C, Šnajdrová L, Jeanneau C, Koča J, Imberty A. Structures and mechanisms of glycosyltransferases. *Glycobiology* (2006) 16:29R−37R. doi: 10.1093/glycob/cwj016
38. Frias AH, Jones RM, Fifadara NH, Vijay-Kumar M, Gewirtz AT. Rotavirus-induced IFN-β promotes anti-viral signaling and apoptosis that modulate viral replication in intestinal epithelial cells. *Innate Immun.* (2012) 18:294–306. doi: 10.1177/1753425911401930
39. Sen A, Rothenberg ME, Mukherjee G, Feng N, Kalisky T, Nair N, et al. Innate immune response to homologous rotavirus infection in the small intestinal villous epithelium at single-cell resolution. *Proc Natl Acad Sci USA*. (2012) 109:20667–72. doi: 10.1073/pnas.1212188109
40. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* (2006) 22:561–9. doi: 10.1016/j.molcel.2006.05.012
41. Liang SL, Quirk D, Zhou A. RNase L: Its biological roles and regulation. *IUBMB Life (International Union Biochem Mol Biol Life)* (2006) 58:508–14. doi: 10.1080/15216540600838232
42. Mitchell PS, Patzina C, Emerman M, Haller O, Malik HS, Kochs G. Evolution-guided identification of antiviral specificity determinants in the broadly acting interferon-induced innate immunity factor MxA. *Cell Host Microbe*. (2012) 12:598–604. doi: 10.1016/j.chom.2012.09.005
43. Hosoya S, Villena J, Shimazu T, Tohno M, Fujie H, Chiba E, et al. Immunobiotic lactic acid bacteria beneficially regulate immune response triggered by poly(I:C) in porcine intestinal epithelial cells. *Vet Res*. (2011) 42:111. doi: 10.1186/1297-9716-42-111
44. Saitoh T, Yamamoto M, Miyagishi M, Taira K, Nakanishi M, Fujita T, et al. A20 is a negative regulator of IFN regulatory factor 3 signaling. *J Immunol*. (2005) 174:1507–12. doi: 10.4049/jimmunol.174.3.1507
45. Koyama S, Ishii KJ, Coban C, Akira S. Innate immune response to viral infection. *Cytokine* (2008) 43:336–41. doi: 10.1016/j.cyto.2008.07.009
46. Tsukida K, Takahashi T, Iida H, Kanmani P, Suda Y, Nochi T, et al. Immunoregulatory effects triggered by immunobiotic *Lactobacillus jensenii* TL2937 involve efficient phagocytosis in porcine dendritic cells. *BMC Immunol.* (2016) 17:21. doi: 10.1186/s12865-016-0160-1
47. Kanmani P, Albarracin L, Kobayashi H, Iida H, Komatsu R, Humayun Kober AKM, et al. Exopolysaccharides from *Lactobacillus delbrueckii* OLL1073R-1 modulate innate antiviral immune response in porcine intestinal epithelial cells. *Mol Immunol.* (2018) 93:253–65. doi: 10.1016/j.molimm.2017.07.009

Conflict of Interest Statement: TM was employed by the company Marusan-Ai Co. Ltd. (Okazaki, Japan).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 September 2018
doi: 10.3389/fimmu.2018.02223

Receptors and Signaling Pathways for Recognition of Bacteria in Livestock and Crops: Prospects for Beneficial Microbes in Healthy Growth Strategies

Julio Villena[1,2]*, *Haruki Kitazawa*[2,3]*, *Saskia C. M. Van Wees*[4], *Corné M. J. Pieterse*[4]* and *Hideki Takahashi*[5,6]*

[1] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [2] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [3] Livestock Immunology Unit, International Education and Research Center for Food Agricultural Immunology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Plant–Microbe Interactions, Department of Biology, Science4life, Utrecht University, Utrecht, Netherlands, [5] Laboratory of Plant Pathology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [6] Plant Immunology Unit, International Education and Research Center for Food Agricultural Immunology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

OPEN ACCESS

Edited by:
Emilio Jirillo,
Università degli Studi di Bari, Italy

Reviewed by:
Anastasia N. Vlasova,
The Ohio State University,
United States
Thea Magrone,
Università degli Studi di Bari, Italy

***Correspondence:**
Julio Villena
jcvillena@cerela.org.ar
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp
Corné M. J. Pieterse
C.M.J.Pieterse@uu.nl
Hideki Takahashi
hideki.takahashi.d5@tohoku.ac.jp

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: 28 May 2018
Accepted: 07 September 2018
Published: 27 September 2018

Citation:
Villena J, Kitazawa H, Van Wees SCM, Pieterse CMJ and Takahashi H (2018) Receptors and Signaling Pathways for Recognition of Bacteria in Livestock and Crops: Prospects for Beneficial Microbes in Healthy Growth Strategies. Front. Immunol. 9:2223. doi: 10.3389/fimmu.2018.02223

Modern animal and crop production practices are associated with the regular use of antimicrobials, potentially increasing selection pressure on bacteria to become resistant. Alternative approaches are needed in order to satisfy the demands of the growing human population without the indiscriminate use of antimicrobials. Researchers have brought a different perspective to solve this problem and have emphasized the exploitation of animal- and plant-associated microorganisms that are beneficial to their hosts through the modulation of the innate immune system. There is increasing evidence that plants and animals employ microbial perception and defense pathways that closely resemble each other. Formation of pattern recognition receptor (PRR) complexes involving leucine-rich repeat (LRR)-containing proteins, mitogen-activated protein kinase (MAPK)-mediated activation of immune response genes, and subsequent production of antimicrobial products and reactive oxygen species (ROS) and nitric oxide (NO) to improve defenses against pathogens, add to the list of similarities between both systems. Recent pioneering work has identified that animal and plant cells use similar receptors for sensing beneficial commensal microbes that are important for the maintenance of the host's health. Here, we reviewed the current knowledge about the molecular mechanisms involved in the recognition of pathogenic and commensal microbes by the innate immune systems of animal and plants highlighting their differences and similarities. In addition, we discuss the idea of using beneficial microbes to modulate animal and plant immune systems in order to improve the resistance to infections and reduce the use of antimicrobial compounds.

Keywords: beneficial microbes, pattern recognition receptors, animal immunity, plant immunity, agricultural immunology

THE USE OF ANTIMICROBIALS IN LIVESTOCK AND CROPS: A GLOBAL PROBLEM

The increasing prevalence of antimicrobial resistance in pathogenic microorganisms of clinical importance exerts a tremendous pressure on human healthcare systems globally. There is a dramatic rise in the prevalence of infections caused by multidrug- or extremely drug-resistant pathogens, which is estimated to cause several hundred thousand deaths annually (1, 2). Perhaps the best examples are the infections caused by multidrug-resistant bacteria belonging to the *Enterobacteriaceae* group. This fact implies a great concern since these pathogens are common natural inhabitants of human and animal microbiomes. Moreover, infections caused by this group of bacteria are often associated with prolonged hospitalization, elevated costs and high mortality rates (1, 2). Resistance to antimicrobials is a naturally occurring phenomenon. However, the increasing use of antimicrobials by the mankind has created a strong and unnaturally high selection pressure for resistant microorganisms. The emergence and spread of antibiotics resistance in microorganisms has been accelerated around the world by several human behaviors, including inappropriate use of antimicrobial substances, poor prevention of infectious diseases, defective control of infected patients within healthcare systems, and the insufficient control of antibiotics release into the environment.

Agricultural and food industries have been benefited from the availability of antimicrobial compounds for animal production and crop protection. Antibiotics have been widely used in livestock diets during the past several decades due to their therapeutic effects (3, 4). Antibiotics are able to reduce the frequency of diarrhea and improve performance parameters like body weight gain or feed conversion ratio. These beneficial effects of feed antibiotics are generally explained by modifications of the intestinal bacteria and their interaction with the animal host, including bacterial interactions with intestinal tissues and the immune system (3, 4). Another class of agents used in agriculture are cationic metals that can be included in animal diets as nutritional supplements or spread on pastures to support crop growth and protection. Heavy metals, in particular, give rise to concerns among public health professionals, as they can persist in the environment for prolonged periods. Moreover, bacteria can also exhibit resistance to these chemical elements and the genes encoding this phenotype can be physically localized on plasmids that may also contain one or more antimicrobial resistance-encoding genes (5).

On the other hand, chemical pesticides including antimicrobials for protection of crops against bacterial plant diseases are limited in availability, use, and efficacy, and their affectivity is limited. Such antimicrobials are used for managing bacterial plant diseases of fruit trees, for which it has been proven to be economically feasible (6). Although the amount of antibacterial antibiotics used on plants is small compared to medical, veterinary, and livestock production uses, antibiotics-resistant bacterial plant pathogens have emerged, which further complicates the control of bacterial diseases of crops, especially fruit trees. In addition, the pollution with antimicrobial, mutagenic and carcinogenic compounds in aquatic and soil environments caused by the discharge industrial wastes, atmospheric deposition, and fertilizers is an emerging public health concern because of the potential in producing drug-resistant microbes that can be up-taken by food crops (7).

Antibiotics-resistant microorganisms of agricultural origin have significant public health implications since they can be transmitted to humans through the environment (8), and food products (9), and to agricultural workers by direct contact (10). It was suggested that repeated exposure to low doses of antimicrobial agents, that is the context in which growth-promoting antimicrobials are administered, creates ideal conditions for the emergence and spread of antibiotics-resistant bacteria (11).

Because of the concern that the use of antimicrobials in agricultural industry might contribute to a rise in bacterial antibiotics resistance, the use of some types of antibiotics have been restricted by some countries since the 1970's. In this regard, the European Union introduced a total ban on the application of antibiotics as feed additives from 2006 onwards (3, 12). These regulatory issues about the ban of antibiotic growth promoters together with the consumer's demand for a safe food production system have stimulated the search for alternative strategies to improve resistance against pathogens, promote growth and health of livestock (13, 14), and minimize the impact of the industry on the environment (3, 4).

In order to control infections, scientists poned that the modulation of animal and plant immune systems by using beneficial microbes able to confer health-promoting activities would be an interesting alternative. Plant and animal innate immune systems respond to pathogen infections but also regulate beneficial interactions with commensal and symbiotic microbes (15). Recent pioneering work revealed striking similarities between the molecular organization of animal and plant systems for non-self-recognition and anti-microbial defense. Studies have also identified that animal and plant cells use similar receptors for sensing beneficial commensal microbes that are important for the maintenance of the host's health. In this review we highlight current knowledge on the molecular mechanisms involved in the recognition of pathogenic and commensal microbes by the innate immune systems of animal and plants highlighting their differences and similarities. In addition, we discuss the idea of using beneficial microbes to modulate animal and plant immune systems to improve resistance to pathogen infections and to reduce the use of antimicrobial compounds in a biological way. The progress in our understanding of the cellular and molecular mechanisms of beneficial microbes-host interaction is reviewed in order to give a scientific basis for the design of new intervention strategies that can improve immune fitness of animals and plants in a more sustainable way.

INNATE IMMUNITY IN ANIMALS AND PLANTS

Animals and plants have acquired the ability to recognize conserved microbial molecules that are characteristic of microorganisms but are not found in animal or plant hosts. The recognition of these microbial molecules is a key step in innate immune defenses, and is mediated by a set of receptors referred to as pattern-recognition receptors (PRRs) that recognize the microbe-associated molecules (16). "Pathogen-associated molecular patterns" (PAMPs) is the term generally used when referring to the molecules that elicit innate immune responses. As classically defined, PAMPs are evolutionarily conserved pathogen-derived molecules that distinguish hosts from pathogens. PAMPs include, among others, lipopolysaccharide (LPS), peptidoglycan, bacterial flagellin, and yeast mannans (17). However, because non-pathogenic microbes also possess such molecules, the term "pathogen-associated" is a misnomer and a more precise term would seem to be "microbe-associated molecular patterns" (MAMPs) (16). Therefore, to avoid confusion here, the term "MAMP" is used instead of "PAMP."

Remarkable similarities have been uncovered in the molecular mode of MAMP perception in animals and plants, including the discovery of plant receptors resembling mammalian Toll-like receptors (TLRs) or cytoplasmic nucleotide binding domain (NBD) and leucine-rich repeat (LRR) superfamily proteins (NLR) (15, 18). Changes in cytoplasmic Ca^{2+} levels, the production of reactive oxygen species (ROS) and nitric oxide (NO) as well as the post-translational activation of mitogen-activated protein kinase (MAPK) cascades are commonly reported to signal the activation of innate immune responses in plants (19). Intriguingly, most of these components have also been described to be of central importance to MAMP-induced activation of innate immune responses in animal cells (20). In addition, both plants and animals synthesize a wide range of small antimicrobial peptides and both produce an oxidative burst via conserved gp91phox NADPH oxidases after the pathogen encounter (16). Therefore, common features of innate immunity in animals and plants include defined receptors for microbe-associated molecules, conserved MAPK signaling cascades and the production of antimicrobial peptides and oxidative compounds (**Figures 1**, **2**).

Animal Innate Immune System

In animals, epithelial cells from the skin and mucosal surfaces, including those lining the respiratory, urogenital and gastrointestinal tracts, provide a physicochemical barrier between the host cells and the outside world including microorganisms. The mucosal surfaces and the skin also have an intricate network of immune cells that perform surveillance functions and have the ability to trigger defense mechanisms against invading pathogens. Antigen presenting cells including dendritic cells and macrophages reside in tissues throughout the body and are especially abundant in areas where infections are likely to arise. In addition, epithelial cells from skin and mucosal surfaces have also immune functions since they are able to deliver signals to immune cells when potentially dangerous microorganisms have reached the host (21, 22). Therefore, when a pathogen invades a tissue, epithelial and immune cells elicit an inflammatory response in order to limit its replication and dissemination (**Figure 1**). This response is characterized by an initial recognition of MAMPs of pathogens by PRRs expressed in epithelial and immune cells leading to production of immune factors including type I interferons (IFNs), cytokines and chemokines. Those biological mediators are responsible for the recruitment and activation of additional immune cells that participate in the innate immune response (21, 22). Changes in the microenvironment of infected tissue induce the blood vessels to dilate and become permeable to fluid and proteins. At the same time, the endothelial cells lining the local blood vessels are stimulated to express cell adhesion proteins that facilitate the attachment and extravasion of immune cells including neutrophils and monocytes (**Figure 1**). Both types of phagocytes possess an extraordinary capacity to kill pathogens through a wide range of antimicrobial agents including antimicrobial proteins and oxidative compounds. Pathogenic stimuli activate pathways in neutrophils and macrophages that signal for the phosphorylation and assembly of the NADPH oxidase that then produces superoxide and H_2O_2 in a process known as the respiratory burst. In addition, the inducible enzyme NO synthase (iNOS) is expressed in phagocytes leading to NO production that is a gas with highly reactive properties (21, 22).

Plant Innate Immune System

The entry of pathogenic bacteria into the plants' tissues is the first and most important step in infectious diseases. Foliar bacterial pathogens mainly enter into the plant cells through the open stomata, water pores, or physical injuries (23, 24). Bacteria colonize the plant apoplast of a leaf, extensively multiply in the apoplast and inject several immune-suppressive effector molecules into plant cells through the type III secretion system (TTSS) (**Figure 1**). Those effector molecules induce visible disease-associated necrosis and chlorosis (25). On the other hand, bacterial pathogens living in the rhizosphere invade root tissues through small wounds after which they colonize intercellular spaces and stem tissues (26). Then, plants develop symptoms, such as bacterial wilt that are caused by suppressed water fluxes (26), or damping-off and root rot symptoms caused by degradation enzymes and toxins secreted by bacteria in vascular tissues (27).

In order to protect themselves against bacterial pathogens, plants have developed highly effective defense systems. In plants, there are no specialized immune cells such as macrophages, neutrophils, or dendritic cells that are the key players of the animal immune system. In contrast, plants are autonomously capable of recognizing the presence of pathogens and trigger defense responses at the level of each single cell (**Figure 1**). As plants are lacking in mobile immune cells and the cellular adaptive immune systems, they are mainly dependent on innate immunity for protection against pathogens including efficient signaling mechanisms, which is now so-called the plant immune system (15).

Once the pathogen breaks the primary defense barriers, e.g., the stomata-mediated defense system, plants can detect several

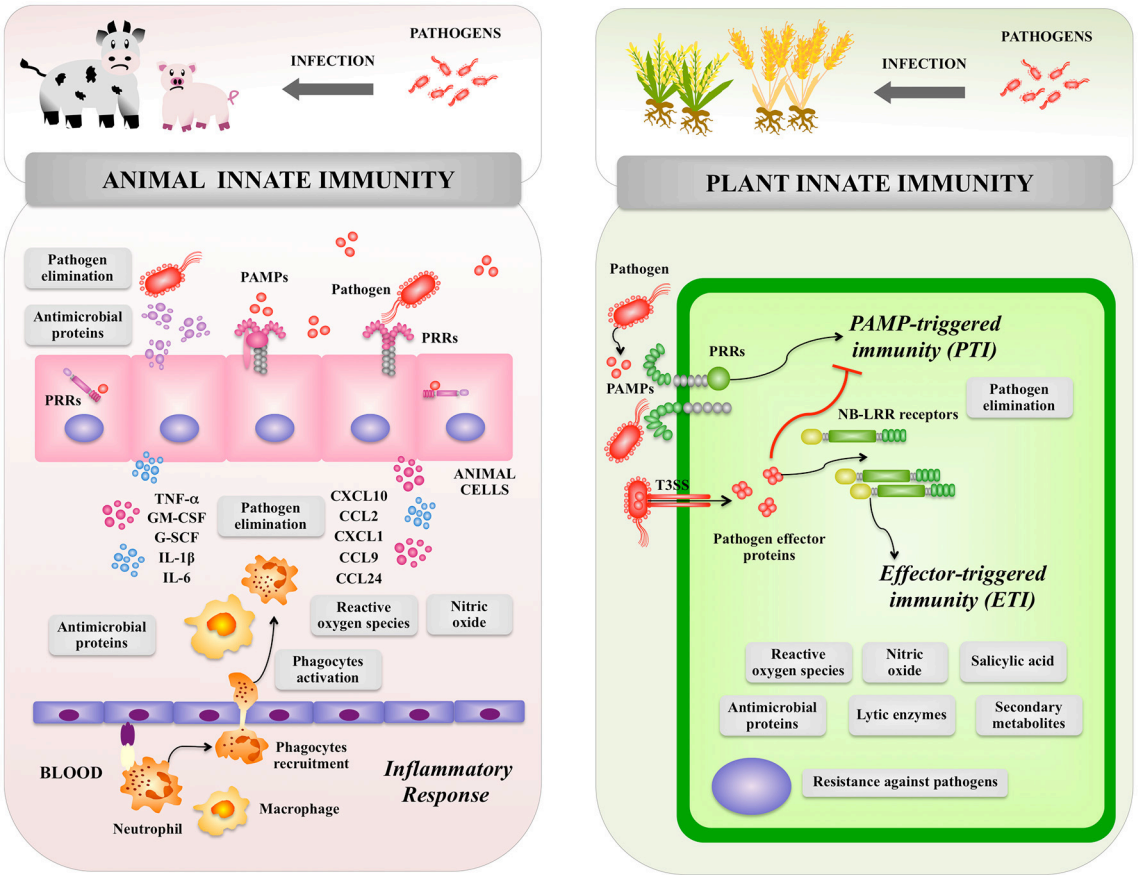


FIGURE 1 | Global overview of animal and plant innate immune systems. The inflammatory response triggered by pathogens in the animal host as well as pattern recognition receptor-triggered immunity and effector triggered immunity triggered by pathogens in the plant host are shown. Pattern recognition receptor (PRR), pathogen-associated molecular patterns (PAMPs), type III secretion system (T3SS), PAMP triggered immunity (PTI), effector-triggered immunity (ETI), tumor necrosis factor alpha (TNF-α), interleukin (IL), chemokine (C–X–C motif) ligand (CXCL), C-C motif chemokine ligand (CCL), granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF).

MAMPs including flagellin, translational elongation factor Tu (EF-Tu), cold-shock protein (CSP) or LPS (27–29). Recognition of MAMPs by PRRs locating at the plasma membrane of plant cells activates downstream signaling cascade and a series of defense responses including the synthesis of phytoalexins, cell wall strengthening, and accumulation of pathogenesis-related proteins such as lytic enzymes (chitinases, glucanases, and proteases) (**Figure 1**) (30–32). The MAMP-triggered immune system, which is named "PAMP triggered immunity" (PTI), prevents the establishment of infection in non-host plants.

Bacterial pathogens have various virulence strategies that inactivate PTI. For example, toxins produced by pathogenic bacteria are able to change plant metabolism in order to establish an advantageous environment for bacterial colonization. In addition, several bacterial pathogens have developed strategies to evade PTI. Multiple effector molecules are delivered by bacterial pathogens into plant cells through the TTSS in order to suppress PTI at various steps of the defense signaling pathways that confer disease resistance (33).

Plants have a second class of immune receptors that include intracellular immune receptors called resistance proteins (NB-LRR receptors). These intracellular receptors directly or indirectly recognize effectors secreted by pathogens into the host intracellular environment and activate effector-triggered immunity (ETI), which is often associated with rapid cell death, production of ROS and salicylic acid (SA), and the expression of defense-related genes (**Figure 1**) (34). Activation of ETI enables plants to respond rapidly and efficiently to virulent pathogens (35). In PTI and ETI, the production of ROS is an important early defense mechanism as in the innate immune response of animals (26, 33). Extracellular generation of ROS during the oxidative burst of plants depends on transient increases of cytosolic Ca^{2+} levels and appears to be mechanistically similar to the respiratory burst of animal phagocytes, which is catalyzed by an NADPH oxidase protein complex. Plants harbor a family of genes with significant homology to the human gene encoding the catalytic subunit gp91 of the NADPH oxidase complex. In addition, NO was found to be produced upon treatment of plants with MAMPs

as well as upon pathogen infection, suggesting that it may be important for the activation of innate defense mechanisms (36).

RECEPTORS FOR BACTERIAL RECOGNITION IN ANIMALS AND PLANTS

The ability to distinguish between self and non-self-antigens is an important feature of all living organisms and forms the basis for the activation of innate defense mechanisms against infections (15). In animals and plants, innate immunity involves both cell surface receptors (19) and intracellular receptors of the NLR superfamily (37) (**Figure 2**).

Pattern Recognition Receptors for Bacteria in Animals

As mentioned before, microbial recognition by the innate immune system of animals occurs via a range of germline-encoded PRRs such as the TLR family, the NLR family, the RIG-I-like RNA helicases, the C-type lectin receptors, and cytosolic DNA sensors (38, 39). The interaction of microbial ligands with PRRs induce the activation of the innate immune system leading to diverse cellular responses including the induction of interferon regulatory factors (IRFs), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) that regulate the expression of cytokines, chemokines, and type I IFNs.

TLRs were the first PRRs discovered and they are the best-characterized family of PRRs. Initially, the Toll pathway was described in the pattern formation in early drosophila embryo development (40). Later, the cytoplasmic domain of Toll (the Toll–interleukin 1 (IL-1) receptor (TIR) domain) was found to have homology with the cytoplasmic domain of human IL-1 (41). In addition, the study of antimicrobial peptides genes in drosophila and their promoters suggested that they were regulated by NF-κB-like transcription factors that also function in the Toll pathway (42). Meanwhile, a human homolog of Toll was shown to activate expression of NF-κB controlled genes (43), and a year later TLR4 was identified as the LPS sensor (44).

TLRs are characterized by an extracellular LRR domain and an intracellular TIR protein-protein interaction domain. TLRs are coupled to signaling adaptors such as MyD88, which also have TIR domains. Activation of the TLR signaling cascade results in the nuclear translocation of NF-κB-like transcription factors, leading to the production of antimicrobial peptides in both insects and vertebrates and signaling molecules such as cytokines and chemokines in vertebrates (16) (**Figure 3**).

Upon interaction with their ligands, TLRs dimerize and initiate two signaling pathways: the MyD88-dependent and MyD88-independent pathways. These two types of cellular responses are mediated by a selective use of adaptor molecules recruited to the TIR domains. Four adaptor molecules have been identified so far: MyD88, TIR-associated protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN-β (TRIF), and TRIF-related adaptor molecules (TRAM). MyD88 and TIRAP are responsible for the induction of pro-inflammatory genes, and TRIF and TRAM induce IFNs (45, 46).

All TLRs, except TLR3, signal through MyD88. In MyD88-dependent signaling, MyD88 is recruited to and associates with the cytoplasmic domain of the TLRs via interaction with the TIR domains (**Figure 3**). Then IL-1R-associated kinase 1 (IRAK-1) and IRAK-4 are recruited and activated by phosphorylation. Activated IRAK-4 phosphorylates IRAK-1, which subsequently associates with Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). TRAF6 activates transforming growth factor (TGF)-activating kinase 1 (TAK1) and this factor then phosphorylates IKK-b and MAPK kinase 6 (MKK6), leading to degradation of I-kB and NF-kB nuclear translocation. The final response is the induction of genes involved in innate defense mechanisms. Activation of the MyD88-dependent pathway also results in the activation of MAPK-p38, MAPK-ERK, and MAPK-JNK, which leads to the activation of AP-1 (45–47).

In addition to the transmembrane TLRs, mammals have a family of cytosolic PRRs that belong to a family of proteins referred to as NLR proteins that are involved in apoptotic and inflammatory responses (48). NLR proteins are characterized by a tripartite domain architecture consisting of a variable N-terminal domain, a central nucleotide-binding domain and C-terminal LRRs (**Figure 4**).

The NOD1 and NOD2 receptors were the first members of the NLR family to be reported as intracellular sensors for microorganisms in mammals. The interaction of NLRs with their ligands triggers signaling cascades that induce the translocation of NF-kB, and the production of cytokines and chemokines (49). NOD1 and NOD2 receptors are located in the cytosol of host cells. However, after their interaction with their ligands they undergo redistribution to the plasma membrane from where they initiate signaling pathways (50). It should be mentioned that differences in the expression of the two receptors exists. NOD2 expression is limited to cells of hematopoietic origin and some types of epithelial cells (specially the gastrointestinal mucosa) while NOD1 is ubiquitously expressed in most cell types (51). Interestingly, the expression of NOD1 and NOD2 in macrophages can be increased by stimulation with LPS or proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (52). Both, NOD1 and NOD2 have three domains: a C-terminal domain containing LRRs, a central nucleotide-binding (NACHT) domain, and a N-terminal effector-binding domain composed of caspase-activated recruitment domains (CARD) (53). The C-terminal domain is responsible for ligand interaction while NACHT domain allows self-oligomerization and is necessary for self-activation. NOD1 has one CARD domain while NOD2 has two series-wound CARDs that mediate the interaction with intracellular proteins that form the signaling platform (53). Similar to TLRs, the major outcome of NOD1 and NOD2 signaling pathways are the activation of NF-κB, MAPK-p38, MAPK-JNK, and MAPK-ERK, with the subsequent production of inflammatory factors (54) (**Figure 4**). Signaling through NOD pathways involves the recruitment of the receptor-interacting protein 2 (RIP2). The adaptor protein RIP2 is a serine/threonine protein kinase that possesses a C-terminal CARD domain that allows its interaction with NOD1 and NOD2 (55). The kinase activity of RIP2 is regulated by ubiquitination and phosphorylation. Ubiquitination

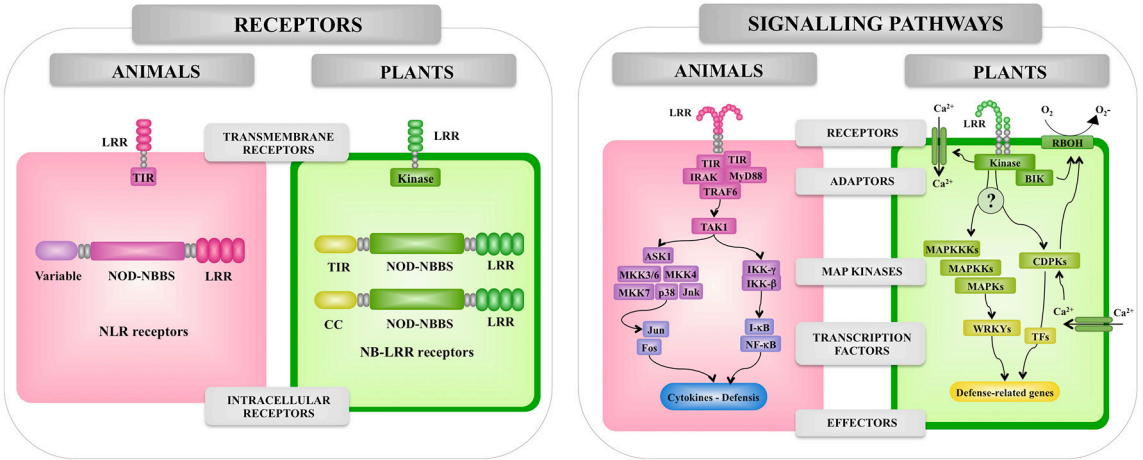


FIGURE 2 | Comparison of the pattern recognition receptors and signaling pathways involved in the recognition of pathogenic microorganisms by animal and plant cells. Receptors, adaptors, signaling pathways, and effectors involved in the response of animal and plant cells to microbes are shown. Leucine-rich repeat (LRR), Toll/interleukin-1 receptor (TIR), nucleotide-binding oligomerization domain (NOD), myeloid differentiation primary response 88 (MyD88), TNF receptor associated factor (TRAF), interleukin-1 receptor-associated kinase (IRAK), mitogen-activated protein kinase (MAPK), (MAPKK), mitogen-activated protein kinase (MAPKKK), WRKY transcription factor (WRKY), nuclear factor kappa B (NF-kB), calcium-dependent protein kinase (CDPK), transcription factor (TF). NADPH oxidases are designated as RBOH in plants.

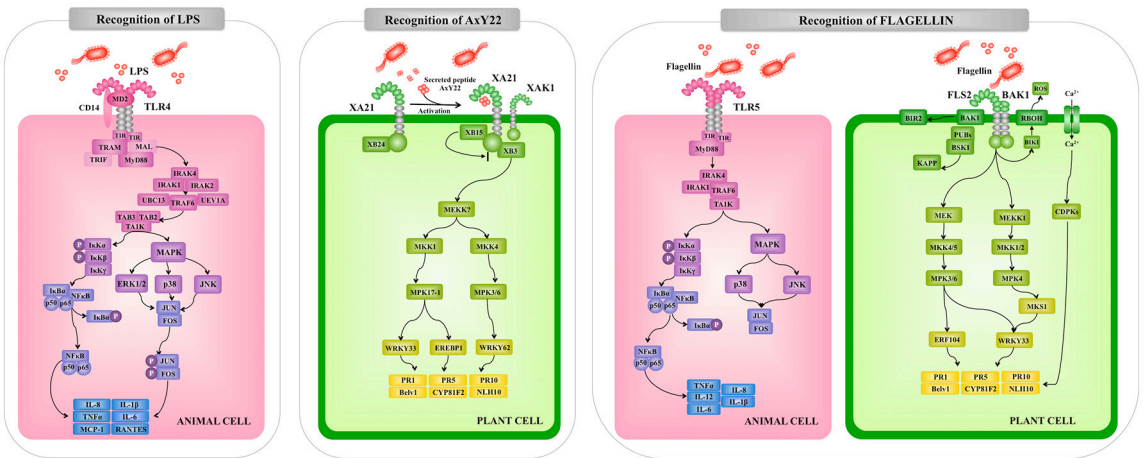


FIGURE 3 | Comparison of the extracellular pattern recognition receptors and signaling pathways involved in the recognition of pathogenic microorganisms by animal and plant cells. Recognition and signaling pathway mediated by animal Toll-like receptor (TLR)-4 and TLR5 and plant receptors XA21 and FLS2 are shown as examples of extracellular microbial recognition. Toll/interleukin-1 receptor (TIR), myeloid differentiation primary response 88 (MyD88), TNF receptor associated factor (TRAF), interleukin-1 receptor-associated kinase (IRAK), mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase (MKK), nuclear factor kappa B (NF-kB), tumor necrosis factor alpha (TNF-α), interleukin (IL), *Arabidopsis thaliana* receptor kinase FLS2 (FLS2), rice receptor kinase XA21 (XA21), reactive oxygen species (ROS), calcium-dependent protein kinase (CDPK), pathogenesis-related protein (PR).

of RIP2 induces the recruitment of TAK1 and the subsequent recruitment of IKK kinase complexes (IKK-α, IKK-β, and IKK-γ) leading to phosphorylation and degradation of IκBα (56).

Animal NLR proteins also participate in the formation of inflammasomes (**Figure 4**). The inflammasomes are multiprotein platforms with cytosolic sensors for a wide range of MAMPs or damage-associated molecular patterns (DAMPs) (57, 58). Inflammasomes participate in the defense against bacterial pathogens through the activation of inflammatory caspases. As mentioned before, flagellin can be recognized by the membrane expressed-TLR5. However, this MAMP can also be delivered into the cytosol by the secretion systems present in pathogenic bacteria such as the type III (T3SS) and type IV (T4SS) secretion systems present in *Salmonella typhimurium* or *Legionella pneumophila*, respectively. Studies have established that the neuronal apoptosis inhibitory protein (NAIP)-NLRC4 inflammasome plays a critical role in anti-bacteria defenses (58). In the NAIP-NLRC4 inflammasome, NAIPs are the cytosolic

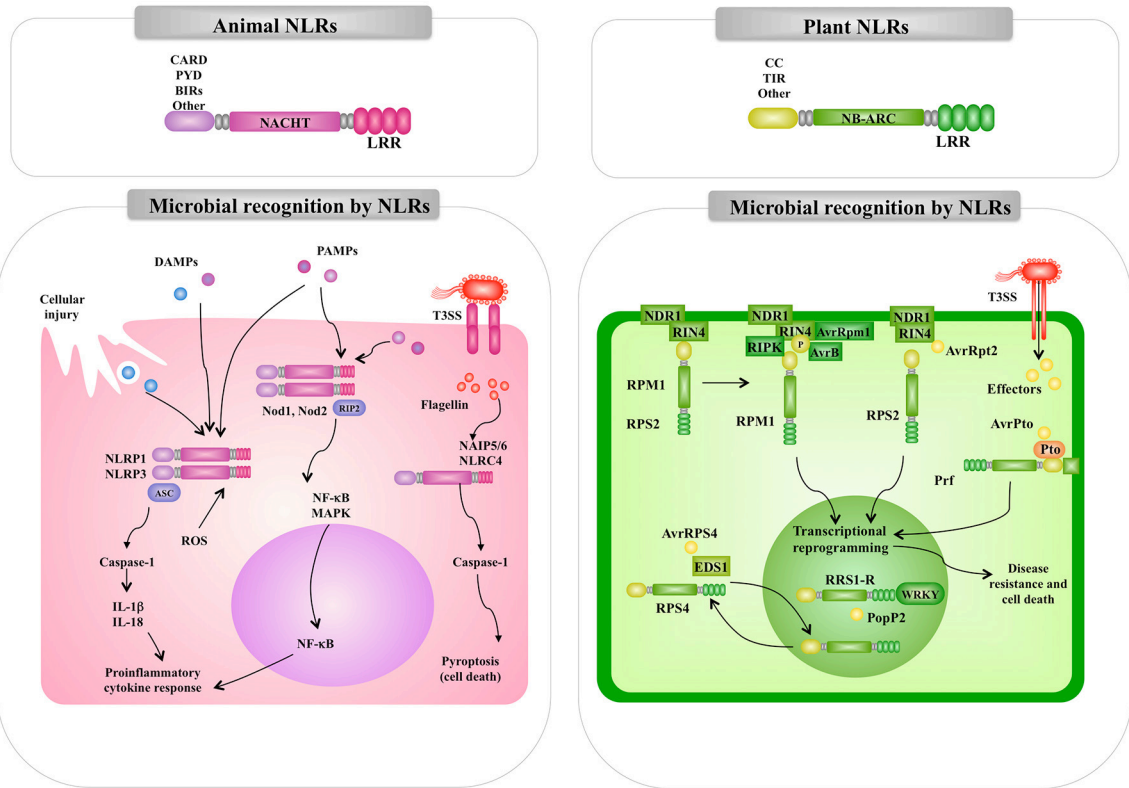


FIGURE 4 | Comparison of the intracellular pattern recognition receptors and signaling pathways involved in the recognition of pathogenic microorganisms by animal and plant cells. Recognition and signaling pathway mediated by animal and plant nucleotide binding domain (NBD) and leucine-rich repeat (LRR) superfamily proteins (NLR) are shown as examples of intracellular microbial recognition. Caspase-activated recruitment domains (CARD), central nucleotide-binding (NACHT), pathogen-associated molecular patterns (PAMPs), microbe-associated molecular patterns (MAMPs), danger-associated molecular patterns (DAMPs), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-kB), interleukin (IL), NLR family pyrin domain containing (NLPR), reactive oxygen species (ROS).

receptors for flagellin and secretion systems proteins while the NLRC4 is the adapter for caspase-1 activation (57). It has been established that component of flagella are sensed by NAIP5 and NAIP6, whereas components from bacterial secretion systems are detected by NAIP1 and NAIP2 (57). Inflammasome-dependent caspase-1 activation participates in the maturation and secretion of the inflammatory factors IL-1β and IL-18, and triggers a proinflammatory form of cell death referred as to pyroptosis. Several other animal NLR proteins are involved in inflammasomes formation and innate immune responses against bacterial pathogens [for a review see (59)].

Pattern Recognition Receptors for Bacteria in Plants

Plant cells encounter a variety of microbial-associated signals when interacting with microorganisms *in vivo*, and the plant's ability to recognize complex MAMPs is likely to determine its efficiency in inducing innate defense mechanisms. Various pathogenic Gram-negative bacteria harbor LPS and flagellin, which stimulate plant defenses (60). These MAMPs bind to PRRs and trigger the expression of immune response genes and the production of antimicrobial compounds (61).

Arabidopsis FLS2 and EFR, which can recognize a conserved 22-amino acid peptide sequence in bacterial flagellin and active epitope of bacterial elongation factor (EF)-Tu, respectively, are typical LRR-receptor-like kinases (RLKs) acting as PRRs (62, 63). Rice receptor kinase XA21 has been identified as a rice resistance gene product conferring race-specific resistance to *Xanthomonas oryzae* pv *oryzae* (64). It has been shown to perceive ax21 protein which seems to play a role in quorum sensing (65). Interestingly, the plant RLKs have structural and functional similarity to animal TLR proteins that can recognize MAMPs of bacterial pathogens in animals thereby inducing innate immunity (18). For example, FLS2 and TLR5 are equally perceptive to bacterial flagellin, suggesting that both PRRs are conserved by convergent evolution. Interestingly, bacterial *Pseudomonas* pathogens have been shown to evade animal and plant immunity through the activity of the protease AprA, which degrades flagellin monomers, therewith escaping detection by the host's immune system (66). On the other hand, the downstream signaling pathways activated by recognition of MAMPs through PRRs are diversified between plants and animals, while the production of ROS, transient increases of cytosolic Ca^{2+} levels followed by activation of calcium-dependent protein kinase (CDPKs), activation of MAPK cascades

and NO-mediated signaling seem to be common signaling events (**Figure 1**).

The best-characterized plant immune receptors are a large class of intracellular receptors often called NBS-LRR pathogen-resistance proteins, which have an overall tripartite structure similar to that of the mammalian NLR proteins (67). In general, plants have large families of these NLR proteins; *Arabidopsis* has 140 (68) and rice has over 500 (69). Most of the NLR pathogen-resistance proteins have either a TIR or a coiled-coil (CC) N-terminal domain (**Figure 4**). In contrast to the animal NOD1, NOD2, and NALP3 proteins, which respond to peptidoglycan degradation products, the plant NLR pathogen-resistance proteins directly or indirectly recognize pathogen effector molecules thereby activating downstream signaling pathways for induction of defense response.

It should be noted that a differential aspect between plant immune system and innate immune system in animals is that PRRs and NOD receptors equally contribute to recognize PAMPs/MAMPs in animals (**Figures 3**, **4**). However, the plant immune system consists of two-layered defense system: first PTI is mediated through recognition of MAMPs by PRRs and second ETI is mediated through recognition of pathogen effector molecules by NB-LRR receptors (**Figure 1**).

MODULATION OF THE IMMUNE SYSTEM BY BENEFICIAL MICROBES IN ANIMALS AND PLANTS

Today, the world faces the enormous challenge of improving the production of livestock and crops without the indiscriminate use of antimicrobials (70). Thus, alternative approaches are needed in order to satisfy the demands of the growing human population. Scientists have brought a different perspective to solve this problem and have emphasize on the exploitation of animal- and plant-associated microorganisms that are beneficial to their hosts through the modulation of the innate immune system.

Beneficial Microbes for Animals

Studies in humans and animals have shown that beneficial microbes in the gut are able to confer several health benefits (**Figure 5**), including the stimulation of intestinal epithelial cell proliferation, the reinforcement and maintenance of tight junctions, the expression of antimicrobial factors, and the modulation of the mucosal immune system (46, 71). It has been demonstrated that for most of these beneficial effects PRRs play a key role in the interaction of microbes with host cells (46, 71, 72).

It was reported that commensal bacteria, through the activation of TLR2, modulate the organization of tight junctions proteins (73), improve transepithelial resistance (74), and induce a rapid reshaping and stretching of epithelial cells after injury (75). In addition, activation of TLR2 and TLR4 in the animal gut have been shown to be involved in the expression of trefoil factor 3 (TFF3), epidermal growth factor receptor (EGFR), amphiregulin, and prostaglandin E2, which are important factors in wound healing and repair of the intestinal mucosa (75, 76). These studies demonstrated an important role of animal beneficial microbes in the maintenance of intestinal barrier function.

Antimicrobial peptides are constitutively produced in the animal gastrointestinal tract. However, their expression can be improved by commensal MAMPs through the activation of TLRs or NLRs (77). Antimicrobial compounds, such as regenerating islet-derived 3 (Reg3)-β protein, Reg3-γ, CRP-ductin, resistin-like molecule-β, and β-defensins are induced in intestinal epithelial cells and Paneth cells by microbial products through MyD88-dependent signaling (78–80). Interestingly, it was recently reported that the metabolic activity of intestinal microbiota also influences the production of antimicrobial factors (81). The study demonstrated that short-chain fatty acids produced by microbiota promoted the production of Reg3-γ and β-defensins in intestinal epithelial cells. The effect of short-chain fatty acids were dependent on G protein-coupled receptor (GPR), mammalian target of rapamycin (mTOR), and signal transducer and activator of transcription protein (STAT)-3 signaling. The data thereby provided a novel pathway by which animal beneficial microbes modulate the expression of antimicrobial compounds in the gut (81).

On the other hand, a plethora of evidence supports the active role of commensal bacteria in the development and maintenance of intestinal immune homeostasis in the animal host (21, 82, 83). The molecular communication between microbes and intestinal epithelial cells, and the role of this interaction in the promotion of immune homeostasis have been subjects of intense research (21). PRR signal transduction triggered by pathogens induce proinflammatory responses by intestinal epithelial cells that significantly influence the behavior of the underlying lamina propria immune cells. This PRR signaling is crucial to protect the animal hosts against infections (80, 84). In addition, intestinal epithelial cells are able to sense commensal and beneficial microbes that help to maintain immune status and inhibit excessive inflammation. Products derived from animal intestinal microbiota activate TLRs in intestinal epithelial cells, and increase the expression of negative regulators of the TLRs signaling pathway such as IRAK-M, TOLLIP, SIGIRR, A20, and peroxisome proliferator-activated receptor-γ (PPARγ). Through this mechanism, animal beneficial microbes help to control intestinal inflammatory responses (85, 86). Microbial products also stimulate the expression of proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) (87). Both, APRIL and BAFF promote IgA class-switching responses in the intestine and are involved in the maintenance of the appropriate levels of secretory IgA antibodies that protect mucosal surfaces. In addition, interactions of animal beneficial microbes with intestinal epithelial cells modulate the function of antigen presenting cells. Intestinal dendritic cells are influenced by factors produced by the intestinal epithelium including transforming growth factor (TGF)-β, retinoic acid and thymic stromal lymphopoietin to acquire a tolerogenic phenotype (88).

Scientists have isolated and select specific microbial strains in order to improve immune functions in human and animals. The strains used to improve the health of human and animals through the modulation of the immune system are referred to as immunomodulatory probiotics or immunobiotics (46, 71,

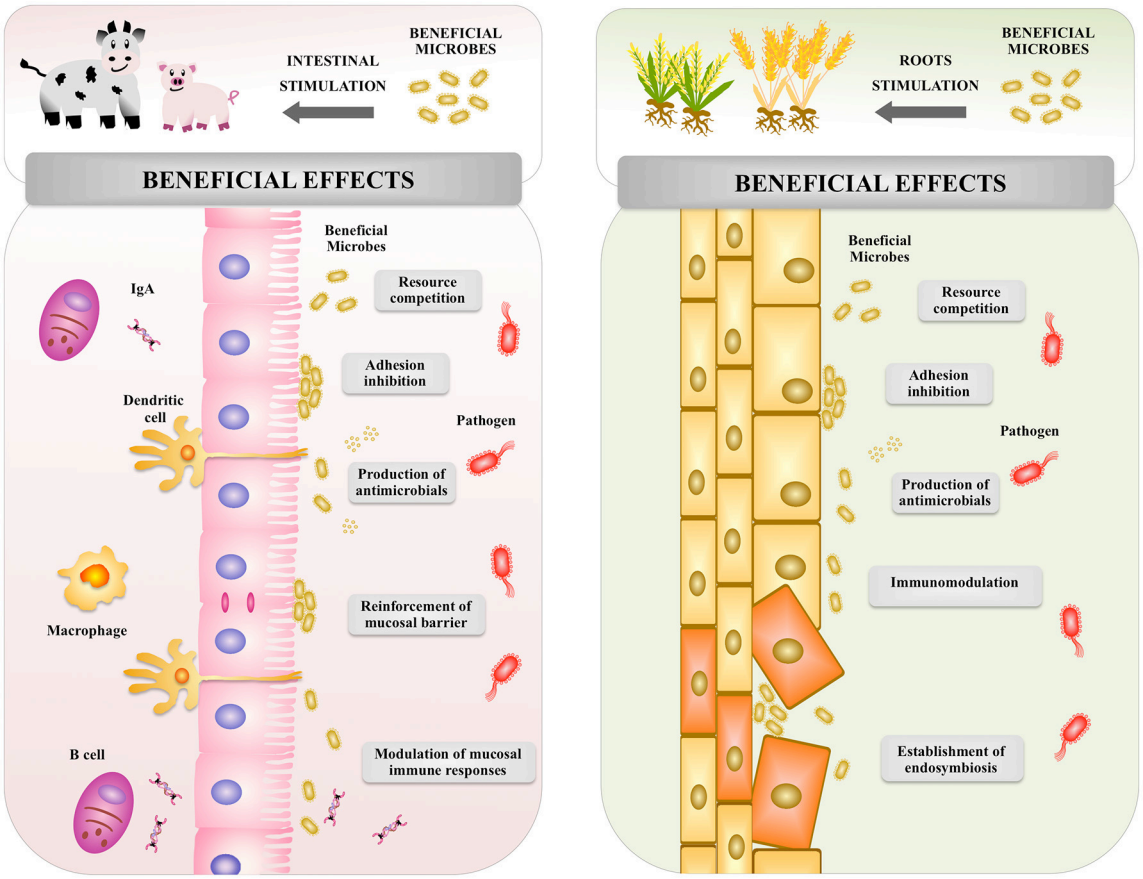


FIGURE 5 | Global overview of the effect of beneficial microbes on animal and plant hosts. Beneficial microbes in both animal and plants are able to increase resistance to pathogenic microorganisms by using similar mechanisms: competition for nutrients, inhibition of adhesion to host's cells, production of antimicrobial molecules, and modulation of host's immune system.

72, 89, 90). Studies from the last decades have shown that immunobiotics are able to beneficially modulate the intestinal immune system of human (90, 91), porcine (89, 90, 92), and bovine (46, 72) hosts. Immunobiotics allow an efficient control of inflammatory responses in the gut and an improved protection against infectious diseases.

In addition, researchers have demonstrated the immunomodulatory beneficial effect of commensal microbes and immunobiotics can be extended beyond the intestinal tract. It was reported that orally administered immunobiotics are able to differentially modulate immune responses in distal mucosal tissues such as the respiratory tract (93, 94) or mammary glands (95). The release of microbial immunomodulatory molecules in the intestine that are transported to distal sites (11, 96, 97); the mobilization of immune cells from the gut into the blood and distal tissues (98–100), and the systemic metabolic reprogramming that induce the production of immunomodulatory metabolites (101) have been proposed to explain the effects of gut animal beneficial microbes on systemic and distal mucosal tissue responses.

Beneficial Microbes for Plants

The interaction of plants with particular microorganisms can be beneficial (**Figure 5**). Microbial populations that influence the growth, development, and health of plants can be found below and above the ground, as well as within the plant (102). Commensal and mutualistic microbes are able to provide plants essential benefits including nitrogen fixation, enhanced mineral uptake, and growth promotion (103). Microbes also help plants to resist a variety of stresses, including toxins, heavy metals, drought, salinity, and extreme temperature (70). Of interest, plants and microbes interaction modulates the plant innate immune system and improves protection from pathogens (104–106).

Pseudomonas simiae WCS417r (formerly known as *Pseudomonas fluorescens* WCS417r) is a non-pathogenic rhizobacterial strain that colonizes the rhizosphere in the regions where plants produce exudates and lysates (107). This strain is capable to suppress soil-borne diseases caused by infection with a broad range of pathogens (108). Beneficial rhizobacteria strains have the potential to reduce the incidence of infectious diseases through the activation of a plant-mediated

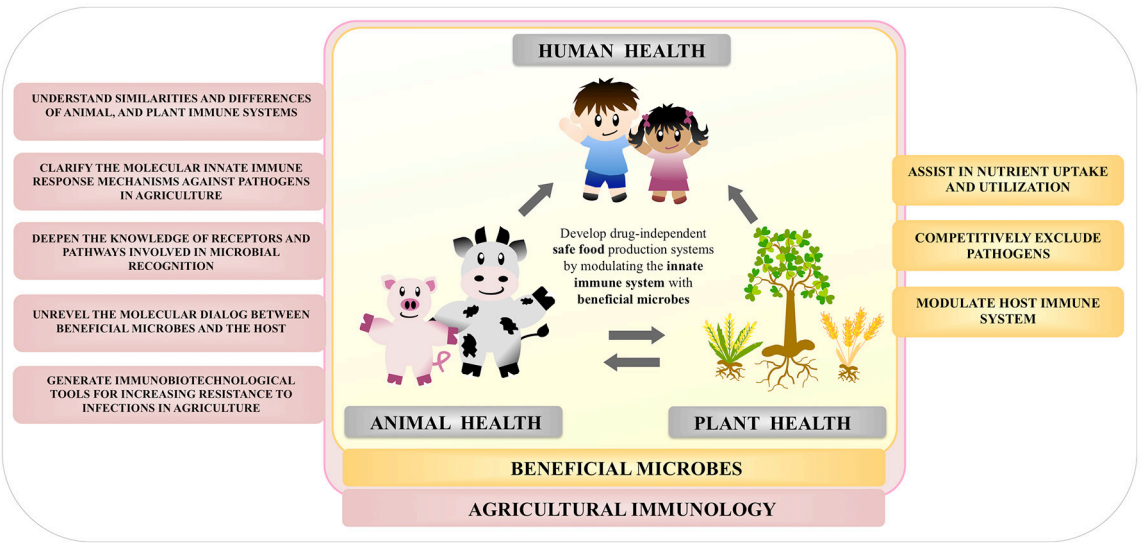


FIGURE 6 | Overview of the importance of the study of beneficial microbes within the field of agricultural immunology.

defense system termed "induced systemic resistance" (ISR) (109). *Arabidopsis* activation of ISR by treatment of the roots with *P. simiae* WCS417r is not accompanied by salicylic acid (SA)-responsive PR-protein gene expression, indicating that WCS417r-mediated ISR functions independently of this plant hormone (110). This is in contrast to findings in other plant species including rice, tobacco, cucumber and tomato where SA-independent ISR has been demonstrated (11, 111, 112). Indeed, a series of studies on signaling pathways required for *P. simiae* WCS417r-mediated ISR using *Arabidopsis* mutants indicated that jasmonic acid (JA)- and ethylene (ET)-dependent signaling play a central role in the regulation of ISR (113–115). Indeed, ET accumulation is a well-known response to MAMP recognition (28). Furthermore, transcriptional co-activator NPR1 and the root-specific transcriptional regulator MYB72 have also been implicated in JA/ET-dependent ISR by *P. simiae* WCS417r (113, 116, 117). Recently, it was shown that MYB72 plays an important role in the rhizobacteria-induced excretion of antimicrobial coumarins that shape the assembly of the microbiome in the rhizosphere, potentially to optimize associations with ISR-inducing rhizobacteria (118).

While colonization of the roots by rhizobacteria is not generally accompanied with up- or down-regulation of defense-related gene expression or increases in the production of JA and ET, plants have enhanced defensive capacity to a broad-range of pathogens. This enhanced ability to induce basal defense system is termed "priming" (119, 120). Global gene expression analysis of *P. simiae* WCS417r-mediated ISR revealed that JA- and/or ET-responsive defense-related gene were primed for enhanced expression in response to challenge infection by the phytopathogenic bacteria *P. syringae* (121–123). In *Arabidopsis*, the levels of transcription factors of the AP2/ERF family including MYC2 are especially increased in the ISR-primed state (124). Since rhizobacteria-mediated ISR was compromised in MYC2-muagenized *jin1 Arabidopsis* mutants (125), MYC2 seems to play a key role to regulate priming during ISR. Furthermore, epigenetic regulation of defense-related gene expression including DNA methylation and chromatin re-modulation seems to be associated with the priming phenomenon (126, 127). However, the relation of rhizobacteria colonization and epigenetic regulator mechanism remains to be investigated.

Other beneficial non-pathogenic microorganisms including bacteria, fungi, and oomycetes including *Bacillus* spp. *Serratia liquefaciens*, *Penicillum* spp. *Phoma* spp. *Trichderma* spp. and *Pythium oligandrum* also induce systemic resistance including ISR to a broad range of pathogens through activation of SA, JA, or ET-responsive defense-related genes in plants. (128–133). In this regard, when tomato roots are treated with a mycelial homogenate of the non-pathogenic oomycete *P. oligandrum*, bacterial wilt disease caused by *Ralstonia solanacearum* is suppressed (134). In addition, the treatment of tomato root cells with *P. oligandrum* elicitin also induced JA/ET-responsive defense-related gene expression and inhibited the occurrence of bacterial wilt disease (132–134). Moreover, this response can be also obtained in *jai1* mutants in which JA-signaling pathway is impaired (112). Thus, the elicitin of *P. oligandrum* seems to be recognized as MAMP by tomato cells thereby activating PTI. Hence, activation of defense system mediated by recognizing MAMPs of beneficial microorganisms seems to contribute their disease suppressive activity.

Recently, it was reported that insect pathogens colonizing the surface of plant leaves or natural soils have the potential to activate the plant defense system (135). *Bacillus thuringiensis* is a well-known pathogen that causes disease in caterpillars of various types of moths and butterflies by producing δ-endotoxins. The treatment of tomato roots with cell-free culture filtrate medium of *B. thuringiensis* suppressed bacterial wilt disease caused by *R.*

solanacearum with systemic induction of ET-responsive defense-related gene expression in tomato (135–137). The cell-free culture filtrate medium of the fungal pathogens *Paecilomyces tenuipes* and *Beauveria bassiana* are also able to induce ET-responsive defense-related gene expression in tomato roots and suppress bacterial wilt disease (Takahashi et al., unpublished results). Hence, insect pathogen-mediated activation of plant defense system to pathogens seems to be consistent with a potential role of insect pathogens to protect plants against the attack of plant pathogens in nature, which has been proposed as the "Bodyguard hypothesis" (138).

Since activation of PTI in plants by recognizing MAMPs of non-pathogenic microorganisms seems to be widely distributed phenomenon (16), PTI would contribute to reduce disease incidence through the defense system activated by environmental microorganisms in nature. In recent years, research on harnessing beneficial functions of members of the plant microbiome to make them useful in sustainable crop protection emerged as one of the frontiers in plant science research (103, 139–141). Besides MAMPs, many other microbe-associated small molecules have been identified as being important for beneficial host-microbe interactions (118), providing useful tools for sustainable protection of future crops.

PERSPECTIVE OF HEALTHY GROWTH STRATEGY BY BENEFICIAL MICROBES IN ANIMALS AND PLANTS

Modern animal and crop production practices are associated with the regular use of antimicrobials, potentially increasing selection pressure on bacteria to become resistant. Considering the global intention of organizations to significantly reduce the use of antimicrobials in agriculture, the need for novel strategies to improve resistance of animal and plants against pathogens became a top priority. Agricultural Immunology is a developing research field that fuses animal, marine, and plant immunology, all of which have been previously regarded as separate topics. Agricultural Immunology therefore aims to plant the seeds for developing drug-independent safe food production systems by modulating animal and plant innate immune system (**Figure 6**).

Deciphering animal–microbe and plant–microbe interactions is a promising aspect to understand the benefits and the pathogenic effect of microbes in the agricultural field. The advancement in sequencing technologies and various "omics" tool has impressively accelerated the research in biological sciences in this area. The development of new techniques in the post–genomic era has greatly enhanced our understanding of the regulation of animal and plant defense mechanisms against pathogens, and also their interaction with beneficial microbes. Thus, animal–microbe and plant–microbe associations can now be studied at a speed and depth as never before. However, a major gap in our knowledge is how recognition of beneficial microbes at the gut or root-soil interface drives the whole animal or plant body toward enhanced growth and elevated stress resistance. The first steps toward unraveling the molecular dialog between hosts and beneficial microbes eliciting distal immunological effects have been made, but major questions still need to be resolved.

The aim of the "agricultural immunobiotic approach" is to repair the deficiencies in the microbiota and restore the host's resistance to disease through the use of beneficial immunomodulatory microorganisms. Such treatments do not introduce any foreign chemicals into the animal gut or plant root and does not run the risk of contaminating and introducing hazardous chemicals into the food chain. We hope to convey the enthusiasm of this rapidly advancing field as an area of active basic and applied research that is at the cusp of exploitation to address pressing plant and animal health problems worldwide.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by a Grant-in-Aid for Scientific Research (B)(2) (16H05019) and Core-to-Core Program, A. Advanced Research Networks entitled Establishment of international agricultural immunology research-core for a quantum improvement in food safety from the Japan Society for the Promotion of Science (JSPS). This study was also supported by grants for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Science, Sports and Technology (MEXT) of Japan (16H06429, 16K21723, and 16H06435).

REFERENCES

1. Giske CG, Monnet DL, Cars O. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother.* (2008) 52:813–21. doi: 10.1128/AAC.01169-07
2. Birgand G, Zahar JR, Lucet JC. Insight into the complex epidemiology of multidrug-resistant *Enterobacteriaceae*. *Clin Infect Dis.* (2018) 66:494–6. doi: 10.1093/cid/cix826
3. Cho JH, Zhao PY, Kim IH. Probiotics as a dietary additive for pigs. *J Animal Vet Adv.* (2011) 10:2127–34. doi: 10.3923/javaa.2011.2127.2134
4. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci USA*. (2015) 112:5649–54. doi: 10.1073/pnas.1503141112
5. Yu Z, Gunn L, Wall P, Fanning S. Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production. *Food Microbiol.* (2017) 64:23–32. doi: 10.1016/j.fm.2016.12.009
6. McManus PS, Stockwell VO, Sundin GW, Jones AL. Antibiotic use in plant agriculture. *Annu Rev Phytopathol.* (2002) 40:443–65. doi: 10.1146/annurev.phyto.40.120301.093927
7. Bueno I, Williams-Nguyen J, Hwang H, Sargeant JM, Nault AJ, Singer RS. Systematic review: impact of point sources on antibiotic-resistant bacteria in the natural environment. *Zoonoses Public Health* (2018) 65:e162–84. doi: 10.1111/zph.12426
8. Graham JP, Evans SL, Price LB, Silbergeld EK. Fate of antimicrobial-resistant enterococci and staphylococci and resistance determinants in stored poultry litter. *Environ Res.* (2009) 109:682–9. doi: 10.1016/j.envres.2009.05.005

9. Price LB, Johnson E, Vailes R, Silbergeld E. Fluoroquinolone-resistant *Campylobacter* isolates from conventional and antibiotic-free chicken products. *Environ Health Perspect*. (2005) 113:557–60. doi: 10.1289/ehp.7647
10. Smith TC, Gebreyes WA, Abley MJ, Harper AL, Forshey BM, Male MJ, et al. Methicillin-resistant *Staphylococcus aureus* in pigs and farm workers on conventional and antibiotic-free swine farms in the USA. *PLoS ONE* (2013) 8:e63704. doi: 10.1371/journal.pone.0063704
11. You Y, Silbergeld EK. Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion. *Front Microbiol*. (2014) 5:284. doi: 10.3389/fmicb.2014.00284
12. Hughes P, Heritage J. Antibiotic growth-promoters in food animals. In: Jutzi S, editor. *Assessing Quality and Safety of Animal Feeds*. Rome: Food and Agriculture Organization of the United Nations (2002). p. 129.
13. Simon O. Micro-organisms as feed additives–probiotics. *Adv Pork Produc*. (2005) 16:2161. Available Online at: https://www.banffpork.ca/documents/BO07-SimonO.pdf
14. Piva A, Galvano F, Biagi G, Casadei G. Intestinal Fermentation: dietary and microbial interactions. In: Mosenthin R, Zentek J, Ebrowska TZ, editors. *Biology of Nutrition in Growing Animals*. Philadelphia, PA: Elsevier (2006). p. 1–31.
15. Jones JDG, Vance RE, Dangl JL. Intracellular innate immune surveillance devices in plants and animals. *Science* (2016) 354:aaf63951–8 doi: 10.1126/science.aaf6395
16. Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol*. (2005) 6:973–9. doi: 10.1038/ni1253
17. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol*. (2002) 14:103–10. doi: 10.1016/S0952-7915(01)00304-1
18. Ronald PC, Beutler B. Plant and animal sensors of conserved microbial signatures. *Science* (2010) 330:1061–4. doi: 10.1126/science.1189468
19. Jonak C, Okresz L, Bogre L, Hirt H. Complexity, cross talk and integration of plant MAP kinase signalling. *Curr Opin Plant Biol*. (2002) 5:415–24. doi: 10.1016/S1369-5266(02)00285-6
20. Barton GM, Medzhitov R. Toll-like receptor signalling pathways. *Science* (2003) 300:1524–5. doi: 10.1126/science.1085536
21. Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Vallance BA. The intestinal epithelium: central coordinator of mucosal immunity. *Trends Immunol*. (2018) 39:677–96. doi: 10.1016/j.it.2018.04.002
22. Eyerich S, Eyerich K, Traidl-Hoffmann C, Biedermann T. Cutaneous barriers and skin immunity: differentiating a connected network. *Trends Immunol*. (2018) 39(4):315–27. doi: 10.1016/j.it.2018.02.004
23. Imam J, Singh PK, Shukla P. Plant microbe interactions in post genomic era: perspectives and applications. *Front Microbiol*. (2016) 7:1488. doi: 10.3389/fmicb.2016.01488
24. Melotto M, Underwood W, Koczan J, Nomura K, He SY. Plant stomata function in innate immunity against bacterial invasion. *Cell* (2006) 126:969–80. doi: 10.1016/j.cell.2006.06.054
25. Alfano JR, Collmer A. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu Rev Phytopathol*. (2004) 42:385–414 doi: 10.1146/annurev.phyto.42.040103.110731
26. Peeters N, Guidot A, Vailleau F, Valls M. *Ralstonia solanacearum*, a widespread bacterial plant pathogen in the post-genomic era. *Mol Plant Pathol*. (2013) 14:651–62. doi: 10.1111/mpp.12038
27. Yadeta KA, Thomma BP. The xylem as battleground for plant hosts and vascular wilt pathogens. *Front Plant Sci*. (2013) 4:97. doi: 10.3389/fpls.2013.00097
28. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol*. (2009) 60:379–406. doi: 10.1146/annurev.arplant.57.032905.105346
29. Pelt MJC, Pieterse CMJ. Microbial recognition and evasion of host immunity. *J Exp Bot*. (2013) 64:1237–48. doi: 10.1093/jxb/ers262
30. Alvarez ME. Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant Mol Biol*. (2000) 44:429–42. doi: 10.1023/A:1026561029533
31. Bolwell GP, Bindschedler LV, Blee KA, Butt VS, Davies DR, Gardner SL, et al. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *J Exp Bot*. (2002) 53:1367–76. doi: 10.1093/jxb/53.372.1367
32. Kombrink E, Somssich IE. Pathogenesis-related proteins and plant defense. In: Carroll GC, Tudzynski P, editors. *Plant Relationships. Part A*. Berlin-Heidelberg: Springer-Verlag (1997). p. 107–28.
33. Pfeilmeier S, Caly DL, Malone JG. Bacterial pathogenesis of plants: future challenges from a microbial perspective: challenges in bacterial molecular plant pathology. *Mol Plant Pathol*. (2016) 17:1298–313. doi: 10.1111/mpp.12427
34. Win J, Chaparro-Garcia A, Belhaj K, Saunders DGO, Yoshida K, Dong S. Effector biology of plant-associated organisms: concepts and perspectives. *Cold Spring Harb Sym Quant Biol*. (2012) 77:235–47. doi: 10.1101/sqb.2012.77.015933
35. Roux F, Voisin D, Badet T, Balagué C, Barlet X, Huard-Chauveau C. Resistance to phytopathogens e tutti quanti: placing plant quantitative disease resistance on the map. *Mol Plant Pathol*. (2014) 15:427–32. doi: 10.1111/mpp.12138
36. Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J*. (2000) 24:667–77. doi: 10.1046/j.1365-313x.2000.00911.x
37. Jacob F, Vernaldi S, Maekawa T. Evolution and conservation of plant NLR functions. *Front Immunol*. (2013) 4:297. doi: 10.3389/fimmu.2013.00297
38. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol*. (2005) 17:1–14. doi: 10.1093/intimm/dxh186
39. Hornung, V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* (2009) 458:514–51. doi: 10.1038/nature07725
40. Belvin MP, Anderson KV. A conserved signaling pathway: the Drosophilatoll-dorsal pathway. *Annu Rev Cell Dev Biol*. (1996) 12:393–416. doi: 10.1146/annurev.cellbio.12.1.393
41. Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. *Nature* (1991) 351:355–6. doi: 10.1038/351355b0
42. Sun SC, Lindstrom I, Lee JY, Faye I. Structure and expression of the attacin genes in *Hyalophora cecropia*. *Eur J Biochem*. (1991) 196:247–54. doi: 10.1111/j.1432-1033.1991.tb15811.x
43. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* (1997) 388:394–97. doi: 10.1038/41131
44. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in tlr4 gene. *Science* (1998) 282:2085–8. doi: 10.1126/science.282.5396.2085
45. Kawai T, Akira S. TLR signaling. *Cell Death Differ* (2006) 13:816–25. doi: 10.1038/sj.cdd.4401850
46. Villena J, Aso H, Kitazawa H. Regulation of toll-like receptors-mediated inflammation by immunobiotics in bovine intestinal epitheliocytes: role of signaling pathways and negative regulators. *Front Immunol*. (2014) 4:1–12. doi: 10.3389/fimmu.2014.00421
47. Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. *J Int Med*. (2007) 261:511–28. doi: 10.1111/j.1365-2796.2007.01821.x
48. Athman R, Philpott D. Innate immunity via Toll-like receptors and Nod proteins. *Curr Opin Microbiol*. (2004) 7:25–32. doi: 10.1016/j.mib.2003.12.013
49. Fritz JH, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol*. (2006) 7:1250–7. doi: 10.1038/ni1412
50. Lecine P, Esmiol S, Metais JY, Nicoletti C, Nourry C, McDonald C, et al. The NOD2-RICK complex signals from the plasma membrane. *J Biol Chem*. (2007) 282:15197–207. doi: 10.1074/jbc.M606242200
51. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhães JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*. (2010) 11:55–62. doi: 10.1038/ni.1823
52. Takahashi Y, Isuzugawa K, Murase Y, Imai M, Yamamoto S, Iizuka M, et al. Up-regulation of NOD1 and NOD2 through TLR4 and TNF-α

in LPS-treated murine macrophages. *J Vet Med Sci*. (2006) 68:471–8. doi: 10.1292/jvms.68.471
53. Boyle JP, Mayle S, Parkhouse R, Monie TP. Comparative genomic and sequence analysis provides insight into the molecular functionality of NOD1 and NOD2. *Front Immunol*. (2013) 4:317. doi: 10.3389/fimmu.2013.00317
54. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol*. (2006) 6:9–20. doi: 10.1038/nri1747
55. Zhang D, Lin J, Han J. Receptor-interacting protein (RIP) kinase family. *Cell Mol Immunol*. (2010) 7:243–9 doi: 10.1038/cmi.2010.10
56. Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Núñez G, Inohara N. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation. *EMBO J*. (2008) 27:373–83. doi: 10.1038/sj.emboj.7601962
57. Lage SL, Longo C, Branco LM, da Costa TB, Buzzo Cde L, Bortoluci KR. Emerging Concepts about NAIP/NLRC4 inflammasomes. *Front Immunol*. (2014) 5:309. doi: 10.3389/fimmu.2014.00309
58. Zhao Y, Shao F. The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. *Immunol Rev*. (2015) 265:85–102. doi: 10.1111/imr.12293
59. Man SM, Kanneganti TD. Converging roles of caspases in inflammasome activation, cell death and innate immunity. *Nat Rev Immunol*. (2016) 16:7–21. doi: 10.1038/nri.2015.7
60. Lee J, Klessig DF, Nürnberger T. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell* (2001) 13:1079–93. doi: 10.1105/tpc.13.5.1079
61. Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, et al. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* (2001) 291:1544–7. doi: 10.1126/science.291.5508.1544
62. Gomez-Gomez L, Boller T. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* (2000) 5:1003–11. doi: 10.1016/S1097-2765(00)80265-8
63. Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* (2006) 125:749–60. doi: 10.1016/j.cell.2006.03.037
64. Song WY, Wang GL, Chen LL, Kim HS. A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* (1995) 270:1804–6. doi: 10.1126/science.270.5243.1804
65. Lee SW, Han SW, Sririyanum M, Park CJ, Seo YS, Ronald PC. A type I–secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* (2009) 326:850–3. doi: 10.1126/science.1173438
66. Bardoel BW, Van der Ent S, Pel MJC, Tommassen J, Pieterse CMJ, Van Kessel KPM, et al. *Pseudomonas* evades immune recognition of flagellin in both mammals and plants. *PLoS Pathog.* (2011) 7:e1002206. doi: 10.1371/journal.ppat.1002206
67. Holt BF, Hubert DA, Dangl JL. Resistance gene signaling in plants–complex similarities to animal innate immunity. *Curr Opin Immunol*. (2003) 15:20–5. doi: 10.1016/S0952-7915(02)00014-6
68. Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW. Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. *Plant Cell* (2003) 15:809–34. doi: 10.1105/tpc.009308
69. Zhou T, Wang Y, Chen JQ, Araki H, Jing Z, Jiang K, et al. Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. *Mol Genet Genomics* (2004) 271:402–15. doi: 10.1007/s00438-004-0990-z
70. Reid A. Microbes helping to improve crop productivity. *Microbe* (2011) 6:435–9. doi: 10.1128/microbe.6.435.1
71. Villena J, and Kitazawa H. Role of Toll-like receptors in the modulation of intestinal inflammation by immunobiotics. In: Kitazawa H, Villena J, Alvarez S, editors. *Probiotics: Immunobiotics and Immunogenics.* Boca Raton, FL: Science Publishers; CRC Press; Taylor & Francis Group Company (2013). p. 89–127.
72. Villena J, Aso H, Rutten VPMG, Takahashi H, van Eden W, Kitazawa H. Immunobiotics for the bovine host: their interaction with intestinal epithelial cells and their effect on antiviral immunity. *Front. Immunol.* (2018) 9:326. doi: 10.3389/fimmu.2018.00326
73. Cario E, Gerken G, Podolsky PK. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology* (2004) 127:224–38. doi: 10.1053/j.gastro.2004.04.015
74. Gibson DL, Ma C, Rosenberger CM, Bergstrom KS, Valdez Y, Huang JT, et al. Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during *Citrobacter rodentium*-induced colitis. *Cell Microbiol*. (2008) 10:388–40. doi: 10.1111/j.1462-5822.2007.01052.x
75. Podolsky DK, Gerken G, Eyking A, Cario E. Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. *Gastroenterology* (2009) 137:209–20. doi: 10.1053/j.gastro.2009.03.007
76. Giraud ASX. Trefoil peptide and EGF receptor/ ligand transgenic mice. *Am J Physiol Gastrointest Liver Physiol.* (2000) 278:G501–6. doi: 10.1152/ajpgi.2000.278.4.G501
77. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*. (2003) 3:710–20. doi: 10.1038/nri1180
78. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA*. (2008) 105:20858–63. doi: 10.1073/pnas.0808723105
79. Vora P, Youdim A, Thomas LS, Fukata M, Tesfay SY, Lukasek K, et al. Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J Immunol*. (2004) 173:5398–405. doi: 10.4049/jimmunol.173.9.5398
80. Wells JM, Loonen LM, Karczewski JM. The role of innate signaling in the homeostasis of tolerance and immunity in the intestine. *Int J Med Microbiol*. (2010) 300:41–8. doi: 10.1016/j.ijmm.2009.08.008
81. Zhao Y, Chen F, Wu W, Sun M, Bilotta AJ, Yao S, et al. GPR43 mediates microbiota metabolite SCFA regulation of antimicrobial peptide expression in intestinal epithelial cells via activation of mTOR and STAT3. *Mucosal Immunol*. (2018) 11:752–62. doi: 10.1038/mi.2017.118
82. Hand TW. The role of the microbiota in shaping infectious immunity. *Trends Immunol.* (2016) 37:647–58. doi: 10.1016/j.it.2016.08.007
83. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. *Nat Rev Immunol.* (2017) 17:219–32. doi: 10.1038/nri.2017.7
84. Abreu MT. Toll-like receptor signaling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol*. (2010) 10:131–44. doi: 10.1038/nri2707
85. Shibolet O, Podolsky DK. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. *Am J Physiol Gastrointest Liver Physiol*. (2007) 292:G1469–73. doi: 10.1152/ajpgi.00531.2006
86. Xiao H, Gulen MF, Qin J, Yao J, Bulek K, Kish D, et al. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity* (2007) 26:461–75. doi: 10.1016/j.immuni.2007.02.012
87. Cerutti A, Rescigno M. The biology of intestinal immunoglobulin A responses. *Immunity* (2008) 28:740–50. doi: 10.1016/j.immuni.2008.05.001
88. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. *In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* (2001) 413:639. doi: 10.1038/35098100
89. Villena J, Kitazawa H. Editorial: immunobiotics-interactions of beneficial microbes with the immune system. *Front Immunol.* (2017) 8:1580. doi: 10.3389/fimmu.2017.01580
90. Villena J, Vizoso Pinto MG, Kitazawa H. Intestinal innate antiviral immunity and immunobiotics: beneficial effects against rotavirus infection. *Front Immunol.* (2016) 7:563. doi: 10.3389/fimmu.2016.00563
91. Carvalho RDDO, do Carmo FLR, de Oliveira Junior A, Langella P, Chatel J-M, Bermúdez-Humarán LG, et al. Use of wild type or recombinant lactic acid bacteria as an alternative treatment for gastrointestinal inflammatory diseases: a focus on inflammatory bowel diseases and mucositis. *Front Microbiol.* (2017) 8:800. doi: 10.3389/fmicb.2017.00800
92. Valeriano VD, Balolong MP, Kang DK. Probiotic roles of *Lactobacillus* sp. in swine: insights from gut microbiota. *J Appl Microbiol*. (2017) 122:554–67. doi: 10.1111/jam.13364
93. Zelaya H, Alvarez S, Kitazawa H, Villena J. Respiratory antiviral immunity and immunobiotics: beneficial effects on inflammation-coagulation interaction during influenza virus infection. *Front Immunol.* (2016) 7:633. doi: 10.3389/fimmu.2016.00633

94. Kitazawa H, Villena J. Modulation of respiratory TLR3-antiviral response by probiotic microorganisms: lessons learned from *Lactobacillus rhamnosus* CRL1505. Special issue: Toll-like receptor activation in immunity vs. tolerance. *Front Immunol.* (2014) 5:1–15. doi: 10.3389/fimmu.2014.00201
95. Cross ML. Immune-signalling by orally delivered probiotic bacteria: effects on common mucosal immunoresponses and protection at distal mucosal sites. *Int J Immunopathol Pharmacol.* (2004) 17:127–34. doi: 10.1177/039463200401700204
96. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med.* (2010) 16:228–31. doi: 10.1038/nm.2087
97. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci USA.* (2011) 108:5354–9. doi: 10.1073/pnas.1019378108
98. Kikuchi Y, Kunitoh-Asari A, Hayakawa K, Imai S, Kasuya, K., Abe K, et al. Oral administration of *Lactobacillus plantarum* strain AYA enhances IgA secretion and provides survival protection against influenza virus infection in mice. *PLoS ONE* (2014) 9:e86416. doi: 10.1371/journal.pone.0086416
99. Salva S, Villena, J, Alvarez S. Immunomodulatory activity of *Lactobacillus rhamnosus* strains isolated from goat milk: impact on intestinal and respiratory infections. *Int J Food Microbiol.* (2010) 141:82–9. doi: 10.1016/j.ijfoodmicro.2010.03.013
100. Villena J, Chiba E, Tomosada Y, Salva S, Marranzino G, Kitazawa H, et al. Orally administered *Lactobacillus rhamnosus* modulates the respiratory immune response triggered by the viral pathogen-associated molecular pattern poly(I:C). *BMC Immunol.* (2012) 13:53. doi: 10.1186/1471-2172-13-53
101. Fonseca W, Lucey K, Jang S, Fujimura KE, Rasky A, Ting HA, et al. *Lactobacillus johnsonii* supplementation attenuates respiratory viral infection via metabolic reprogramming and immune cell modulation. *Mucosal Immunol.* (2017) 10:1569–80. doi: 10.1038/mi.2017.13
102. Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ. Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends Microbiol.* (2010) 18:365–73. doi: 10.1016/j.tim.2010.06.002
103. Bakker PAHM, Pieterse CMJ, De Jonge R, Berendsen RL. The soil-borne legacy. *Cell* (2018) 172:1178–80. doi: 10.1016/j.cell.2018.02.024
104. Mendes R, Kruijt M, De Bruijn I, Dekkers E, Van der Voort M, Schneider JHM. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* (2011) 332:1097–100. doi: 10.1126/science.1203980
105. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. *Trends Plant Sci.* (2012) 17:478–86. doi: 10.1016/j.tplants.2012.04.001
106. Berendsen RL, Vismans G, Yu K, Song Y, Burgman W, Burmølle M, et al. Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* (2018) 12:1496–507. doi: 10.1038/s41396-018-0093-1
107. Doornbos RF, Van Loon LC, Bakker PAHM. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. *Agron Sustain Dev.* (2012) 32:227–243. doi: 10.1007/s13593-011-0028-y
108. Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM. Induced systemic resistance by beneficial microbes. *Ann Rev Phytopathol.* (2014) 52:347–75. doi: 10.1146/annurev-phyto-082712-102340
109. Van Loon LC, Bakker PAHM, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol.* (1998) 36:453–83. doi: 10.1146/annurev.phyto.36.1.453
110. Pieterse, CMJ, Van Wees SCM, Hoffland E, Van Pelt JA, Van Loon LC. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* (1996) 8:1225–37. doi: 10.1105/tpc.8.8.1225
111. Takenaka S, Nakamura Y, Ono T, Sekiguchi H, Masunaka A, Takahashi H. Novel elicitin-like proteins isolated from the cell wall of the biocontrol agent *Pythium oligandrum* induce defense-related genes in sugar beet. *Mol Plant Pathol.* (2006) 7:325–39. doi: 10.1111/j.1364-3703.2006.00340.x
112. Hase S, Takahashi S, Takenaka S, Nakaho K, Arie T, Seo S, et al. Jasmonic acid signaling is required for bacterial wild disease resistance induced by biocontrol agent *Pythium oligandrum* in tomato. *Plant Pathol.* (2008) 57:870–6. doi: 10.1111/j.1365-3059.2008.01858.x
113. Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* (1998) 10:1571–80 doi: 10.1105/tpc.10.9.1571
114. Knoester M, Van Loon, LC, Van Den Heuvel J, Hennig J, Bol JF, Linthorst HJM. Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. *Proc Natl Acad Sci USA.* (1998) 95:1933–337. doi: 10.1073/pnas.95.4.1933
115. Van Loon LC, Bakker PAHM. Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. In: Siddiqui ZA, editor. *PGPR: Biocontrol and Biofertilization.* Dordrecht: Springer Netherlands (2005). p. 39–66.
116. Van der Ent S, Verhagen BWM, Van Doorn R, Bakker D, Verlaan MG, Pel MJC, et al. MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiol.* (2008) 146:1293–304. doi: 10.1104/pp.107.113829
117. Zamioudis C, Hanson J, Pieterse CMJ. ß-Glucosidase BGLU42 is a MYB72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in *Arabidopsis* roots. *New Phytol.* (2014) 204:368–79. doi: 10.1111/nph.12980
118. Stringlis IA, Yu K, Feussner K, De Jonge R, Van Bentum S, Van Verk MC, et al. MYB72-dependent coumarin exudation shapes root microbiome. *Proc Natl Acad Sci USA.* (2018) 115:E5213–22. doi: 10.1073/pnas.1722335115
119. Conrath U, Beckers GJ, Flors V, García-Agustín P, Jakab G, Mauch F, et al. Priming: getting ready for battle. *Mol Plant Microbe Interact.* (2006) 19:1062–71. doi: 10.1094/MPMI-19-1062
120. Martinez-Medina A, Flors V, Heil M, Mauch-Mani B, Pieterse CMJ, Pozo MJ, et al. Recognizing plant defense priming. *Trends Plant Sci.* (2016) 21:818–22. doi: 10.1016/j.tplants.2016.07.009
121. Verhagen BWM, Glazebrook J, Zhu T, Chang HS, Van Loon LC, Pieterse CMJ. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Mol Plant Microbe Interact.* (2004) 17:895–908. doi: 10.1094/MPMI.2004.17.8.895
122. Van Wees SCM, Luijendijk M, Smoorenburg I, Van Loon LC, Pieterse CMJ. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. *Plant Mol Biol.* (1999) 41:537–49. doi: 10.1023/A:1006319216982
123. Hase S, Van Pelt JA, Van Loon LC, Pieterse CMJ. Colonization of Arabidopsis roots by Pseudomonas fluorescens primes the plant to produce higher levels of ethylene upon pathogen infection. *Physiol Mol Plant Pathol.* (2003) 62:219–26. doi: 10.1016/S0885-5765(03)00059-6
124. Van der Ent S, Van Hulten MHA, Pozo MJ, Czechowski T, Udvardi MK, Pieterse CMJ, et al. Priming of plant innate immunity by rhizobacteria and β-aminobutyric acid: differences and similarities in regulation. *New Phytol.* (2009) 189:419–31. doi: 10.1111/j.1469-8137.2009.02851.x
125. Pozo MJ, Van der Ent S, Van Loon LC, Pieterse CMJ. Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytol.* (2008) 180:511–23. doi: 10.1111/j.1469-8137.2008.02578.x
126. Conrath U. Molecular aspects of defence priming. *Trends Plant Sci.* (2011) 16:524–31. doi: 10.1016/j.tplants.2011.06.004
127. Jaskiewicz M, Conrath U, Peterhänsel C. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* (2011) 12:50–55. doi: 10.1038/embor.2010.186
128. Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, et al. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environ Microbiol.* (2007) 9:1084–90. doi: 10.1111/j.1462-2920.2006.01202.x
129. Djonović S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. *Mol Plant Microbe Interact.* (2006) 19:838–53. doi: 10.1094/MPMI-19-0838
130. Hossain MM, Sultana F, Kubota M, Koyama H, Hyakumachi M. The plant growth-promoting fungus *Penicillium simplicissimum* GP17-2 induces resistance in Arabidopsis thaliana by activation of multiple defense signals. *Plant Cell Physiol.* (2007) 48:1724–36. doi: 10.1093/pcp/pcm144

131. Schuhegger R, Ihring A, Gantner S, Bahnweg G, Knappe C, Vogg G, et al. Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* (2006) 29:909–18. doi: 10.1111/j.1365-3040.2005.01471.x
132. Hase S, Shimizu A, Nakaho K, Takenaka S, Takahashi H. Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. *Plant Pathol.* (2006) 55:537–43. doi: 10.1111/j.1365-3059.2006.01396.x
133. Takahashi H, Ishihara T, Hase S, Chiba A, Nakaho K, Arie T, et al. Beta-cyanoalanine synthase as a molecular marker induced resistance by fungal glycoprotein elicitor and commercial plant activators. *Phytopathology* (2006) 96:908–16. doi: 10.1094/PHYTO-96-0908
134. Takenaka S, Yamaguchi K, Masunaka A, Hase S, Inoue T, Takahashi H. Implications of oligomeric forms of POD-1 and POD-2 proteins isolated from cell walls of the biocontrol agent *Pythium oligandrum* in relation to their ability to induce defense reactions in tomato. *J Plant Physiol.* (2011) 168:1972–9. doi: 10.1016/j.jplph.2011.05.011
135. Hyakumachi M, Takahashi H, Matsubara Y, Someya N, Shimizu M, Kobayashi K, et al. Recent study on biological control of plant diseases in Japan. *J General Plant Pathol.* (2014) 80:287–302. doi: 10.1007/s10327-014-0524-4
136. Takahashi H, Nakaho K, Ishihara T, Ando S, Wada T, Kanayama Y, et al. Transcriptional profile of tomato roots exhibiting *Bacillus thuringiensis*-induced resistance to *Ralstonia solanacearum*. *Plant Cell Rep.* (2014) 33:99–110. doi: 10.1007/s00299-013-1515-1
137. Hyakumachi M, Nishimura M, Arakawa T, Asano S, Yoshida S, Tsushima S, et al. *Bacillus thuringiensis* suppresses bacterial wilt disease caused by Ralstonia solanacearum with systemic induction of defense-related gene expression in tomato. *Microb Environ.* (2013) 28:128–34. doi: 10.1264/jsme2.ME12162
138. Elliot SJ, Sabelis MW, Janssen A, Van der Geest LPS, Beerling EAM, Fransen J. Can plants use entomopathogens as bodyguards? *Ecol Lett.* (2000) 3:228–35. doi: 10.1046/j.1461-0248.2000.00137.x
139. Finkel OM, Castrillo G, Paredes SH, Gonzalez IS, Dangl JL. Understanding and exploiting plant beneficial microbes. *Curr Opin Plant Biol.* (2017) 38:155-163. doi: 10.1016/j.pbi.2017.04.018
140. Oyserman BO, Medema MH, Raaijmakers JM. Road MAPs to engineer host microbiomes. *Curr Opin Microbiol.* (2018) 43:46–54. doi: 10.1016/j.mib.2017.11.023
141. Pieterse CMJ, De Jonge R, Berendsen RL. The soil-borne supremacy. *Trends Plant Sci.* (2016) 21:171–3. doi: 10.1016/j.tplants.2016.01.018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer TM and handling editor declared their shared affiliation.

frontiers in Nutrition

REVIEW
published: 02 October 2018
doi: 10.3389/fnut.2018.00090

How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture

Wakako Ikeda-Ohtsubo[1], Sylvia Brugman[2], Craig H. Warden[3], Johanna M. J. Rebel[4], Gert Folkerts[5] and Corné M. J. Pieterse[6]*

[1] Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2] Cell Biology and Immunology Group, Wageningen University and Research, Wageningen, Netherlands, [3] Departments of Pediatrics, Neurobiology Physiology and Behavior, University of California, Davis, Davis, CA, United States, [4] Wageningen Livestock Research, Wageningen University and Research, Wageningen, Netherlands, [5] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Netherlands, [6] Plant–Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Raquel Hontecillas, Virginia Tech, United States

Reviewed by:
Abdel Qawasmeh, Hebron University, Palestine
Julio Villena, Centro de Referencia para Lactobacilos, Argentina

****Correspondence:***
Wakako Ikeda-Ohtsubo
wakako.ohtsubo@tohoku.ac.jp

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *09 June 2018*
Accepted: *07 September 2018*
Published: *02 October 2018*

Citation:
Ikeda-Ohtsubo W, Brugman S, Warden CH, Rebel JMJ, Folkerts G and Pieterse CMJ (2018) How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture. Front. Nutr. 5:90. doi: 10.3389/fnut.2018.00090

All multicellular organisms benefit from their own microbiota, which play important roles in maintaining the host nutritional health and immunity. Recently, the number of studies on the microbiota of animals, fish, and plants of economic importance is rapidly expanding and there are increasing expectations that productivity and sustainability in agricultural management can be improved by microbiota manipulation. However, optimizing microbiota is still a challenging task because of the lack of knowledge on the dominant microorganisms or significant variations between microbiota, reflecting sampling biases, different agricultural management as well as breeding backgrounds. To offer a more generalized view on microbiota in agriculture, which can be used for defining criteria of "optimal microbiota" as the goal of manipulation, we summarize here current knowledge on microbiota on animals, fish, and plants with emphasis on bacterial community structure and metabolic functions, and how microbiota can be affected by domestication, conventional agricultural practices, and use of antimicrobial agents. Finally, we discuss future tasks for defining "optimal microbiota," which can improve host growth, nutrition, and immunity and reduce the use of antimicrobial agents in agriculture.

Keywords: microbiota, agriculture, animal husbandry, aquaculture, rhizosphere, phyllosphere, agricultural immunology

INTRODUCTION

Today, biologists in agricultural science, regardless of the organism of their interest, focus significant attention on the microbiota, i.e., the complex communities of microorganisms colonizing host animals, fish, and plants (1). Meta-analyses of 16S rRNA genes from different body parts of animals, fish, and plants are frequently performed expecting that some changes of microbiota will explain the effectiveness of treatments such as feed changes, fertilizer amendment, or gene knockouts on host organisms, which have been conducted with aims to improve productivity and sustainability in agriculture (2). However, it is often the case that no apparent

changes are observed in the microbial structure corresponding to the specific treatment, or if present, the functions of the responding microorganisms are not well-known [e.g., (3–5)]. Besides, it is often difficult for researchers in agricultural sciences to exploit the microbial data to improve the host factors because of the lack of definition and criteria of "optimal microbiota" in animals, fish, and plants.

Compared to a large body of studies on microbiota of human subjects (6) or experimental models using rodents(7), zebrafish (8), or *Arabidopsis* (9), there are a very limited number of studies on economically important animals, fish, and plants. Microbiota datasets obtained from livestock animals, aquaculture fish, and crop plants grown may significantly be affected by complex environmental factors such as climates, cultivation scales, and uses of antibiotics and fertilizers, which can vary between different countries and regions. Besides, the microbiota of agricultural organisms may also reflect the great variability of host species and genotypes, biological functions at different developmental stages, and macro- and microstructures of the colonizing sites, which are not thoroughly studied as the laboratory models. Due to the overall limited understanding of the microbiota in agricultural ecosystems at this point, it is not an easy task to define "optimal microbiota," which can optimize the growth, host nutrition, and immunity of agricultural organisms.

The importance of understanding the structure and functions of microbiota in agriculture is also widely discussed in the context of the spread of antimicrobial resistance (AMR) from agricultural sites to human society (10). While manipulation of microbiota is a promising strategy to tackle the AMR (11), it is prerequisite for researchers to interpret and exploit the rapidly expanding datasets of the microbiota in animals, fish, and plants in agriculture with a more generalized view. By sharing knowledge on the ecophysiology of microbiota in different host organisms with respect to their structure and metabolites and understanding how the host factors and ambient conditions can alter them, we would be able to refine targets of microbial manipulation and reduce uses of chemicals and antimicrobial agents in agricultural fields.

The aim of this review is to summarize and generalize the current knowledge on the microbiota on animals, fish, and plants in agriculture with emphasis on structure and functions of bacterial communities, which may contribute to the health of the host organisms and can strongly be impacted by agricultural practices such as uses of antimicrobial agents. We finally provide important yet overlooked aspects of microbiota in animals, fish, and plants in agriculture, which should be considered in future studies to reach the goal of defining the "optimal microbiota."

STRUCTURE AND FUNCTION OF MICROBIOTA OF ANIMALS, FISH, AND PLANTS

General Overview of Microbiota of Animals, Fish, and Plants

The body of organisms provides a wide variety of ecological niche, in which the environmental conditions such as temperature, pH, and oxygen level as well as nutrition availability affect the composition of microbiota residing there. While archaea and eukaryotic microorganisms such as fungi and protozoa account for a significant proportion of microbiota in the plant rhizosphere (12) and cow rumen (13), bacterial communities have been primarily focused on in many studies in agricultural science in terms of their functional contribution to host nutrition and health. The 16S rRNA gene-based approach with a next-generation sequencing platform has revealed diversity and dynamics of bacterial communities colonizing animals, fish, and plants in agriculture, which have enabled us to grasp a general overview of compositional similarities and differences of microbiota among these organisms (**Figure 1**). Microbiota of animal, fish, and plants are highly diverse and can harbor up to 20 bacterial phyla, but it is a common trait that three phyla: Proteobacteria, Firmicutes, and Bacteroidetes, dominate the bacterial community (**Figure 1**). Less abundant phyla include Actinobacteria, which are commonly found but variable at lower taxonomic levels (e.g., Streptomycetaceae, Microbacteriaceae, and Corynebacteriaceae), while Fusobacteria and Acidobacteria are more specified to animal/fish and plants, respectively (**Figure 1**). Fusobacteria can represent a major bacterial group of "core gut microbiome" of some marine and freshwater fish (8, 27). Chloroflexi, Cyanobacteria, Planctomycetes, Spirochaetes, and Verrucomicrobia sporadically occur as subdominant phyla (**Figure 1**).

The high abundance of Proteobacteria in animals, fish, and plants (**Figure 1**) reflects the advantages of facultative anaerobes in the host proximity, where strict anaerobes are exposed to the risk of oxygen toxicity but strict aerobes may face a severe competition over oxygen as an electron acceptor. Such oxic-anoxic interface is ubiquitous as microenvironments in and around the host organism and is an important determinant of the composition of microbiota (28). Facultative anaerobic bacteria have highly flexible metabolic properties; they are able to generate energy by fermentation or use inorganic nitrogen compounds such as nitrate as an alternative electron acceptor when oxygen is depleted from the environment. Under oxic conditions, they grow rapidly using oxygen and break down and build up a wide variety of organic compounds, which essentially change the surrounding organochemical conditions (29). Such exceptional adaptability to multiple environmental conditions, which have been characterized by their high genetic and phenotypic plasticity, enable Proteobacteria to be specialists of host association, as represented by major target symbionts and pathogens in agriculture (30–32).

While Proteobacteria are ubiquitous and their association is often described to be opportunistic, they show apparent host specificities in some microbiota. In fish intestinal microbiota, Aeromonadaceae (Gammaproteobacteria) represents the most abundant symbionts in freshwater fish, while Vibrionaceae (Gammaproteobacteria) replace the occupation in marine fish (27, 33). In microbiota of livestock animals, the proteobacterial community is predominated by Enterobacteriaceae, followed by Campylobacteriaceae and Helicobacteraceae, which are a major source of foodborne diseases of human (3, 34, 35).

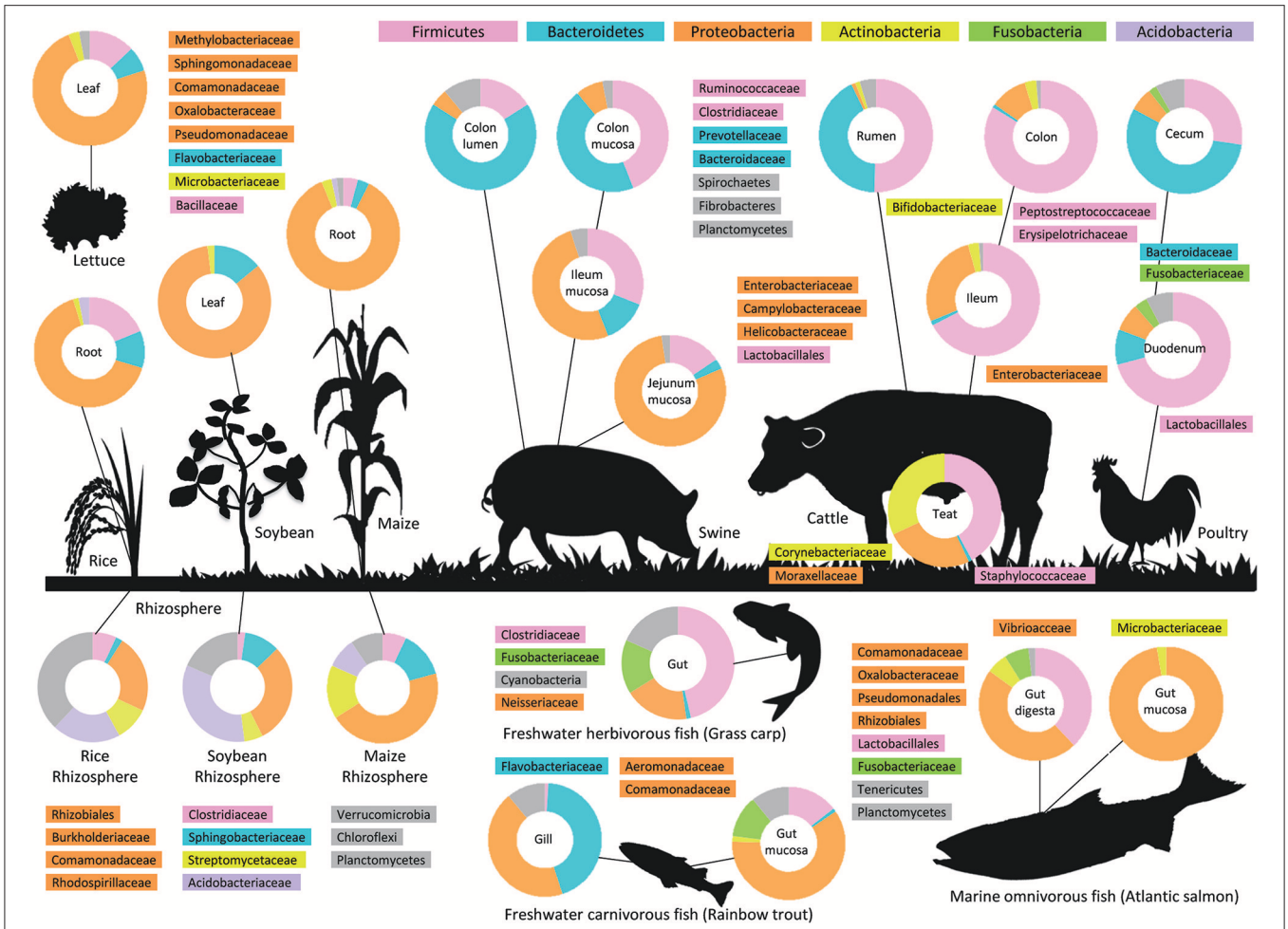


FIGURE 1 | Microbiota in agriculture. The figure provides an overview of the bacterial composition of the microbiota of different parts of livestock animals, gill and intestines of fish, and phyllosphere and rhizosphere of plants at the phylum-level (pie-charts) and lower taxonomic levels. The data sources are 16S rRNA or metagenomic analyses of intestinal samples from pigs (14, 15), cattle (3, 16), chicken (17), Atlantic salmon (18), grass carp (19), gill and mucosal samples from rainbow trout (20), leaf samples from lettuce (21), leaf and rhizosphere samples from soybean (22, 23), root and rhizosphere samples from maize (24), rice (25, 26).

Plant phyllosphere has been found to be dominated by strict aerobes, represented by Methylobacteriaceae and Sphingomonadaceae (Alphaproteobacteria), but their abundance is low in the plant root, being replaced by a large diversity of facultative anaerobes (36). Interestingly, microbiota of plants and fish have several groups of facultative anaerobes in common. Multiple apertures of fish, i.e., skin, gills, and gut, are constantly in contact with ambient water, each of which is covered with thick mucus biofilm, which bears resemblance to the plant root system. Comamonadaceae and Oxalobacteraceae (Betaproteobacteria) as well as Flavobacteriaceae (Bacteroidetes), which dominate the leaf-to-root microbiota of plants, are also abundant in fish mucus, especially in gill microbiota [**Figure 1**; (36, 37)]. Also, bacteria known as plant growth-promoting microbes (PGPM) such as Pseudomonadaceae (Gammaproteobacteria) and Rhizobiales (Alphaproteobacteria) are also frequent colonizers in fish intestinal microbiota (33, 38). In contrast, these bacterial groups are not commonly found in animal intestinal microbiota.

Firmicutes population in animals, fish, and plants can be roughly classified into two groups: Lactic acid bacteria from the orders Bacillales and Lactobacillales, and anaerobic fermentative bacteria affiliated with Clostridiales such as Clostridiaceae and Ruminococcaceae. The former represents microbiota in oxic- to microoxic regions like plant phyllosphere, fish mucosa, and small intestines of animals, while the latter represents anoxic fermentative digestive tracts like rumen and large intestine (**Figure 1**). Lactobacillales is one of the most frequently found and most studied bacterial groups in animal and fish microbiota with some family-level variations, as Lactobacillaceae is a stable and important colonizer in the small intestine of pigs (39, 40) and chickens (17), Carnobacteriaceae [rainbow trout; (20)], and Leuconostocaceae [Atlantic salmon; (18)] are characteristic in fish microbiota, and Enterococcaceae and Streptococcaceae are generally found both in animals and fish microbiota (41). Clostridiaceae is phylogenetically and functionally diverse and widely distributed in anaerobic environments from plant

rhizosphere to animal and fish intestines (42). Studies on *Clostridium* spp. from various origins have shown their metabolic versatility and dexterous switching of their fermentation pathway in response to environmental changes (43, 44).

Bacteroidetes colonizing animals, fish, and plants can also be affiliated with two types: aerobic Flavobacteriaceae, which is adapted to the oxic interface of plants and fish as mentioned above, and anaerobic fermentative bacteria such as Bacteroidaceae and Prevotellaceae (**Figure 1**). Flavobacteriaceae is often recognized as threatening pathogens of animals and fish (45), but it also represents PGPM and has been introduced to industrial applications (46). Bacteroidaceae and Prevotellaceae are important primary fermenters in animal and fish intestinal tracts, through which complex carbohydrates derived from plants and undigested proteins enter the microbial metabolic network and provide soluble sugars and amino acids available for all type of cells (47). They dominate the rumen and colonal microbiota of animals (48, 49) but are rarely found in fish and plants, which suggests their speciation to animal digestive tracts.

Relationship Between Intestinal Microbiota Composition and Host Nutrition of Animals and Fish

Microbiota of animals and fish associated with different host physiological conditions have been widely studied to elucidate the relationship between the structure and functions of microbiota and host nutritional health. Although results are variable across studies, which may be attributed to different experimental designs, analytical methods, or individual variations, a few general aspects can be inferred from recent studies.

In animal husbandry, feed efficiency and growth performance are often focused on as the most important physiological factors. Recent studies on pig microbiota have reported the enrichment of Clostridiales, as well as microbial functional genes involved in fermenting dietary polysaccharides and amino acid metabolism, are positively associated with porcine feed efficiency (50, 51). Similarly, positive correlations of Clostridiales (family Lachnospiraceae) to good feed efficiency have also been found in the cattle rumen (49) and chicken caeca (52). In fish, the intestinal microbiota of prebiotics-treated fish with improved growth performance also showed an increased number of *Clostridium* spp. (53). All of these studies have attributed the positive effects of Clostridiales on the host feed efficiency and growth performance to the high energy yields by the production of short-chain fatty acids (SCFA) such as butyrate, which has also been suggested in human gut microbiota studies (54).

Lactobacillales are thought to improve feed efficiency of animals and multiple *Lactobacillus* strains are widely used as feed additives especially in pig farming (55). While studies have suggested a positive correlation of *Lactobacillus* to a better feed efficiency of cattle, chickens, and fish (17, 49, 53), contrasting effects of *Lactobacillus* spp. on growth performance have been also reported on chicken (17, 52, 56). Therefore, species- and strain-level variation should be considered when the abundance of *Lactobacillus* strains is used as a criterion for evaluating the health and growth performance of animals and fish.

Both in animals and fish, intestinal microbial colonization has been shown to promote epithelial cell turnover and regulate transcription of genes involved in nutrient metabolism and immunity, and the corresponding gene modules are universally conserved between mammals and fish (57–59).

Rhizosphere vs. Phyllosphere: Difference of Microbiota Composition

Plant microbiota significantly differed from those of animals and fish, in that in addition to the complex bacterial community, a large variety of archaea as well as eukaryotic macro- and microorganisms can directly and constantly affect the health of the host plant (12, 60). In response to this challenge, plants have a finely regulated immunological capacity, which recognizes different exogenous molecules and responds by activating specific defense mechanisms (61). The plant rhizosphere is home of a high density [10^{10}-10^{12} cells per gram soil; (62)] of microorganisms and a large pool of microbial metabolites influence the nutritional conditions of the host plant as well as the composition of microbial populations (63). In contrast, microbiota in the plant phyllosphere, i.e., leaf and root, are enriched with restricted groups of bacteria. As mentioned already, Proteobacteria and Flavobacteria have been found as endophytes or epiphytes of the host plant, while any other bacterial phyla dominating the rhizosphere, such as the phylum Acidobacteria and Firmicutes, or prominent rhizobacteria such as Streptomycetaceae (Actinobacteria) and Burkholderiaceae (Betaproteobacteria) are segregated at different levels of the phyllosphere (64). This suggests the existence of a "selecting gate" between the rhizosphere and the phyllosphere, or different compartments of the plant root (65).

Studies across different plant species including the *Arabidopsis* model indicate that the enrichment of Proteobacteria is a common trait in the plant phyllosphere (**Figure 1**). However, the enriched bacterial species, i.e., members selected by the host plant, seem to differ significantly between plant species. Pseudomonadaceae (Gammaproteobacteria) and Streptomycetaceae (Actinobacteria), which are frequent colonizers in the root of *Arabidopsis*, are not found in grass plants such as barley, maize, and wheat (24, 66). Also, leaf microbiota between plant species can be very different, as exemplified that Enterobacteriaceae, Bacillaceae, and *Pantoea* spp. dominating spinach and lettuce leaves are not abundant in *Arabidopsis* (21, 67). While the bacterial composition of microbiota of the phyllosphere of economically important plants is very limited compared to that of the rhizosphere (36, 68), the composition of microbiota in different plant compartment may provide useful insights into site-specific selection mechanisms of the host plant.

Acquisition of Microbiota in the Early Life of Animals, Fish, and Plants

In animals, the intestinal immunity is known to be developed in the course of frequent interaction with microbiota, which are

formed and fluctuated in response to the host dynamics (69). It has been shown that the early-life transfer of microbiota from the mother to the child via the birth channel and colostrum milk can impact on subsequent intestinal microbial diversity and immune processes in piglets (70). The transition from nursing, weaning to conventional diets can dramatically affect intestinal microbiota. Milk provides immunological factors such as Immunoglobulin A (IgA), leukocytes, and peptides, which suppress inflammatory cytokine expression, and lactose and oligosaccharides contained in milk can stimulate the growth of early-colonizing microorganisms such as lactic acid bacteria. (71). Comparative analyses of the intestinal microbiota of nursing and weaning piglets have shown that the dietary change from sow milk to a starter diet composed of plant and animal-based components has a significant impact on the microbial structure as well as its functional capacities (34). In their study, Enterobacteriaceae, Bacteroidaceae, and Clostridiaceae dominating the nursing piglets almost disappeared as the piglet diet shifted to a starter diet, which has been characterized by the dominance of Prevotellaceae and Ruminococcaceae associated with plant polysaccharide degradation. The early colonization and subsequent disappearance of Enterobacteriaceae as well as the maturation of microbiota associated with the domination of plant-polysaccharide degraders in the early life have also been commonly found in other mammals including human (72) and also in chickens (73). Proliferation of pathogenic members of Enterobacteriaceae can be regulated by selective binding activities of host-derived IgA, which seems to be one of the most important mechanisms affecting early development of intestinal microbiota in animals (74).

Fish develop from eggs that are directly exposed to microorganisms in their surroundings. The eggs are quickly coated with microorganisms present in the surrounding water, of which some have been shown to protect the eggs from infection with oomycete *Saprolegnia*, a deleterious pathogen causing economic loss in the salmon industry (75). Since fish represents the largest number of vertebrates (>28,000 species), a lot of interspecies variation may exist based on the receptors or binding moieties on the egg surface. Although microbiota of fish larvae is poorly understood (76, 77), some studies have suggested that the microbiota composition of fish larva greatly depend on the microorganisms present on the eggs, in the live feed and rearing water (78, 79). Since microorganisms are able to enter the fish larvae before it starts feeding (3–4 days after fertilization), initial microbial infection in the larval intestine probably occurs before the feed specific species grow to abundance [(76, 80) Lopez Nadal, unpublished observations)]. In early life stages of Coho salmon, *Pseudomonas* sp. present on the eggs has been predominantly found in the juvenile gastrointestinal tract, but not in the culture water or food, which suggests that a maternal transfer may occur in the early developmental stages of the salmon (81).

The development of seedlings from largely sterile plant seeds is one of the most critical stages of a plant's life cycle. Yet, very little is known about the role of microbiota in the early life of plants (82). Starting inoculum on the ripening seed may be important for the establishment of microbiota and preliminary enrichment of the soil microbiota by the parental plant will form ideal environments for germination of seeds in the same soil. Interestingly, it has recently been shown that diseased plants can recruit themselves a consortium of beneficial, immune-stimulatory microbes from the soil environment and let them colonize germinating seedlings, which suggests that plants are capable of selecting soil microbiota for protecting a successive generation of plants against the causal agent of the disease (83, 84).

Post-translational Host Modulation by Microbiota

Host epigenomics has recently been shown to be one of the most important factors significantly affected by microbiota. Anti-inflammatory effects of some intestinal microorganisms such as *Clostridium* spp. have been attributed to their metabolite butyrate, an epigenetic substance known to inhibit activities of histone deacetylases and modulate gene expression patterns of host animals (85). Also in fish, promotion of resistance to viral infection of conventionally reared zebrafish has been shown to be associated with microbe-induced epigenetic changes in the host (86). In plants, not only bacterial pathogens but also fungal and other eukaryotic organisms have been known to manipulate their host epigenetically to favor themselves (87). While low-molecular-weight microbial metabolites such as SCFAs and polyamines from mammalian intestinal microbiota have been shown to be involved in various epigenomic mechanisms in the mammalian host (88), modulatory effects of microbial structural components such as LPS, peptidoglycan, and exopolysaccharides from microbiota in most agricultural organisms have not yet been well-studied except for those from some pathogens and probiotics (see below). Molecular mechanisms how microbiota modulates host epigenomics have recently been attracting major attention, which may also contribute to understanding functions of microbiota in animals, fish, and plants in agriculture (89).

MICROBIAL METABOLITES: BENEFICIAL AND DELETERIOUS EFFECTS OF METABOLITES PRODUCED BY MICROBIOTA OF ANIMALS, FISH, AND PLANTS

"Optimal microbiota" of agricultural organisms are expected to provide beneficial effects on their host nutritional health and immunological resistance. Microorganisms influence the host health by producing a large variety of metabolites, which can have both beneficial effects and detrimental effects on the host physiology (**Table 1**).

Short Chain Fatty Acid (SCFA)

Fermentative microorganisms break down carbohydrates and proteins into SCFA. In the gut of animals and fish, major SCFAs produced by fermentative microorganisms are acetate, propionate, and butyrate, while relatively low amounts of formate, valerate, caproate, and branched-chain SCFAs, i.e., isobutyrate, 2-methyl-butyrate, and isovalerate, which are used as a marker of undesired intestinal protein fermentation (90), are also present (91, 92). SCFAs can modulate the gene

TABLE 1 | Important microbial metabolites and their effects on host animals, fish, and plants.

Microbial metabolites	Examples	Hosts[a]	Beneficial effects	Detrimental effects
Short-chain fatty acids (SCFAs)	Butyrate	A, F	Energy homeostasis anti-inflammatory effect, improve intestinal barrier	Mucosal disruption
	Propionate			Neurotoxicity
Organic acids	Lactate	A, F,	Increase butyrate production	Acidosis, inflammation, neurotoxicity,
	Succinate	A, F, P	Glycemic control, feed PGPM, mineral solubilization	Feed pathogens
Ammonia and amino acid derivatives	Ammonia, ammonium	A, F, P	Nitrogen nutritional source, pH neutralization	Inflammation, mucosal damages, increase oxidative stress
	Biogenic amines	A, F	Synthesis of neurotransmitter (serotonin)	Production of uremic toxins, carcinogenesis
Signaling molecules acting on the host	IAA, 2,4-DAPG, GABA	A, F, P	Growth promotion, anti-inflammation	
Signaling molecules acting on other microbes	AHL, AI-2		Maintenance of microbial structure (e.g., biofilm formation), cell-to-cell communication between microbes	
Antimicrobial compounds	Bacteriocins, RiPPs	A, F, P	Defense against pathogens, immunomodulatory effects	Cytotoxicity
Vitamins	Vitamin B_{12}, vitamin K, D	A, F	Provisioning of host nutrition, immunomodulation	
Microbial cellular components	LPS, Polysaccharide A	A, F, P	Immunomodulation, maintenance of intestinal homeostasis	Inflammation

[a] *A, animals; F, fish; P, plants.*

expression of the host epithelial cells in multiple ways and their physiological concentrations may significantly affect the host nutritional health and immunity (93, 94). SCFAs produced by gut microbiota are known to serve as a major energy source for ruminant animals, which consume cellulose fibers and complex carbohydrates as the main diet, but also play a crucial role for young monogastric animals for maintaining the body weight after weaning (95, 96). Additional roles of SCFA include defense mechanisms, mineral solubilization, and the anti-inflammatory effects (97, 98). SCFA produced by intestinal microbiota improve intestinal barrier functions and suppress inflammation through signaling pathways such as activating G-protein coupled receptors, inhibiting histone deacetylase, stimulation of histone acetyltransferase activity, and stabilizing hypoxia-inducible factor (HIF), which have been extensively studied with rodent models (99, 100).

While SCFAs produced by intestinal microbiota are generally considered to be beneficial to the host, excessive SCFAs can cause intestinal injuries in animals with premature or weakening mucosal conditions (101, 102). Formate, which concentration increases along with dysbiosis, can enhance the growth of unwanted enterobacterial pathogens (103). High levels of propionate are often found in human and animals with psychological and behavioral disorders and thought to have a neurotoxic potential (104, 105).

Lactate and Other Organic Acids

Lactate is an important intermediate in anaerobic fermentation of carbohydrates. While host-derived lactate has been known for regulatory functions on the energy homeostasis and brain metabolism (106, 107), lactate produced by microbiota may also play important roles in the intestinal ecosystem, such as turnover of host epithelial cells (108), in addition to their role as a major food source for other SCFA producing bacteria (109). In the small intestine of animals and fish, lactic acid bacteria such as Lactobacillales (**Figure 1**) are known to produce lactate as a primary metabolite, while *Turicibacter* (Erysipelotrichaceae) represent the major lactate producers in the large intestine. Residuous oxygen may increase relative abundance of intestinal lactic acid bacteria, which generally show high tolerance against oxygen (110, 111), and lactate productions and consumption profiles may differ significantly between upper and lower intestines (112).

Succinate is another major organic acid released from microbiota during carbohydrate fermentation. Prevotellaceae and Veillonellaceae, which are predominant bacterial groups in the rumen and in the colon of pigs, are major succinate producers. A large variety of bacteria including Enterobacteriaceae and Clostridiaceae can grow on succinate, and succinate accumulation would increase a risk of infection by pathogenic bacteria (113). Recent studies have reported succinate production by gut microbiota is strongly correlated to the metabolic fluctuation of host animals (114, 115).

Accumulation of lactate and succinate has been reported in the intestine of pigs with gastric problems (116, 117), which has been shown to be inversely related to the SCFA concentrations (118). Increased concentrations of lactate and succinate can cause a decline in pH and drastic changes in metabolic patterns in

animal and fish intestines, which can lead to deleterious outcomes such as acidosis and inflammation (119). To avoid this, a rapid turnover of lactate by gut microbiota seems to be crucial for intestinal homeostasis in animals and fish (28).

Plant root exudates contain a high amount of organic acids, such as citrate, succinate, and malate, which can significantly affect the composition of the microbial community in the rhizosphere (120, 121). The high amount of organic acids exudated from the host plant feed and control proximal microbiota consisting of plant growth-promoting microorganisms (PGPM) as well as pathogens, and the microbiota in rhizosphere may also affect the concentration of organic acids excreted from the plant host by modulating their regulatory genes (122, 123). As organic acids can affect the growth and plant-promoting activities of PGPM, e.g., suppressing phosphate stabilization (124), the concentration of organic acids should be well fine-tuned by the host-microbe regulatory network (125).

Ammonia and Amino Acid Derivatives

Ammonia (NH_3) and ammonium (NH_4^+) play an important physiological role in the body of animals, fish, and plants as it provides usable forms of nitrogen required for the synthesis of DNA, RNA, and proteins. Ammonia not only serves as a major nitrogen source but also are responsible for buffering the ecosystem such as rumen by neutralizing excess acids. Many bacteria are able to generate ammonia via protein or peptide degradation and N_2 fixation. Fixed atmospheric N_2 in the NH_4^+ form is an important source of nitrogen in the soil ecosystem, which concentration in agricultural soils is approximately between 20 and 200 μM (126), and many plants are highly dependent on endophytic or rhizospheric nitrogen-fixing bacteria for their nitrogen demands. Ammonia is also an important metabolite in the microbiota of animals and fish and millimolar level concentration of ammonia can be generally found in the intestinal ecosystem [e.g., 10–70 mM in colonic lumen; (127)]. Many bacteria such as *E. coli* and *Bacteroides* spp. are known to require ammonia or ammonium for their growth in the intestinal system, while they are able to provide amino acids and their derivatives to other intestinal bacteria and the host (29, 128).

Toxicity of ammonia (NH_3) and ammonium (NH_4^+) from microbiota poses a risk to the host as well. When excess protein is present in the intestine, ammonia production by microbial deamination will exceed microbial ammonia assimilation (129). Urea produced by the host animals is also converted to ammonia and further to ammonium hydroxide by microbiota, which can elevate luminal pH at the level of causing mucosal damage and irritation (130). Accumulated ammonia has multiple adverse effects on host epithelial cells (129). Ammonium toxicity is also documented in plants, but the cause for this phenomenon and involvement of microbiota is still unknown (131).

Increased protein and peptide concentrations in a microbial ecosystem may facilitate active amino acid conversion to various nitrogenous derivatives. Many facultative and obligate anaerobic bacteria ferment amino acids into a wide variety of intermediate metabolites such as indoles, phenols, cresols, and their derivatives as well as biogenic amines (132). Biogenic amines such as tyramine, putrescine, histamine, methylamine, and tryptamine, are produced by decarboxylation of amino acids, which have significant physiological and toxicological functions in eukaryotic cells (132, 133). Biogenic amines serve as precursors of various bioactive compounds, which can directly regulate physiology and behavior of the host. For example, tryptamine, a β-arylamine neurotransmitter derived from tryptophan metabolism, influences modes of serotonin production in enterochromaffin (EC) cells and therefore affect host behavior (134, 135).

Secondary Metabolites

Secondary metabolites from microbiota such as tryptamine, which can serve as hormones or signaling molecules (136) to "control" the host physiology and behavior, are also known for plants. Indole-3-acetic acid (IAA), one of the most important plant growth regulators, is also derived from the tryptophan metabolism of PGPM such as Pseudomonadaceae (137). Pseudomonadaceae are also known to produce a wide variety of secondary metabolites including antibiotic compounds and siderophores, which can protect the host plant from invasive pathogens not only in the rhizosphere but also in phyllosphere (138, 139). Specific secondary metabolites of Pseudomonadaceae such as 2,4-diacetylphloroglucinol (2,4-DAPG) are of special interests for controlling specific plant–microbe interaction (140). In animals and fish, secondary metabolites produced by gut microbiota such as gamma-aminobutyric acid (GABA) are likely to have more general but significant influence on physiological and psychological properties of the host (141).

Antimicrobial compounds such as bacteriocins, siderophores, and lipidopeptide biosurfactants enable some microorganisms to outcompete and eliminate pathogens and shape the structure of microbiota by also affecting the host immunity (139, 142, 143). Although bacteriocins and siderophores have been well-documented in some beneficial or pathogenic strains, genes encoding these compounds could be commonly found in a wide range of microorganisms (144). In human microbiota, ribosomally synthesized post-translationally modified peptides (RiPPs), which include lantibiotics, thiazole/oxazole-modified microcins (TOMMs) as well as thiopeptides antibiotics, are one of the most widely distributed and variable microbial metabolites (145).

Signaling molecules known as autoinducers play important roles in cell-to-cell communication between microorganisms and shape the synchronized behavior of microbial community such as biofilm formation (146). In contrast to the well-known quorum-sensing molecule AHL (N-acyl homoserine lactone), which are produced as virulence factors by many gram-negative pathogenic bacteria and probably uncommon in healthy intestinal microbiota in animals and fish (147, 148), AI-2 (autoinducer 2) are present in many intestinal bacteria such as Firmicutes and Bacteroidetes and known to modify the structure and behavior of intestinal microbiota (149, 150).

Vitamins

Animals depend on their gut microbiota for various vitamins, which are often deficient in their normal diet. Deficiencies in vitamin B_{12} and other B-complex vitamins, as well as vitamin K and D in animals and fish has been correlated to the absence of intestinal microorganisms producing those vitamins (151, 152). In addition to the crucial role for the host nutritional health (153–155), vitamins formed by microbiota are also provisioned to other microorganisms in proximity thereby supporting the cross-feeding metabolic network in gut microbiota (152). Some vitamins are also known to participate in host epigenomic mechanisms by altering the transcriptional machinery of the host cells (88). While most plants can synthesize vitamins and do not depend on their microbiota for their vitamin requirements, some algae have been known to benefit from the microbial provision of vitamin B_{12} (156).

Microbial Cellular Components

Microbial metabolites affecting host health also include structural compounds of microorganisms themselves. Exo- and lipopolysaccharides (LPS), peptidoglycan, flagellin, and some unique peptides and nucleic acids released from the microbial community, which are often collectively called as microorganism-associated molecular patterns (MAMPs), are specifically detected as "non-self" and distinguished by pattern recognition receptors (PRRs) of the host cells and trigger immune responses in animals, fish (86), and plants (157, 158). It has long been recognized that MAMPs from pathogens play a crucial role for host immunity in animals and plant (96, 159–161), but recent studies have revealed that MAMPs from commensal microbiota may also control the host immune system to maintain intestinal homeostasis (162). Common intestinal residential bacteria such as *Clostridium* and *Bacteroides* have been shown to stimulate the production of cytokines such as IL-6 and TNFα that protect intestinal tissues from injury (163), and also to induce the proliferation of immune cells such as $FOXP3^+$ regulatory T (Treg) cells (164). Although MAMPs required for induction of each host factor are not well-understood, species-specific polysaccharides such as Polysaccharide A found on the capsule of *Bacteroides fragilis* may play important roles for initial binding and recognition to the host cells (165).

In the model plant *Arabidopsis thaliana* it was recently shown that MAMPs from beneficial root microbiota members are similarly recognized by the plant immune system as MAMPs from pathogens, but the downstream immune response was suppressed by so far unknown mechanisms (89).

INFLUENCE OF AGRICULTURAL MANAGEMENT PRACTICES ON MICROBIOTA IN ANIMALS, FISH, AND PLANTS

Recent comparative studies on gut microbiota between urban and hunter-gatherer human population have suggested continuous decreases in microbial diversity over generations during worldwide industrialization (166–168). Similar changes, i.e., loss of diversity in domestic vs. wild counterparts, have been documented in primates and Przewalski's horses (169, 170), but the diversity level of gut microbiota has been found to be consistent in mice (171) and a vice versa situation has also been observed in cloacal microbiota in parrots (172). Nevertheless, many studies have shown that the reduced diversity of gut microbiota is characteristic to many diseases and disorders in human [e.g., (173, 174)], therefore the loss of diversity in gut microbiota over generations may have negatively affected the health of not only human but also other animals and fish. Although reduced microbial diversity is not often discussed for plants, the long-term agricultural practices may have served as strong selective pressures on the microbiota of the phyllosphere and rhizosphere (175, 176). Supplementation of the "lost" population could improve host fitness, as has been shown in mice (177), but the cause of the loss of certain microbial groups and its consequences are not fully understood. For optimizing microbiota in agricultural organisms, it is important to evaluate how domestication and agricultural management practices can affect the microbiota and host nutritional health and immunity.

Domestication

While some livestock animals and farmed fish have evolved into domestic species distinct from wild relatives, studies on the microbiota of wild representatives of agricultural organisms provide insights into how domestication may have affected the microbial composition of agricultural organisms. For example, a comparative study of domestic pig microbiota with that of wild boars has revealed that *Lactobacillus* spp. and Enterobacteriaceae, which are considered to be dominant bacterial groups in pig intestinal microbiota (178), are not common in wild boars (179). Interestingly, recently domesticated wild boars have been found to harbor Enterobacteriaceae as a major group, which collectively suggests that gut microbiota of domestic pigs may reflect the recent agricultural management practices (179). Since the increased abundance of Enterobacteriaceae has been reported to be correlated to post-weaning diarrhea (180), agricultural management is likely to have a significant impact on the health of domestic pigs via the fluctuation of gut microbiota. In cattle, inoculation with bison rumen contents has been shown to increase protein digestibility and nitrogen retention but not fiber digestibility, which suggests that microbiota of ancestors of livestock animals may have had higher capacities to extract nitrogen nutrition from crude materials (181).

While a study on gut microbiota of laboratory-reared and recently-caught zebrafish has shown little influence from domestication on intestinal microbiota of fish and shrimps (8, 182), some bacterial groups in wild fish have been found to disappear upon captivity (183) and therefore careful investigation should be needed in future studies.

Plant microbiota can also be affected by domestication, i.e., plant breeding in combination with yield-increasing agricultural practices and the use of chemical fertilizers and pesticides, which has resulted in the selection of specific plant traits maximizing profitable functions from the root microbiome (184).

Studies have shown distinct features of the microbial community associated with wild and domesticated crop species such as rhizosphere microbiota from sugar beets (185) and endophytic population from grapevines (186). Nevertheless, plant hosts respond to various microbial factors by changing their physiology and thereby can modulate their microbial composition (187), it is important to obtain more insights into the physiological and structural differences between wild and domesticated plant species.

Agricultural Management Practices

Agricultural management practices include multiple and long-term stress factors such as selective breeding, confinement, nutritional changes, close contact with people, and antimicrobial usage, all of which can affect the composition of microbiota to a greater extent.

Selective breeding produces a new type of organism with a phenotype different from its parental organisms, which can affect the composition of the host-specific microbiota. Gut microbiota in livestock animals including cattle and pigs have been reported to show a host-specificity and habitability over generations (188, 189), which suggests that the host genetics are correlated to microbial structure and functions (190). Although fish gut microbiota are largely affected by ecological factors, several studies have shown host selection plays an important role in shaping intestinal and gill microbiota (191, 192). In plants, rhizosphere microbiota has been shown to have specific profiles unique to its host plant species, genotype, and cultivar (193, 194).

Confinement such as indoor breeding and aquaculture has been reported to affect microbiota of animals and fish to various extents. There is no clear evidence on how housing systems (indoor vs. outdoor) can affect microbiota of animals, since previously reported changes in gut microbiota in response to different housing methods can be better explained by dietary changes (195, 196). As intestinal tracts of fish are constantly exposed to water and a large number of microorganisms in their surroundings, it is not surprising that the conditions of aquaculture such as water quality (e.g., salinity) and external microbial community significantly affect gut microbiota of fish (197–199).

Nutritional changes including grazing, feeding, and weaning in animals or fertilizer amendment for plants, are one of the most important factors shaping the structure and functions of microbiota in agriculture. Availability of microbiota-accessible organic compounds is a crucial determinant for the survivability of individual microorganisms in the host systems (200). In livestock animals, starch grains, plant fibers and crude proteins in feed are digested by rumen or colonic microbiota to different degrees, which can essentially change the structure and functions of gut microbiota and the host nutritional health as already mentioned above (51, 201, 202). Weaning can cause serious fluctuation of rumen and intestinal microbiota of young animals (34, 203), which can occasionally lead to dysbiosis and post-weaning diarrhea (204). Also similar to animals, dietary changes such as feeding high-cellulose diet has been shown to increase cellulolytic bacteria in the fish intestine (205, 206), and relationships between the dietary components and gut microbiota are extensively studied (207). Continuous cropping and fertilizer amendment can modulate nutritional status in the agricultural soil and affect plant microbiota (208). The growth inhibition caused by the continuous cropping, such as the imbalance of inorganic nutrients and prevalence of pathogenic fungi, could be mitigated by native microbiota (209), which suggests the resilience of agricultural soils highly depend on their microbial activities.

Continuous close contact with human and animals seems to allow inter-species transmission of certain bacterial groups even between the intestinal microbiota. As the microbial composition in human gut has been found to be affected by adjacent livestock animals or companion animals (210, 211), the microbiota of animals, fish, and plants could be affected by the human microbial assemblage. The predominance of *Bifidobacterium* spp. in the modern human gut microbiota reflecting the dietary transition from fiber-rich plant-based diet to western diet (212), has also been observed in animals, which have experienced close contact with humans (213).

Influence of Usage of Antimicrobial Agents on Microbiota in Agriculture

Antimicrobial agents have been used as a common agricultural practice over the decades, which aims not only to treat infectious diseases of animals and fish but also to promote growth and improve productivity (214). The worldwide overuse of antimicrobial agents has already brought major concerns: the spread of AMR in microorganisms through the global ecosystem. Microbiota of animals, fish, and plants, which have been treated with antimicrobial agents, can serve as a reservoir of resistance genes where commensal microorganisms may confer AMR to pathogenic microorganisms by horizontal transfer events (215). Use of low-dose antibiotics as antimicrobial growth promoters (AGPs) in livestock farming is still a common practice in many countries, which poses a great risk to accelerate emergence and spread of antibiotic-resistant bacteria (216).

It has been shown that antimicrobial agents can alter intestinal microbiota of animals in location-specific ways, (i) structural and functional disruption of foregut microbiota, and (ii) increase of AMR in hindgut microbiota (217). The major risk of disruption of foregut microbiota in piglet is characterized by the increased number of *Streptococcus suis* and Enterobacteriaceae, which are known to cause infectious diseases like pneumonia and post-weaning diarrhea (218). Such effects by the early-life exposure to antimicrobial agents can be retained throughout the life of animals (70, 219). Reduced diversity of intestinal microbiota by antimicrobial treatments, which can increase the host susceptibility to pathogens, has also been documented in fish (220).

The risk of AMR in agriculture is not restricted to livestock farms. Livestock manure is frequently used for composting and eventually amended to agricultural soils as fertilizers and the high frequency of AMR in manure can be transferred to the microbial community in the soil, which can also affect the plant microbiota (221, 222). Aquaculture ponds are considered to be a significant

reservoir of AMR (223), especially in countries where livestock manure is used for feeding fish in farming ponds (224). Once entering to the agricultural food chain, AMR is transmitted and exchanged between microbiota associated with animals, fish, and plants and spread over agricultural food products, which can be eventually introduced to human microbiota (225–227).

FUTURE TASKS FOR DEFINING "OPTIMAL MICROBIOTA" OF ANIMALS, FISH, AND PLANTS

The cross-sectional view of the microbiota of animals, fish, and plants reviewed here may provide an idea of what aspects should be particularly considered in the future investigation for elucidating structure and functions of "optimal microbiota" and applying the knowledge for improving host nutrition and immunology to maximize productivity and sustainability in agriculture.

Quantitative Understanding of Microbiota

Many past studies employ 16S rRNA gene sequencing approach to study microbiota of animals, fish, and plants. While the composition of microbiota, which can be estimated by the number of sequencing reads, allows understanding of the diversity and distribution of specific microbial groups, the density of microorganisms is often overlooked. Microbial density is especially important when studying a specific host region where microbial activities play crucial roles or the host immunological factors respond to a certain density threshold. The microbiota of animal rumen, hindguts or rhizosphere with the extremely high density of microorganisms [10^{11}-10^{12} cells per mL or gram; (62, 228)] and animal foreguts or the plant phyllosphere with a smaller number of microbial cells in several orders of magnitude (10^4-10^7 cells) should be considered as a separate ecosystem themselves, i.e., habitats with different types and levels of microbial structure and functions, which could be differently recognized by the host immunity (162). Therefore, using fecal samples for studying gut microbiota should be done with a special caution, since the foregut microbiota are highly underrepresented in the feces and its compositional and functional changes can be completely masked by the hindgut microbiota (229).

While no direct counts of the number of microbial metabolites produced are available for any host organism, as their composition constantly changes depending on environmental, host and microbial factors, attempts have been made to use sequencing information to estimate the number of compounds that may be produced from human microbiota. Donia et al. have identified 14,000 predicted small-molecule biosynthetic gene clusters (BGCs) by shotgun sequencing human gut metagenome where they have shown that 3,118 BCGs have been found in the healthy human microbiota, among which 599 clusters can be affiliated with typical human gut microbiota while 1,061 clusters with the typical oral cavity (90). They reported that gene cluster classes in the human microbiota differed from those in non-human microbiota, which suggests that species-specific analyses of BCGs will also be useful for agricultural organisms. To make the best use of such useful approach, it is worth summarizing what kind of microbial metabolites can occur and how they affect physiology and growth properties of the host animals, fish, and plants.

Cultivation of the Uncultured Majority

The limitation of our current knowledge on microbiota of animals, fish, and plants can be largely attributed to the predominance of uncultured microorganisms in each microbiota. For example, a study by Stanley et al. has been able to identify several bacterial phylotypes in chicken caeca, which are negatively correlated with growth performance of the host chicken, but all of these phylotypes have been affiliated with unknown and uncultured bacterial groups of the phylum Firmicutes (35). In cattle, 44.6% of all microbial sequences obtained from gastrointestinal tracts have failed to be identified at the genus level (3). Similarly, eggs at the fertilization stage of grass carp have been reported to be colonized by a large proportion (>50%) of uncultured bacteria (230). In maize rhizosphere, important functional genes for microbial nitrogen metabolism such as nitrogen-fixation and denitrification have been mainly affiliated with uncultured bacteria (231).

These findings underscore the importance of cultivation of the uncultured members of microbiota colonizing animals, fish, and plants. The difficulties of conventional cultivation techniques are now able to be addressed by modern technologies featured by single-cell (meta)genomics (232) and culturomics (233), which combine the analytical methods such as the index fluorescence-activated cell sorting (FACS) or the matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) with multiple culture conditions and high-throughput 16S rRNA gene sequencing (233, 234). Isotope probing (SIP) (235) has also been used as a powerful method to identify uncultured microorganisms with specific activities such as host-protein utilization in animal gut microbiota (236) or the pesticide degradation in the rhizosphere microbiota (237). Individual profiles of microbial metabolites (**Table 1**) are also an important aspect to understand the role of uncultured microorganisms, which can be extensively assessed by recently advancing metabolomic approach integrated with genomic and proteomic datasets (238).

Systematic Investigation of Microbial Functions

Many well-described Proteobacteria species, which are widely distributed in healthy animals, fish and plants, also behave as opportunistic pathogens (239, 240). *Campylobacter* spp. and *Salmonella* spp., two major food-borne pathobionts have been found to be stable colonizers of livestock animals and human, which are usually unharmful but occasionally cause diseases of the hosts (241). Plant-associated bacteria of the class Gammaproteobacteria such as Pseudomonadaceae, Erwiniaceae, Xanthomonadaceae show species- and strain-level differences in their traits as pathogens, antagonists of the pathogens, or PGPM (240). Most microorganisms consisting the microbiota of animals, fish, and plants seem to be opportunistic symbionts,

which colonization can result in beneficial and detrimental, or no effects on the host, which might be determined not only by genetic properties of each microorganism but also by various environmental conditions and host factors (242, 243). The question whether the identified bacterial groups are beneficial or detrimental cannot be answered only by 16S rRNA gene-based analyses, and the microbial metabolites (244), host-specific selective marker genes including virulence or symbiotic factors in the microbial genomes (145, 245), and metabolites involved in modulations of the host cell immunity (246) should be systematically investigated for elucidating the roles of microbiota. A study on the fruit fly *Drosophila* has shown that occurrence of a single protein of plant pathogenic bacterium *Erwinia carotovora*, i.e., *evf* factor determines the successfulness of persistence in the gut of the host (247). Similar unknown mechanisms may present in microorganisms associated with animals, fish, and plants, which are responsible for the host-specific selection of individual microorganisms.

As the majority is still uncultured, microbial physiology is not fully resolved and many important microbial processes in natural ecosystems have still not well-discussed in microbiota research on animals, fish, and plants.

Nitrogen fixation, as well as ammonia oxidation and denitrification (reduction of nitrate, nitrite, and N_2O), are globally important processes conducted by microorganisms fueling the nitrogen cycle of most ecological systems (248) but have been poorly investigated for animal and fish microbiota. Nitrogen fixation and other inorganic nitrogen conversion have been known to maintain nutritional status of termite gut microbiota, where nitrogen-poor wood polysaccharides (cellulose and hemicellulose) serve as major sources of nutrition (249, 250). Recent findings of a genetic diversity of the nitrogen fixation gene *nifH* in human microbiota indicate that inorganic nitrogen metabolism may play an important role in animal microbiota (251), but it is still unknown which microorganisms are responsible for the processes.

Microbial removal of hydrogen (H_2) generated in the course of fermentation of fiber-rich carbohydrates is a critical process in every anaerobic system, including gut microbiota of animals and fish (252). H_2-consuming intestinal microorganisms such as methanogenic archaea, sulfate-reducing bacteria, and reductive acetogens are therefore as important as primary fermenters such as Bacteroidetes for maintaining the redox balance and conserving energy (253), and are crucial for the stable SCFA production in the ecosystem (254, 255). In contrast to the well-studied rumen microbiota, little is known about H_2-consuming microorganisms for monogastric animal guts, but a study by Rey et al. have shown that genes encoding Wood-Ljundahl pathway, which are key components for reductive acetogenesis, have been shown to be highly represented among expressed RNAs in human gut microbiota than marker genes for methanogenesis or sulfate reduction (256).

Clarification of Optimization Purposes

Recently, Lloyd-Price et al. has suggested "healthy human microbiome" can be defined in terms of microbial composition, function, dynamics, and ecology (6). Although this definition can be applied for defining "optimal microbiota" of animals, fish, and plants in agriculture, the dataset from each target organism may be highly limited compared with that of human gut microbiome (257) and additional criteria should be considered in the context of productivity and sustainability.

In plant science, improving growth speeds, conferring resistance against environmental stresses, or improving nutritional values have been successfully accomplished by inoculating PGPM consisting of specific bacterial groups or amending materials promoting the growth of PGPM (258). In contrast, the impact of inoculation of putatively beneficial microorganisms i.e., probiotics to animals and fish seems to be less pronounced (259). Striking similarities between gut microbiota in antibiotic-treated pigs, which gain weight and have high-feed efficiency, and gut microbiota linked to human obesity (218, 260) indicate that the "optimal microbiota" are not necessarily identical to the "healthy microbiota" in agricultural contexts. Therefore, clarifying purposes of the microbiota optimization, i.e., prevention of specific diseases or addition of nutritional values in the products, is prerequisite for the employment of microbiota manipulation techniques, which have been reviewed by Brugman et al., in this issue (11). As discussed above, the worldwide threat of AMR should be combated by reducing the amount of unnecessary use of antimicrobial agents in agricultural practice and by manipulation of microbiota, which can minimize the risk of diseases and optimize the growth performance of target organisms. Host–microbe interaction in individual agricultural organisms should be studied with close reference to the current knowledge available from laboratory models and humans, through which new ideas for modulating microbiota as alternative strategies to antibiotic use can be shared and discussed interdisciplinarily.

AUTHOR CONTRIBUTIONS

WI-O and CP conceived the idea of the review, WI-O wrote the manuscript, and all authors discussed the contents and contributed to the writing of the manuscript.

FUNDING

This work was financially supported by the Japan Society for the Promotion of Science (JSPS) through JSPS Core-to-Core Program (Advanced Research Networks) entitled Establishment of international agricultural immunology research-core for a quantum improvement in food safety.

ACKNOWLEDGMENTS

This review is based on the workshop The Dawn of Agricultural Immunology hosted and supported by the Lorentz Center in Leiden, the Netherlands, during September 19–23, 2016. The authors would like to thank the Lorentz Center as well as the organizers and participants of the workshop for providing us with the opportunity to discuss the ideas in this review with the aim to integrate knowledge of microbiota of animals, fish, and plants in agriculture.

REFERENCES

1. Hacquard S, Garrido-Oter R, González A, Spaepen S, Ackermann G, Lebeis S, et al. Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* (2015) 17:603–16. doi: 10.1016/j.chom.2015.04.009
2. Mueller UG, Sachs JL. Engineering microbiomes to improve plant and animal health. *Trends Microbiol.* (2015) 23:606–17. doi: 10.1016/j.tim.2015.07.009
3. Mao S, Zhang M, Liu J, Zhu W. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci Rep.* (2015) 5:16116. doi: 10.1038/srep16116
4. Thibodeau A, Letellier A, Yergeau É, Larrivière-Gauthier G, Fravalo P. Lack of evidence that selenium-yeast improves chicken health and modulates the caecal microbiota in the context of colonization by *Campylobacter jejuni*. *Front Microbiol.* (2017) 8:451. doi: 10.3389/fmicb.2017.00451
5. Wemheuer F, Kaiser K, Karlovsky P, Daniel R, Vidal S, Wemheuer B. Bacterial endophyte communities of three agricultural important grass species differ in their response towards management regimes. *Sci Rep.* (2017) 7:40914. doi: 10.1038/srep40914
6. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med.* (2016) 8:51. doi: 10.1186/s13073-016-0307-y
7. Xiao L, Feng Q, Liang S, Sonne SB, Xia Z, Qiu X, et al. A catalog of the mouse gut metagenome. *Nat Biotechnol.* (2015) 33:1103. doi: 10.1038/nbt.3353
8. Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, et al. Evidence for a core gut microbiota in the zebrafish. *ISME J.* (2011) 5:1595–608. doi: 10.1038/ismej.2011.38
9. Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, et al. Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* (2015) 528:364. doi: 10.1038/nature16192
10. Thanner S, Drissner D, Walsh F. Antimicrobial resistance in agriculture. *mBio* (2016) 7:e02227-15. doi:10.1128/mBio.02227-15
11. Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CMJ, Bakker PAHM. A comparative review on microbiota manipulation: lessons from fish, plants, livestock and human research. *Front Nutr.* 5:80. doi: 10.3389/fnut.2018.00080
12. Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E. The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil.* (2009) 321:189–212. doi: 10.1007/s11104-009-9991-3
13. Henderson G, Cox F, Ganesh S, Jonker A, Young W, Collaborators GRC, et al. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci Rep.* (2015) 5:14567. doi: 10.1038/srep14567
14. Burrough ER, Arruda BL, Plummer PJ. Comparison of the luminal and mucosa-associated microbiota in the colon of pigs with and without swine dysentery. *Front Vet Sci.* (2017) 4:139. doi: 10.3389/fvets.2017.00139
15. Kelly J, Daly K, Moran AW, Ryan S, Bravo D, Shirazi-Beechey SP. Composition and diversity of mucosa-associated microbiota along the entire length of the pig gastrointestinal tract; dietary influences. *Environ Microbiol.* (2017) 19:1425–38. doi: 10.1111/1462-2920.13619
16. Braem G, De Vliegher S, Verbist B, Heyndrickx M, Leroy F, De Vuyst L. Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. *Vet Microbiol.* (2012) 157:383–90. doi: 10.1016/j.vetmic.2011.12.031
17. Yan W, Sun C, Yuan J, Yang N. Gut metagenomic analysis reveals prominent roles of *Lactobacillus* and cecal microbiota in chicken feed efficiency. *Sci Rep.* (2017) 7:45308. doi: 10.1038/srep45308
18. Gajardo K, Rodiles A, Kortner TM, Krogdahl Å, Bakke AM, Merrifield DL, et al. A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research. *Sci Rep.* (2016) 6:30893. doi: 10.1038/srep30893
19. Ni J, Yan Q, Yu Y, Zhang T. Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiol Ecol.* (2014) 87:704–14. doi: 10.1111/1574-6941.12256
20. Lyons P, Turnbull J, Dawsonl K, Crumlish M. Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS). *Aquacult Res.* (2017) 48:77–91. doi: 10.1111/are.12863
21. Rastogi G, Sbodio A, Tech JJ, Suslow TV, Coaker GL, Leveau JHJ. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J.* (2012) 6:1812–22. doi: 10.1038/ismej.2012.32
22. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, et al. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci USA.* (2009) 106:16428–33. doi: 10.1073/pnas.0905240106
23. Liang J, Sun S, Ji J, Wu H, Meng F, Zhang M, et al. Comparison of the rhizosphere bacterial communities of Zigongdongdou soybean and a high-methionine transgenic line of this cultivar. *PLoS ONE* (2014) 9:e103343. doi: 10.1371/journal.pone.0103343
24. Niu B, Paulson JN, Zheng X, Kolter R. Simplified and representative bacterial community of maize roots. *Proc Natl Acad Sci USA* (2017) 114:E2450–9. doi: 10.1073/pnas.1616148114
25. Lu Y, Rosencrantz D, Liesack W, Conrad R. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. *Environ Microbiol.* (2006) 8:1351–60. doi: 10.1111/j.1462-2920.2006.01028.x
26. Breidenbach B, Conrad R. Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Front Microbiol.* (2014) 5:752. doi: 10.3389/fmicb.2014.00752
27. Tarnecki AM, Burgos FA, Ray CL, Arias CR. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *J Appl Microbiol.* (2017) 123:2–17. doi: 10.1111/jam.13415
28. Brune A, Frenzel P, Cypionka H. Life at the oxic-anoxic interface: microbial activities and adaptations. *FEMS Microbiol Rev.* (2000) 24:691–710. doi: 10.1111/j.1574-6976.2000.tb00567.x
29. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat Chem Biol.* (2009) 5:593–9. doi: 10.1038/nchembio.186
30. Unden G, Becker S, Bongaerts J, Holighaus G, Schirawski J, Six S. O_2-sensing and O_2-dependent gene regulation in facultatively anaerobic bacteria. *Arch Microbiol.* (1995) 164:81–90. doi: 10.1007/s002030050238
31. Dobrindt U, Hochhut B, Hentschel U, Hacker J. Genomic islands in pathogenic and environmental microorganisms. *Nat Rev Microbiol.* (2004) 2:414. doi: 10.1038/nrmicro884
32. Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev.* (2011) 35:652–80. doi: 10.1111/j.1574-6976.2011.00269.x
33. Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, Knight R, et al. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol Ecol.* (2012) 21:3363–78. doi: 10.1111/j.1365-294X.2012.05552.x
34. Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* (2015) 3:28. doi: 10.1186/s40168-015-0091-8
35. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl Microbiol Biotechnol.* (2014) 98:4301–10. doi: 10.1007/s00253-014-5646-2
36. Müller DB, Vogel C, Bai Y, Vorholt JA. The plant microbiota: systems-level insights and perspectives. *Annu Rev Genet.* (2016) 50:211–34. doi: 10.1146/annurev-genet-120215-034952
37. Lowrey L, Woodhams DC, Tacchi L, Salinas I. Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a diverse bacterial community with antifungal properties in the skin. *Appl Environ Microbiol.* (2015) 81:6915–25. doi: 10.1128/AEM.01826-15
38. Estruch G, Collado MC, Peñaranda DS, Tomás Vidal A, Jover Cerdá M, Pérez Martínez G, et al. Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16s rRNA gene. *PLoS ONE* (2015) 10:e0136389. doi: 10.1371/journal.pone.0136389
39. Levesque CL, Hooda S, Swanson KS, de Lange K. Alterations in ileal mucosa bacteria related to diet complexity and growth performance in young pigs. *PLoS ONE* (2014) 9:e108472. doi: 10.1371/journal.pone.0108472

40. Valeriano VD, Balolong MP, Kang D-K. Probiotic roles of *Lactobacillus* sp. in swine: insights from gut microbiota. *J Appl Microbiol.* (2017) 122:554–67. doi: 10.1111/jam.13364
41. Hardie JM, Whiley RA. Classification and overview of the genera *Streptococcus* and *Enterococcus*. *J Appl Microbiol.* (2003) 83:1S—11S. doi: 10.1046/j.1365-2672.83.s1.1.x
42. Wiegel J, Tanner R, Rainey FA. An introduction to the family Clostridiaceae BT. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. *The Prokaryotes, Vol. 4, Bacteria: Firmicutes, Cyanobacteria*. New York, NY: Springer US (2006). pp. 654–78. doi: 10.1007/0-387-30744-3_20
43. Grupe H, Gottschalk G. Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. *Appl Environ Microbiol.* (1992) 58:3896–902.
44. Fischbach MA, Sonnenburg JL. Eating for two: how metabolism establishes interspecies interactions in the gut. *Cell Host Microbe* (2011) 10:336–47. doi: 10.1016/j.chom.2011.10.002
45. Loch TP, Faisal M. Emerging flavobacterial infections in fish: a review. *J Adv Res.* (2015) 6:283–300. doi: 10.1016/j.jare.2014.10.009
46. Nishioka T, Elsharkawy MM, Suga H, Kageyama K, Hyakumachi M, Shimizu M. Development of culture medium for the isolation of *Flavobacterium* and *Chryseobacterium* from rhizosphere soil. *Microb Environ.* (2016) 31:104–10. doi: 10.1264/jsme2.ME15144
47. El Kaoutari A, Armougom F, Gordon JI, Raoult D, Henrissat B. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol.* (2013) 11:497. doi: 10.1038/nrmicro3050
48. Holman DB, Brunelle BW, Trachsel J, Allen HK. Meta-analysis to define a core microbiota in the swine gut. *mSystems* (2017) 2:e00004-17. doi: 10.1128/mSystems.00004-17
49. Li F, Guan LL. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl Environ Microbiol.* (2017) 83:e00061-17. doi: 10.1128/AEM.00061-17
50. McCormack UM, Curião T, Buzoianu SG, Prieto ML, Ryan T, Varley P, et al. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. *Appl Environ Microbiol.* (2017) 83:e00380-17. doi: 10.1128/AEM.00380-17
51. Yang H, Huang X, Fang S, He M, Zhao Y, Wu Z, et al. Unraveling the fecal microbiota and metagenomic functional capacity associated with feed efficiency in pigs. *Front Microbiol.* (2017) 8:1555. doi: 10.3389/fmicb.2017.01555
52. Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with Improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. *Front Microbiol.* (2016) 7:187. doi: 10.3389/fmicb.2016.00187
53. Geraylou Z, Souffreau C, Rurangwa E, D'Hondt S, Callewaert L, Courtin CM, et al. Effects of arabinoxylan-oligosaccharides (AXOS) on juvenile Siberian sturgeon (*Acipenser baerii*) performance, immune responses and gastrointestinal microbial community. *Fish Shellfish Immunol.* (2012) 33:718–24. doi: 10.1016/j.fsi.2012.06.010
54. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* (2005) 102:11070–5. doi: 10.1073/pnas.0504978102
55. Dowarah R, Verma AK, Agarwal N. The use of *Lactobacillus* as an alternative of antibiotic growth promoters in pigs: a review. *Anim Nutr.* (2017) 3:1–6. doi: 10.1016/j.aninu.2016.11.002
56. Crisol-Martinez E, Stanley D, Geier MS, Hughes RJ, Moore RJ. Understanding the mechanisms of zinc bacitracin and avilamycin on animal production: linking gut microbiota and growth performance in chickens. *Appl Microbiol Biotechnol.* (2017) 101:4547–59. doi: 10.1007/s00253-017-8193-9
57. Savage DC, Siegel JE, Snellen JE, Whitt DD. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. *Appl Environ Microbiol.* (1981) 42:996–1001.
58. Rawls JF, Samuel BS, Gordon JI. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci USA.* (2004) 101:4596-601. doi: 10.1073/pnas.0400706101
59. Sommer F, Nookaew I, Sommer N, Fogelstrand P, Bäckhed F. Site-specific programming of the host epithelial transcriptome by the gut microbiota. *Genome Biol.* (2015) 16:62. doi: 10.1186/s13059-015-0614-4
60. Sapp M, Ploch S, Fiore-Donno AM, Bonkowski M, Rose LE. Protists are an integral part of the *Arabidopsis thaliana* microbiome. *Environ Microbiol.* (2017) 20:30–43. doi: 10.1111/1462-2920.13941
61. Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM. Networking by small-molecule hormones in plant immunity. *Nat Chem Biol.* (2009) 5:308. doi: 10.1038/nchembio.164
62. Foster RC. Microenvironments of soil microorganisms. *Biol Fertil Soils* (1988) 6:189–203. doi: 10.1007/BF00260816
63. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. *Trends Plant Sci.* (2012) 17:478–86. doi: 10.1016/j.tplants.2012.04.001
64. Hernández M, Dumont MG, Yuan Q, Conrad R. Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. *Appl Environ Microbiol.* (2015) 81:2244–53. doi: 10.1128/AEM.03209-14
65. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc Natl Acad Sci USA.* (2015) 112:E911–20. doi: 10.1073/pnas.1414592112
66. Bulgarelli D, Garrido-Oter R, Munch PC, Weiman A, Droge J, Pan Y, et al. Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* (2015) 17:392–403. doi: 10.1016/j.chom.2015.01.011
67. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS ONE* (2013) 8:e56329. doi: 10.1371/journal.pone.0056329
68. Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, et al. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* (2012) 6:1378–90. doi: 10.1038/ismej.2011.192
69. Francino MP. Early development of the gut microbiota and immune health. *Pathogens* (2014) 3:769–90. doi: 10.3390/pathogens3030769
70. Schokker D, Zhang J, Zhang L, Vastenhouw SA, Heilig HGHJ, Smidt H, et al. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. *PLoS ONE* (2014) 9:e100040. doi: 10.1371/journal.pone.0100040
71. Moeser AJ, Pohl CS, Rajput M. Weaning stress and gastrointestinal barrier development: implications for lifelong gut health in pigs. *Anim Nutr.* (2017) 3:313–21. doi: 10.1016/j.aninu.2017.06.003
72. Matsuki T, Yahagi K, Mori H, Matsumoto H, Hara T, Tajima S, et al. A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. *Nat Commun.* (2016) 7:11939. doi: 10.1038/ncomms11939
73. Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, et al. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. *PLoS ONE* (2014) 9:e115142. doi: 10.1371/journal.pone.0115142
74. Okai S, Usui F, Yokota S, Hori-i Y, Hasegawa M, Nakamura T, et al. High-affinity monoclonal IgA regulates gut microbiota and prevents colitis in mice. *Nat Microbiol.* (2016) 1:16103. doi: 10.1038/nmicrobiol.2016.103
75. Liu Y, de Bruijn I, Jack ALH, Drynan K, van den Berg AH, Thoen E, et al. Deciphering microbial landscapes of fish eggs to mitigate emerging diseases. *ISME J.* (2014) 8:2002. doi: 10.1038/ismej.2014.44
76. Tinh NTN, Dierckens K, Sorgeloos P, Bossier P. A review of the functionality of probiotics in the larviculture food chain. *Mar Biotechnol (NY).* (2008) 10:1–12. doi: 10.1007/s10126-007-9054-9
77. Llewellyn MS, Boutin S, Hoseinifar SH, Derome N. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front Microbiol.* (2014) 5:207. doi: 10.3389/fmicb.2014.00207
78. Ringo E, Birkbeck TH. Intestinal microflora of fish larvae and fry. *Aquac Res.* (1999) 30:73–93. doi: 10.1046/j.1365-2109.1999.00302.x
79. Olafsen JA. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* (2001) 200:223–47. doi: 10.1016/S0044-8486(01)00702-5

80. Lauzon HL, Gudmundsdottir S, Petursdottir SK, Reynisson E, Steinarsson A, Oddgeirsson M, et al. Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre- and posthatch stages and the effect of different treatments. *J Appl Microbiol.* (2010) 109:1775–89. doi: 10.1111/j.1365-2672.2010.04806.x
81. Romero J, Navarrete P. 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *Microb Ecol.* (2006) 51:422–30. doi: 10.1007/s00248-006-9037-9
82. Nelson EB. The seed microbiome: origins, interactions, and impacts. *Plant Soil* (2018) 422:7–34. doi: 10.1007/s11104-017-3289-7
83. Bakker PAHM, Pieterse CMJ, de Jonge R, Berendsen RL. The soil-borne legacy. *Cell* (2018) 172:1178–80. doi: 10.1016/j.cell.2018.02.024
84. Berendsen RL, Vismans G, Yu K, Song Y, de Jonge R, Burgman WP, et al. Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* (2018) 12:1496–1507. doi: 10.1038/s41396-018-0093-1
85. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst.* (2000) 92:1210–6. doi: 10.1093/jnci/92.15.1210
86. Galindo-Villegas J, García-Moreno D, de Oliveira S, Meseguer J, Mulero V. Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development. *Proc Natl Acad Sci USA.* (2012) 109:E2605-14. doi: 10.1073/pnas.1209920109
87. Ramirez-Prado JS, Piquerez SJM, Bendahmane A, Hirt H, Raynaud C, Benhamed M. Modify the histone to win the battle: chromatin dynamics in plant–pathogen interactions. *Front Plant Sci.* (2018) 9:355. doi: 10.3389/fpls.2018.00355
88. Bhat MI, Kapila R. Dietary metabolites derived from gut microbiota: critical modulators of epigenetic changes in mammals. *Nutr Rev.* (2017) 75:374–89. doi: 10.1093/nutrit/nux001
89. Stringlis IA, Proietti S, Hickman R, Van Verk MC, Zamioudis C, Pieterse CMJ. Root transcriptional dynamics induced by beneficial rhizobacteria and microbial immune elicitors reveal signatures of adaptation to mutualists. *Plant J.* (2018) 93:166–80. doi: 10.1111/tpj.13741
90. Macfarlane GT, Gibson GR, Beatty E, Cummings JH. Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. *FEMS Microbiol Lett.* (1992) 101:81–8. doi: 10.1111/j.1574-6968.1992.tb05764.x
91. Kihara M, Sakata T. Fermentation of dietary carbohydrates to short-chain fatty acids by gut microbes and its influence on intestinal morphology of a detritivorous teleost tilapia (*Oreochromis niloticus*). *Comp Biochem Physiol Physiol.* (1997) 118:1201–7. doi: 10.1016/S0300-9629(97)00052-2
92. Krajmalnik-Brown R, Ilhan Z-E, Kang D-W, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. *Nutr Clin Pract.* (2012) 27:201–14. doi: 10.1177/0884533611436116
93. Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. *J AOAC Int.* (2012) 95:50–60. doi: 10.5740/jaoacint.SGE_Macfarlane
94. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* (2016) 165:1332–45. doi: 10.1016/j.cell.2016.05.041
95. van Beers-Schreurs HM, Nabuurs MJ, Vellenga L, Kalsbeek-van der Valk HJ, Wensing T, Breukink HJ. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *J Nutr.* (1998) 128:947–53. doi: 10.1093/jn/128.6.947
96. Montagne L, Pluske JR, Hampson DJ. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim Feed Sci Technol.* (2003) 108:95–117. doi: 10.1016/S0377-8401(03)00163-9
97. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* (2013) 341:569LP−73. doi: 10.1126/science.1241165
98. Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr Res Rev.* (2010) 23:366–84. doi: 10.1017/S0954422410000247
99. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Adv Immunol.* (2014) 121:91–119. doi: 10.1016/B978-0-12-800100-4.00003-9
100. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* (2015) 17:662–71. doi: 10.1016/j.chom.2015.03.005
101. Nafday SM, Chen W, Peng L, Babyatsky MW, Holzman IR, Lin J. Short-chain fatty acids induce colonic mucosal injury in rats with various postnatal ages. *Pediatr Res.* (2005) 57:201–4. doi: 10.1203/01.PDR.0000150721.83224.89
102. Peng L, He Z, Chen W, Holzman IR, Lin J. Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. *Pediatr Res.* (2007) 61:37. doi: 10.1203/01.pdr.0000250014.92242.f3
103. Hughes ER, Winter MG, Duerkop BA, Spiga L, Furtado de Carvalho T, Zhu W, et al. Microbial respiration and formate oxidation as metabolic signatures of inflammation-associated dysbiosis. *Cell Host Microbe* (2017) 21:208–19. doi: 10.1016/j.chom.2017.01.005
104. El-Ansary AK, Ben Bacha A, Kotb M. Etiology of autistic features: the persisting neurotoxic effects of propionic acid. *J Neuroinflamm.* (2012) 9:74. doi: 10.1186/1742-2094-9-74
105. Wiley NC, Dinan TG, Ross RP, Stanton C, Clarke G, Cryan JF. The microbiota-gut-brain axis as a key regulator of neural function and the stress response: implications for human and animal health. *J Anim Sci.* (2017) 95:3225–46. doi: 10.2527/jas.2016.1256
106. Sola-Penna M. Metabolic regulation by lactate. *IUBMB Life* (2008) 60:605–8. doi: 10.1002/iub.97
107. Proia P, Di Liegro MC, Schiera G, Fricano A, Di Liegro I. Lactate as a metabolite and a regulator in the central nervous system. *Int J Mol Sci.* (2016) 17: doi: 10.3390/ijms17091450
108. Okada T, Fukuda S, Hase K, Nishiumi S, Izumi Y, Yoshida M, et al. Microbiota-derived lactate accelerates colon epithelial cell turnover in starvation-refed mice. *Nat Commun.* (2013) 4:1654. doi: 10.1038/ncomms2668
109. Bourriaud C, RobinsRJ, Martin L, Kozlowski F, Tenailleau E, Cherbut C, et al. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *J Appl Microbiol.* (2005) 99:201–12. doi: 10.1111/j.1365-2672.2005.02605.x
110. Condon S. Responses of lactic acid bacteria to oxygen*. *FEMS Microbiol Rev.* (1987) 3:269–80. doi: 10.1111/j.1574-6968.1987.tb02465.x
111. Guo T, Zhang L, Xin Y, Xu Z, He H, Kong J. Oxygen-inducible conversion of lactate to acetate in heterofermentative *Lactobacillus brevis* ATCC 367. *Appl Environ Microbiol.* (2017) 83: doi: 10.1128/AEM.01659-17
112. Kahlert S, Junnikkala S, Renner L, Hynönen U, Hartig R, Nossol C, et al. Physiological concentration of exogenous lactate reduces antimycin A triggered oxidative stress in intestinal epithelial cell line IPEC-1 and IPEC-J2 *in vitro*. *PLoS ONE* (2016) 11:e0153135. doi: 10.1371/journal.pone.0153135
113. Ferreyra JA, Wu KJ, Hryckowian AJ, Bouley DM, Weimer BC, Sonnenburg JL. Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance. *Cell Host Microbe* (2014) 16:770–7. doi: 10.1016/j.chom.2014.11.003
114. De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis. *Cell Metab.* (2016) 24:151–7. doi: 10.1016/j.cmet.2016.06.013
115. Serena C, Ceperuelo-Mallafré V, Keiran N, Queipo-Ortuño MI, Bernal R, Gomez-Huelgas R, et al. Elevated circulating levels of succinate in human obesity are linked to specific gut microbiota. *ISME J.* (2018) doi: 10.1038/s41396-018-0068-2
116. Tsukahara T, Ushida K. Organic acid profiles in feces of pigs with pathogenic or non-pathogenic diarrhea. *J Vet Med Sci.* (2001) 63:1351–4. doi: 10.1292/jvms.63.1351
117. Tsukahara T, Ushida K. Succinate accumulation in pig large intestine during antibiotic-associated diarrhea and the constitution of succinate-producing flora. *J Gen Appl Microbiol.* (2002) 48:143–54. doi: 10.2323/jgam.48.143
118. Højberg O, Canibe N, Poulsen HD, Hedemann MS, Jensen BB. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Appl Environ Microbiol.* (2005) 71:2267–77. doi: 10.1128/AEM.71.5.2267-2277.2005
119. Hernández J, Benedito JL, Abuelo A, Castillo C. Ruminal acidosis in feedlot: from aetiology to prevention. *Sci World J.* (2014) 2014:702572. doi: 10.1155/2014/702572

120. Doornbos RF, van Loon LC, Bakker PAHM. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. a review. *Agron Sustain Dev.* (2012) 32:227–43. doi: 10.1007/s13593-011-0028-y
121. Stringlis IA, Yu K, Feussner K, de Jonge R, Van Bentum S, Van Verk MC, et al. MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc Natl Acad Sci USA*. (2018). 115:E5213–22. doi: 10.1073/pnas.1722335115
122. Glick BR. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol Lett.* (2005) 251:1–7. doi: 10.1016/j.femsle.2005.07.030
123. Kamilova F, Kravchenko L V, Shaposhnikov AI, Makarova N, Lugtenberg B. Effects of the tomato pathogen *Fusarium oxysporum* f. sp. radicis-lycopersici and of the biocontrol bacterium *Pseudomonas fluorescens* WCS365 on the composition of organic acids and sugars in tomato root exudate. *Mol Plant Microbe Interact.* (2006) 19:1121–6. doi: 10.1094/MPMI-19-1121
124. Iyer B, Rajput MS, Rajkumar S. Effect of succinate on phosphate solubilization in nitrogen fixing bacteria harbouring chick pea and their effect on plant growth. *Microbiol Res.* (2017) 202:43–50. doi: 10.1016/j.micres.2017.05.005
125. Wu H, Wu L, Zhu Q, Wang J, Qin X, Xu J, et al. The role of organic acids on microbial deterioration in the *Radix pseudostellariae* rhizosphere under continuous monoculture regimes. *Sci Rep.* (2017) 7:3497. doi: 10.1038/s41598-017-03793-8
126. Loqué D, von Wirén N. Regulatory levels for the transport of ammonium in plant roots. *J Exp Bot.* (2004) 55:1293–1305. doi: 10.1093/jxb/erh147
127. Hrnjez BJ, Song JC, Prasad M, Mayol JM, Matthews JB. Ammonia blockade of intestinal epithelial K^+ conductance. *Am J Physiol.* (1999) 277:G521–32.
128. Davila A-M, Blachier F, Gotteland M, Andriamihaja M, Benetti P-H, Sanz Y, et al. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. *Pharmacol Res.* (2013) 68:95–107. doi: 10.1016/j.phrs.2012.11.005
129. Apajalahti J, Vienola K. Interaction between chicken intestinal microbiota and protein digestion. *Anim Feed Sci Technol.* (2016) 221:323–330. doi: 10.1016/j.anifeedsci.2016.05.004
130. Tsujii M, Kawano S, Tsuji S, Fusamoto H, Kamada T, Sato N. Mechanism of gastric mucosal damage induced by ammonia. *Gastroenterology* (1992) 102:1881–8.
131. Podgorska A, Burian M, Gieczewska K, Ostaszewska-Bugajska M, Zebrowski J, Solecka D, et al. Altered cell wall plasticity can restrict plant growth under ammonium nutrition. *Front Plant Sci.* (2017) 8:1344. doi: 10.3389/fpls.2017.01344
132. Premont RT, Gainetdinov RR, Caron MG. Following the trace of elusive amines. *Proc Natl Acad Sci USA*. (2001) 98:9474–5. doi: 10.1073/pnas.181356198
133. Spano G, Russo P, Lonvaud-Funel A, Lucas P, Alexandre H, Grandvalet C, et al. Biogenic amines in fermented foods. *Eur J Clin Nutr.* (2010) 64(Suppl 3):S95–100. doi: 10.1038/ejcn.2010.218
134. Williams BB, Van Benschoten AH, Cimermancic P, Donia MS, Zimmermann M, Taketani M, et al. Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell Host Microbe* (2014) 16:495–503. doi: 10.1016/j.chom.2014.09.001
135. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* (2015) 161:264–76. doi: 10.1016/j.cell.2015.02.047
136. Galligan JJ. Beneficial actions of microbiota-derived tryptophan metabolites. *Neurogastroenterol Motil.* (2018) 30:e13283. doi: 10.1111/nmo.13283
137. Spaepen S, Vanderleyden J, Remans R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol Rev.* (2007) 31:425–48. doi: 10.1111/j.1574-6976.2007.00072.x
138. Dowling DN, O'Gara F. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends Biotechnol.* (1994) 12:133–41. doi: 10.1016/0167-7799(94)90091-4
139. Stringlis IA, Zhang H, Pieterse CMJ, Bolton MD, de Jonge R. Microbial small molecules - weapons of plant subversion. *Nat Prod Rep.* (2018) 35:410–33. doi: 10.1039/c7np00062f
140. Loeschcke A, Thies S. *Pseudomonas putida*—a versatile host for the production of natural products. *Appl Microbiol Biotechnol.* (2015) 99:6197–14. doi: 10.1007/s00253-015-6745-4
141. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. *Cell Metab.* (2014) 20:719–30. doi: 10.1016/j.cmet.2014.10.016
142. Miethke M, Marahiel MA. Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol Rev.* (2007) 71:413–51. doi: 10.1128/MMBR.00012-07
143. Garcia-Gutierrez E, Mayer MJ, Cotter PD, Narbad A. Gut microbiota as a source of novel antimicrobials. *Gut Microbes* (2018) 27:1–21. doi: 10.1080/19490976.2018.1455790
144. Walsh CJ, Guinane CM, Hill C, Ross RP, O'Toole PW, Cotter PD. *In silico* identification of bacteriocin gene clusters in the gastrointestinal tract, based on the Human Microbiome Project's reference genome database. *BMC Microbiol.* (2015) 15:183. doi: 10.1186/s12866-015-0515-4
145. Donia MS, Cimermancic P, Schulze CJ, Wieland Brown LC, Martin J, Mitreva M, et al. A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. *Cell* (2014) 158:1402–14. doi: 10.1016/j.cell.2014.08.032
146. Taga ME, Bassler BL. Chemical communication among bacteria. *Proc Natl Acad Sci USA* (2003) 100(Suppl.):14549–54. doi: 10.1073/pnas.1934514100
147. Bruhn JB, Dalsgaard I, Nielsen KF, Buchholtz C, Larsen JL, Gram L. Quorum sensing signal molecules (acylated homoserine lactones) in gram-negative fish pathogenic bacteria. *Dis Aquat Organ.* (2005) 65:43–52. doi: 10.3354/dao065043
148. Swearingen MC, Sabag-Daigle A, Ahmer BMM. Are there acyl-homoserine lactones within mammalian intestines? *J Bacteriol.* (2013) 195:173–9. doi: 10.1128/JB.01341-12
149. Federle MJ. Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. *Contrib Microbiol.* (2009) 16:18–32. doi: 10.1159/000219371
150. Thompson JA, Oliveira RA, Djukovic A, Ubeda C, Xavier KB. Manipulation of the quorum sensing signal AI-2 affects the antibiotic-treated gut microbiota. *Cell Rep.* (2015) 10:1861–71. doi: 10.1016/j.celrep.2015.02.049
151. Sugita H, Miyajima C, Deguchi Y. The vitamin B12-producing ability of the intestinal microflora of freshwater fish. *Aquaculture* (1991) 92:267–76. doi: 10.1016/0044-8486(91)90028-6
152. Degnan PH, Taga ME, Goodman AL. Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab.* (2014) 20:769–78. doi: 10.1016/j.cmet.2014.10.002
153. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr.* (2002) 22:283–307. doi: 10.1146/annurev.nutr.22.011602.092259
154. Magnúsdóttir S, Ravcheev D, de Crécy-Lagard V, Thiele I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Front Genet.* (2015) 6:148. doi: 10.3389/fgene.2015.00148
155. Biesalski HK. Nutrition meets the microbiome: micronutrients and the microbiota. *Ann N Y Acad Sci.* (2016) 1372:53–64. doi: 10.1111/nyas.13145
156. Smith AG, Croft MT, Moulin M, Webb ME. Plants need their vitamins too. *Curr Opin Plant Biol.* (2007) 10:266–75. doi: 10.1016/j.pbi.2007.04.009
157. Bittel P, Robatzek S. Microbe-associated molecular patterns (MAMPs) probe plant immunity. *Curr Opin Plant Biol.* (2007) 10:335–41. doi: 10.1016/j.pbi.2007.04.021
158. Newman M-A, Sundelin T, Nielsen JT, Erbs G. MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front Plant Sci.* (2013) 4:139. doi: 10.3389/fpls.2013.00139
159. Nürnberger T, Brunner F, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev.* (2004) 198:249–66. doi: 10.1111/j.0105-2896.2004.0119.x
160. Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol.* (2005) 6:973. doi: 10.1038/ni1253
161. Mackey D, McFall A. MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Mol Microbiol.* (2006) 61:1365–71. doi: 10.1111/j.1365-2958.2006.05311.x
162. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio C-W, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature* (2011) 478:250. doi: 10.1038/nature10434

163. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu T-T, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* (2009) 458:351–6. doi: 10.1038/nature07674
164. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* (2011) 331:337–41. doi: 10.1126/science.1198469
165. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* (2005) 122:107–18. doi: 10.1016/j.cell.2005.05.007
166. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. *Nat Commun.* (2014) 5:3654. doi: 10.1038/ncomms4654
167. Obregon-Tito AJ, Tito RY, Metcalf J, Sankaranarayanan K, Clemente JC, Ursell LK, et al. Subsistence strategies in traditional societies distinguish gut microbiomes. *Nat Commun.* (2015) 6:6505. doi: 10.1038/ncomms7505
168. Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-induced extinction in the gut microbiota compounds over generations. *Nature* (2016) 529:212–5. doi: 10.1038/nature16504
169. Clayton JB, Vangay P, Huang H, Ward T, Hillmann BM, Al-Ghalith GA, et al. Captivity humanizes the primate microbiome. *Proc Natl Acad Sci USA*. (2016) 113:10376–81. doi: 10.1073/pnas.1521835113
170. Metcalf JL, Song SJ, Morton JT, Weiss S, Seguin-Orlando A, Joly F, et al. Evaluating the impact of domestication and captivity on the horse gut microbiome. *Sci Rep.* (2017) 7:15497. doi: 10.1038/s41598-017-15375-9
171. Kreisinger J, Dagmar C, Vohánka J, Piálek J. Gastrointestinal microbiota of wild and inbred individuals of two house mouse subspecies assessed using high-throughput parallel pyrosequencing. *Mol Ecol.* (2014) 23:5048–60. doi: 10.1111/mec.12909
172. Xenoulis PG, Gray PL, Brightsmith D, Palculict B, Hoppes S, Steiner JM, et al. Molecular characterization of the cloacal microbiota of wild and captive parrots. *Vet Microbiol.* (2010) 146:320–5. doi: 10.1016/j.vetmic.2010.05.024
173. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Muller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol.* (2011) 128:645–6. doi: 10.1016/j.jaci.2011.04.060
174. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* (2004) 53:685–93. doi: 10.1136/gut.2003.025403
175. O'Donnell AG, Seasman M, Macrae A, Waite I, Davies JT. Plants and fertilisers as drivers of change in microbial community structure and function in soils. *Plant Soil* (2001) 232:135–45. doi: 10.1023/A:1010394221729
176. Williams TR, Marco ML. Phyllosphere microbiota composition and microbial community transplantation on lettuce plants grown indoors. *MBio* (2014) 5:e01564-14. doi: 10.1128/mBio.01564-14
177. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell* (2017) 171:1015.e13–28.e13. doi: 10.1016/j.cell.2017.09.016
178. Castillo M, Martín-Orúe SM, Manzanilla EG, Badiola I, Martín M, Gasa J. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet Microbiol.* (2006) 114:165–70. doi: 10.1016/j.vetmic.2005.11.055
179. Ushida K, Tsuchida S, Ogura Y, Toyoda A, Maruyama F. Domestication and cereal feeding developed domestic pig-type intestinal microbiota in animals of suidae. *Anim Sci J.* (2016) 87:835–41. doi: 10.1111/asj.12492
180. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE* (2017) 12:e0169851. doi: 10.1371/journal.pone.0169851
181. Ribeiro GO, Oss DB, He Z, Gruninger RJ, Elekwachi C, Forster RJ, et al. Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. *Sci Rep.* (2017) 7:1276. doi: 10.1038/s41598-017-01269-3
182. Rungrassamee W, Klanchui A, Maibunkaew S, Chaiyapechara S, Jiravanichpaisal P, Karoonuthaisiri N. Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). *PLoS ONE* (2014) 9:e91853. doi: 10.1371/journal.pone.0091853
183. Dhanasiri AKS, Brunvold L, Brinchmann MF, Korsnes K, Bergh Ø, Kiron V. Changes in the intestinal microbiota of wild atlantic cod *Gadus morhua* L. upon captive rearing. *Microb Ecol.* (2011) 61:20–30. doi: 10.1007/s00248-010-9673-y
184. Pieterse CMJ, de Jonge R, Berendsen RL. The soil-borne supremacy. *Trends Plant Sci.* (2016) 21:171–3. doi: 10.1016/j.tplants.2016.01.018
185. Zachow C, Müller H, Tilcher R, Berg G. Differences between the rhizosphere microbiome of Beta vulgaris ssp. maritima—ancestor of all beet crops—and modern sugar beets. *Front Microbiol.* (2014) 5:415. doi: 10.3389/fmicb.2014.00415
186. Campisano A, Pancher M, Puopolo G, Puddu A, Lòpez-Fernàndez S, Biagini B, et al. Diversity in endophyte populations reveals functional and taxonomic diversity between wild and domesticated grapevines. *Am J Enol Vitic.* (2015) 66:12 LP-21. doi: 10.5344/ajev.2014.14046
187. Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM. Hormonal Modulation of Plant Immunity. *Annu Rev Cell Dev Biol.* (2012) 28:489–521. doi: 10.1146/annurev-cellbio-092910-154055
188. Roehe R, Dewhurst RJ, Duthie C-A, Rooke JA, McKain N, Ross DW, et al. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLOS Genet.* (2016) 12:e1005846. doi: 10.1371/journal.pgen.1005846
189. Camarinha-Silva A, Maushammer M, Wellmann R, Vital M, Preuss S, Bennewitz J. Host genome influence on gut microbial composition and microbial prediction of complex traits in pigs. *Genetics* (2017) 206:1637–44. doi: 10.1534/genetics.117.200782
190. Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J, et al. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA*. (2010) 107:18933–8. doi: 10.1073/pnas.1007028107
191. Li X, Yu Y, Feng W, Yan Q, Gong Y. Host species as a strong determinant of the intestinal microbiota of fish larvae. *J Microbiol.* (2012) 50:29–37. doi: 10.1007/s12275-012-1340-1
192. Pratte ZA, Besson M, Hollman RD, Stewart FJ. The gills of reef fish support a distinct microbiome influenced by host-specific factors. *Appl Environ Microbiol.* (2018) 84:e00063-18. doi: 10.1128/AEM.00063-18
193. Pérez-Jaramillo JE, Mendes R, Raaijmakers JM. Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol Biol.* (2016) 90:635–44. doi: 10.1007/s11103-015-0337-7
194. Mendes LW, Raaijmakers JM, de Hollander M, Mendes R, Tsai SM. Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. *ISME J.* (2018) 12:212–24. doi: 10.1038/ismej.2017.158
195. Doyle CJ, Gleeson D, O'Toole PW, Cotter PD. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. *Appl Environ Microbiol.* (2017) 83:e02694-16. doi: 10.1128/AEM.02694-16
196. Kubasova T, Davidova-Gerzova L, Merlot E, Medvecky M, Polansky O, Gardan-Salmon D, et al. Housing systems influence gut microbiota composition of sows but not of their piglets. *PLoS ONE* (2017) 12:e0170051. doi: 10.1371/journal.pone.0170051
197. Dehler CE, Secombes CJ, Martin SAM. Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Sci Rep.* (2017) 7:13877. doi: 10.1038/s41598-017-13249-8
198. Giatsis C, Sipkema D, Smidt H, Heilig H, Benvenuti G, Verreth J, et al. The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Sci Rep.* (2015) 5:18206. doi: 10.1038/srep18206
199. Schmidt, VT, Smith, KF, Melvin, DW, Amaral-Zettler, LA. Community assembly of a euryhaline fish microbiome during salinity acclimation. *Mol Ecol.* (2015) 24:2537–50. doi: 10.1111/mec.13177
200. Sonnenburg ED, Sonnenburg JL. Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab.* (2014) 20:779–86. doi: 10.1016/j.cmet.2014.07.003
201. Niu Q, Li P, Hao S, Zhang Y, Kim SW, Li H, et al. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Sci Rep.* (2015) 5:9938. doi: 10.1038/srep09938
202. Shanks OC, Kelty CA, Archibeque S, Jenkins M, Newton RJ, McLellan SL, et al. Community structures of fecal bacteria in cattle from different

animal feeding operations. *Appl Environ Microbiol.* (2011) 77:2992–3001. doi: 10.1128/AEM.02988-10
203. Meale SJ, Li S, Azevedo P, Derakhshani H, Plaizier JC, Khafipour E, et al. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front Microbiol.* (2016) 7:582. doi: 10.3389/fmicb.2016.00582
204. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends Microbiol.* (2017) 25:851–73. doi: 10.1016/j.tim.2017.05.004
205. Wu S, Wang G, Angert ER, Wang W, Li W, Zou H. Composition, diversity, and origin of the bacterial community in grass carp intestine. *PLoS ONE* (2012) 7:e30440. doi: 10.1371/journal.pone.0030440
206. Li H, Wu S, Wirth S, Hao Y, Wang W, Zou H, et al. Diversity and activity of cellulolytic bacteria, isolated from the gut contents of grass carp (*Ctenopharyngodon idellus*) (Valenciennes) fed on Sudan grass (*Sorghum sudanense*) or artificial feedstuffs. *Aquac Res.* (2014) 47:153–64. doi: 10.1111/are.12478
207. Ringø E, Zhou Z, Vecino JLG, Wadsworth S, Romero J, Krogdahl Å, et al. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquac Nutr.* (2015) 22:219–82. doi: 10.1111/anu.12346
208. Jangid K, Williams MA, Franzluebbers AJ, Sanderlin JS, Reeves JH, Jenkins MB, et al. Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems. *Soil Biol Biochem.* (2008) 40:2843–53. doi: 10.1016/j.soilbio.2008.07.030
209. Santhanam R, Luu VT, Weinhold A, Goldberg J, Oh Y, Baldwin IT. Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. *Proc Natl Acad Sci USA.* (2015) 112:E5013-20. doi: 10.1073/pnas.1505765112
210. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, et al. Cohabiting family members share microbiota with one another and with their dogs. *Elife* (2013) 2:e00458. doi: 10.7554/eLife.00458
211. Kraemer JG, Ramette A, Aebi S, Oppliger A, Hilty M. Influence of pig farming on the human's nasal microbiota: the key role of the airborne microbial communities. *Appl Environ Microbiol.* (2018) 12:AEM.02470-17. doi: 10.1128/AEM.02470-17
212. Soverini M, Rampelli S, Turroni S, Schnorr SL, Quercia S, Castagnetti A, et al. Variations in the post-weaning human gut metagenome profile as result of *Bifidobacterium* acquisition in the western microbiome. *Front Microbiol.* (2016) 7:1058. doi: 10.3389/fmicb.2016.01058
213. Milani C, Mangifesta M, Mancabelli L, Lugli GA, James K, Duranti S, et al. Unveiling bifidobacterial biogeography across the mammalian branch of the tree of life. *ISME J.* (2017) 11:2834. doi: 10.1038/ismej.2017.138
214. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci.* (2005) 84:634–43. doi: 10.1093/ps/84.4.634
215. van Schaik W. The human gut resistome. *Philos Trans R Soc Lond B Biol Sci.* (2015) 370:20140087. doi: 10.1098/rstb.2014.0087
216. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol.* (2014) 12:465–78. doi: 10.1038/nrmicro3270
217. Mu C, Yang Y, Su Y, Zoetendal EG, Zhu W. Differences in microbiota membership along the gastrointestinal tract of piglets and their differential alterations following an early-life antibiotic intervention. *Front Microbiol.* (2017) 8:797. doi: 10.3389/fmicb.2017.00797
218. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA.* (2012) 109:1691–6. doi: 10.1073/pnas.1120238109
219. Van Vleck Pereira R, Lima S, Siler JD, Foditsch C, Warnick LD, Bicalho RC. Ingestion of milk containing very low concentration of antimicrobials: longitudinal effect on fecal microbiota composition in preweaned calves. *PLoS ONE* (2016) 11:e0147525. doi: 10.1371/journal.pone.0147525
220. Navarrete P, Mardones P, Opazo R, Espejo R, Romero J. Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. *J Aquat Anim Health* (2011) 20:177–83. doi: 10.1577/H07-043.1
221. Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S. Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. *Soil Biol Biochem.* (2008) 40:1583–91. doi: 10.1016/j.soilbio.2008.01.010
222. Liu F, Ying G-G, Tao R, Zhao J-L, Yang J-F, Zhao L-F. Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environ Pollut.* (2009) 157:1636–42. doi: 10.1016/j.envpol.2008.12.021
223. Xiong W, Sun Y, Zhang T, Ding X, Li Y, Wang M, et al. Antibiotics, antibiotic resistance genes and bacterial community composition in fresh water aquaculture environment in China. *Microb Ecol.* (2015) 70:425–32. doi: 10.1007/s00248-015-0583-x
224. Petersen A, Andersen JS, Kaewmak T, Somsiri T, Dalsgaard A. Impact of integrated fish farming on antimicrobial resistance in a pond environment. *Appl Environ Microbiol.* (2002) 68:6036–42. doi: 10.1128/AEM.68.12.6036-6042.2002
225. Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol.* (2006) 8:1137–44. doi: 10.1111/j.1462-2920.2006.01054.x
226. Marshall BM, Levy SB. Food animals and antimicrobials: impacts on human health. *Clin Microbiol Rev.* (2011) 24:718–33. doi: 10.1128/CMR.00002-11
227. Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K. Fate and effects of veterinary antibiotics in soil. *Trends Microbiol.* (2014) 22:536–45. doi: 10.1016/j.tim.2014.05.005
228. Mark Welch JL, Hasegawa Y, McNulty NP, Gordon JI, Borisy GG. Spatial organization of a model 15-member human gut microbiota established in gnotobiotic mice. *Proc Natl Acad Sci USA.* (2017) 114:E9105–14. doi: 10.1073/pnas.1711596114
229. Marteau P, Pochart P, Doré J, Béra-Maillet C, Bernalier A, Corthier G. Comparative study of bacterial groups within the human cecal and fecal microbiota. *Appl Environ Microbiol.* (2001) 67:4939–42. doi: 10.1128/AEM.67.10.4939-4942.2001
230. Wang W, Wu S, Zheng Y, Cheng Y, Li W, Zou H, et al. Characterization of the bacterial community associated with early-developmental stages of grass carp (*Ctenopharyngodon idella*). *Aquac Res.* (2014) 46:2728–35. doi: 10.1111/are.12428
231. Li X, Rui J, Xiong J, Li J, He Z, Zhou J, et al. Functional potential of soil microbial communities in the maize rhizosphere. *PLoS ONE* (2014) 9:e112609. doi: 10.1371/journal.pone.0112609
232. Stepanauskas R. Single cell genomics: an individual look at microbes. *Curr Opin Microbiol.* (2012) 15:613–20. doi: 10.1016/j.mib.2012.09.001
233. Lagier J-C, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat Microbiol.* (2016) 1:16203. doi: 10.1038/nmicrobiol.2016.203
234. Stepanauskas R, Fergusson EA, Brown J, Poulton NJ, Tupper B, Labonté JM, et al. Improved genome recovery and integrated cell-size analyses of individual uncultured microbial cells and viral particles. *Nat Commun.* (2017) 8:84. doi: 10.1038/s41467-017-00128-z
235. Radajewski S, Ineson P, Parekh NR, Murrell JC. Stable-isotope probing as a tool in microbial ecology. *Nature* (2000) 403:646–9. doi: 10.1038/35001054
236. Berry D, Stecher B, Schintlmeister A, Reichert J, Brugiroux S, Wild B, et al. Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. *Proc Natl Acad Sci USA.* (2013) 110:4720–5. doi: 10.1073/pnas.1219247110
237. Jiang B, Jin N, Xing Y, Su Y, Zhang D. Unraveling uncultivable pesticide degraders via stable isotope probing (SIP). *Crit Rev Biotechnol.* (2018) 38:1025–48. doi: 10.1080/07388551.2018.1427697
238. Vernocchi P, Del Chierico F, Putignani L. Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health. *Front Microbiol.* (2016) 7:1144. doi: 10.3389/fmicb.2016.01144
239. Berg G, Eberl L, Hartmann A. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol.* (2005) 7:1673–85. doi: 10.1111/j.1462-2920.2005.00891.x
240. Hardoim PR, van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, et al. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev.* (2015) 79:293–320. doi: 10.1128/MMBR.00050-14
241. Iraola G, Forster SC, Kumar N, Lehours P, Bekal S, García-Peña FJ, et al. Distinct *Campylobacter fetus* lineages adapted as livestock pathogens and human pathobionts in the intestinal microbiota. *Nat Commun.* (2017) 8:1367. doi: 10.1038/s41467-017-01449-9

242. Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heidelberg JF, Read TD, et al. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci USA.* (2002) 99:13148–53. doi: 10.1073/pnas.192319099
243. Rosenberg E, Zilber-Rosenberg I. Pathogens as symbionts BT. In: Rosenberg E, Zilber-Rosenberg I. *The Hologenome Concept: Human, Animal and Plant Microbiota* (Cham: Springer International Publishing), 129–49. doi: 10.1007/978-3-319-04241-1_9
244. Postler TS, Ghosh S. Understanding the holobiont: how microbial metabolites affect human health and shape the immune system. *Cell Metab.* (2017) 26:110–30. doi: 10.1016/j.cmet.2017.05.008
245. Toft C, Andersson SGE. Evolutionary microbial genomics: insights into bacterial host adaptation. *Nat Rev Genet.* (2010) 11:465. doi: 10.1038/nrg2798
246. Hevia A, Delgado S, Sánchez B, Margolles A. Molecular players involved in the interaction between beneficial bacteria and the immune system. *Front Microbiol.* (2015) 6:1285. doi: 10.3389/fmicb.2015.01285
247. Quevillon-Cheruel S, Leulliot N, Muniz CA, Vincent M, Gallay J, Argentini M, et al. Evf, a virulence factor produced by the *Drosophila* pathogen *Erwinia carotovora*, is an S-palmitoylated protein with a new fold that binds to lipid vesicles. *J Biol Chem.* (2009) 284:3552–62. doi: 10.1074/jbc.M808334200
248. Jetten MSM. The microbial nitrogen cycle. *Environ Microbiol.* (2008) 10:2903–9. doi: 10.1111/j.1462-2920.2008.01786.x
249. Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA. Nitrogen fixation by symbiotic and free-living spirochetes. *Science* (2001) 292:2495–8. doi: 10.1126/science.1060281
250. Ngugi DK, Brune A. Nitrate reduction, nitrous oxide formation, and anaerobic ammonia oxidation to nitrite in the gut of soil-feeding termites (*Cubitermes* and *Ophiotermes* spp.). *Environ Microbiol.* (2012) 14:860–71. doi: 10.1111/j.1462-2920.2011.02648.x
251. Igai K, Itakura M, Nishijima S, Tsurumaru H, Suda W, Tsutaya T, et al. Nitrogen fixation and *nifH* diversity in human gut microbiota. *Sci Rep.* (2016) 6:31942. doi: 10.1038/srep31942
252. Morris, BE, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. *FEMS Microbiol Rev.* (2013) 37:384–406. doi: 10.1111/1574-6976.12019
253. Carbonero F, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat Rev Gastroenterol Hepatol.* (2012) 9:504–18. doi: 10.1038/nrgastro.2012.85
254. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev.* (1997) 61:262–80.
255. Chung KT. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum*. *Appl Environ Microbiol.* (1976) 31:342–8.
256. Rey FE, Faith JJ, Bain J, Muehlbauer MJ, Stevens RD, Newgard CB, et al. Dissecting the *in vivo* metabolic potential of two human gut acetogens. *J Biol Chem.* (2010) 285:22082–90. doi: 10.1074/jbc.M110.117713
257. Schmidt TSB, Raes J, Bork P. The human gut microbiome: from association to modulation. *Cell* (2018) 172:1198–215. doi: 10.1016/j.cell.2018.02.044
258. Lucy M, Reed E, Glick BR. Applications of free living plant growth-promoting rhizobacteria. *Anton Van Leeuwen.* (2004) 86:1–25. doi: 10.1023/B:ANTO.0000024903.10757.6e
259. Sachsenröder J, Twardziok SO, Scheuch M, Johne R. The general composition of the faecal virome of pigs depends on age, but not on feeding with a probiotic bacterium. *PLoS ONE* (2014) 9:e88888. doi: 10.1371/journal.pone.0088888
260. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* (2011) 334:105–8. doi: 10.1126/science.1208344

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

CORRECTION
published: 28 November 2018
doi: 10.3389/fnut.2018.00113

Corrigendum: How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture

Wakako Ikeda-Ohtsubo[1], Sylvia Brugman[2], Craig H. Warden[3], Johanna M. J. Rebel[4], Gert Folkerts[5] and Corné M. J. Pieterse[6]*

[1] Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2] Cell Biology and Immunology Group, Wageningen University and Research, Wageningen, Netherlands, [3] Departments of Pediatrics, Neurobiology Physiology and Behavior, University of California, Davis, Davis, CA, United States, [4] Wageningen Livestock Research, Wageningen University and Research, Wageningen, Netherlands, [5] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Netherlands, [6] Plant–Microbe Interactions, Department of Biology, Science4Life, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Approved by:
Frontiers in Nutrition Editorial Office, Frontiers Media SA, Switzerland

****Correspondence:***
Wakako Ikeda-Ohtsubo
wakako.ohtsubo@tohoku.ac.jp

Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *15 October 2018*
Accepted: *08 November 2018*
Published: *28 November 2018*

Citation:
Ikeda-Ohtsubo W, Brugman S, Warden CH, Rebel JMJ, Folkerts G and Pieterse CMJ (2018) Corrigendum: How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture. Front. Nutr. 5:113. doi: 10.3389/fnut.2018.00113

Keywords: microbiota, agriculture, animal husbandry, aquaculture, rhizosphere, phyllosphere, agricultural immunology

A Corrigendum on

How Can We Define "Optimal Microbiota?": A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture
by Ikeda-Ohtsubo, W., Brugman, S., Warden, C. H., Rebel, J. M. J., Folkerts, G., and Pieterse, C. M. J. (2018) Front. Nutr. 5:90. doi: 10.3389/fnut.2018.00090

In the original article, we regret that the following Funding statement was missing:
This work was financially supported by the Japan Society for the Promotion of Science (JSPS) through JSPS Core-to-Core Program (Advanced Research Networks) entitled Establishment of international agricultural immunology research-core for a quantum improvement in food safety.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 06 December 2018
doi: 10.3389/fimmu.2018.02884

A Functionally Different Immune Phenotype in Cattle Is Associated With Higher Mastitis Incidence

Karina Lutterberg[1†], Kristina J. H. Kleinwort[1†], Bernhard F. Hobmaier[1], Stefanie M. Hauck[2], Stefan Nüske[3], Armin M. Scholz[3] and Cornelia A. Deeg[1]*

[1] Chair of Animal Physiology, Department of Veterinary Sciences, LMU Munich, Munich, Germany, [2] Research Unit for Protein Science, Helmholtz Zentrum Munich, German Research Center for Environmental Health GmbH, Munich, Germany, [3] Livestock Center of the Faculty of Veterinary Medicine, LMU Munich, Oberschleißheim, Germany

A novel vaccine against bovine viral diarrhea (BVD) induced pathogenic antibody production in 5–10% of BVD-vaccinated cows. Transfer of these antibodies via colostrum caused Bovine neonatal pancytopenia (BNP) in calves, with a lethality rate of 90%. The exact immunological mechanisms behind the onset of BNP are not fully understood to date. To gain further insight into these mechanisms, we analyzed the immune proteome from alloreactive antibody producers (BNP cows) and non-responders. After *in vitro* stimulation of peripheral blood derived lymphocytes (PBL), we detected distinctly deviant expression levels of several master regulators of immune responses in BNP cells, pointing to a changed immune phenotype with severe dysregulation of immune response in BNP cows. Interestingly, we also found this response pattern in 22% of non-BVD-vaccinated cows, indicating a genetic predisposition of this immune deviant (ID) phenotype in cattle. We additionally analyzed the functional correlation of the ID phenotype with 10 health parameters and 6 diseases in a retrospective study over 38 months. The significantly increased prevalence of mastitis among ID cows emphasizes the clinical relevance of this deviant immune response and its potential impact on the ability to fight infections.

Keywords: bovine neonatal pancytopenia, hyperproliferation, differential immune proteome, Concanavalin A, STAT, T helper pathways, deviant immune phenotype, mastitis

OPEN ACCESS

Edited by:
Willem Van Eden, Utrecht University, Netherlands

Reviewed by:
Sylvia Brugman, Wageningen University & Research, Netherlands
Victor PMG Rutten, Utrecht University, Netherlands

***Correspondence:**
Cornelia A. Deeg
cornelia.deeg@lmu.de

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: *19 May 2018*
Accepted: *23 November 2018*
Published: *06 December 2018*

Citation:
Lutterberg K, Kleinwort KJH, Hobmaier BF, Hauck SM, Nüske S, Scholz AM and Deeg CA (2018) A Functionally Different Immune Phenotype in Cattle Is Associated With Higher Mastitis Incidence. Front. Immunol. 9:2884. doi: 10.3389/fimmu.2018.02884

INTRODUCTION

Vaccines are the most effective and also affordable disease-prevention tools (1) and maternal antibodies protect the offspring from infections in man (2) and animals (3). In cattle, pre-partum vaccination with various virulence factors of bacterial or viral infectious diseases is a standard procedure which effectively stimulates the production of specific antibodies (4, 5). A novel production technology using an allogeneic cell line (6) and the addition of a highly potent adjuvant to PregSure BVD, a new vaccine against bovine viral diarrhea (BVD), induced fatal immune reactions with production of alloreactive antibodies in 5–10% of vaccinated cows (7–9). These alloantibodies were transmitted to calves, regardless of kin, via the colostrum of these dams (8, 10) and caused bovine neonatal pancytopenia (BNP) (7, 11–13), even years after the vaccine has been taken off the market (14). BNP calves suffered from hemorrhagic diathesis, thrombo and leukocytopenia and bone marrow depletion, which resulted in a deadly bleeding disorder (10, 15, 16). Several authors hypothesized alloimmune reactions against the major histocompatibility complex class I (MHC I) as causal for BNP (17–20). This hypothesis is still

controversial since MHC I alloantibodies would be expected to bind all nucleated cells, not only to blood-derived and hematopoietic progenitor cells (12, 16). Also transcriptome analyses provided evidence that MHC I can be excluded as single causal agent for BNP-associated alloantibodies (21). As other authors claimed the possibility of a genetic predisposition of BNP dams for production of BNP inducing alloantibodies (22–24), our studies focused on investigating a general difference in immune responses between PregSure BVD vaccinated control cows and BNP donors. We wanted to analyze if there is evidence for differential expression of master transcription factors of cellular immune response in lymphocyte proteome of controls and BNP dams. Interestingly, we found a markedly different immune phenotype in BNP dams, characterized by significant hyperproliferation of lymphocytes and a deviant immune signaling pathway compared to controls. We were next interested to see if we could find evidence for this immune phenotype in cows that were never vaccinated with PregSure BVD. Further testing showed that this altered immune phenotype was also detectable in 22% of cows from a PregSure BVD unvaccinated cohort. This confirmed its natural, not vaccine-induced, appearance in cattle and gave more evidence for immune deviant (ID) phenotype as a genetic predisposition. In further in-depth experiments we found that Interleukin-2 (IL-2) plays an important role in diverging immune response of ID lymphocytes. In addition to these immune response studies, we analyzed the functional correlation of the ID phenotype with overall 10 health parameters and 6 diseases in a retrospective study over 38 months. Here, a significantly increased mastitis incidence in cows with an ID phenotype became apparent, highlighting the clinical impact of altered immune response.

MATERIALS AND METHODS

In this study, peripheral blood derived lymphocytes (PBL) of a total of 132 cows were used. Eleven of these cows had previously been vaccinated with PregSure BVD, of which six did not produce BNP-inducing antibodies (BVD vaccinated control cows) and five showed alloreactive antibody production (BNP dams) resulting in their calves dying from hemorrhagic diathesis with confirmed thrombocytopenia, leukocytopenia and bone marrow depletion. The remaining 121 cows were healthy controls (female, age: 2–10 years, first to eight lactation period) from one dairy farm never vaccinated with PregSure BVD. No experimental animals were killed through this study. Withdrawal of blood was permitted by the local authority Regierung von Oberbayern, Munich, permit no. 55.2-1-54-2532.3-22-12.

PBL Isolation

Bovine venous blood samples were collected in tubes with heparin sodium 25.000 I.U.. Blood was diluted with equal parts of phosphate buffered saline (PBS; 136,9 mM NaCl, 2,6 mM KCl, 1,4 mM KH_2PO_4, 8,1 mM $Na_2HPO_4 \times 2H_2O$; pH 7,2) and subsequently layered on density gradient separating solution (Pancoll; PanBiotech, Aidenbach, Germany). After density gradient centrifugation (room temperature, 290 × g, 25 min, brake off), PBL were obtained from intermediate phase. Cells were washed twice in PBS (4°C) and either used immediately or stored at −20°C (lysate) for western blot analyses.

Proliferation Assays

PBL were seeded in 96-well plates (1×10^5 cells/well) and stimulated for 48 h with either PWM (Pokeweed mitogen), ConA (Concanavalin A) or BanLec (Banana lectin) (Sigma-Aldrich, Taufkirchen, Germany, 5 µg/ml), bovine IL-2 (Bio-Techne, Wiesbaden, Germany, 1 ng/ml), bovine IL-4 or bovine Interferon gamma (IFNγ) (Thermo Fisher Scientific, Bremen, Germany, 3 ng/ml). For the inhibition assays, cells were incubated with STAT3 inhibitor (WP 1,066, Santa Cruz, Heidelberg, Germany, 50 ng/ml) 12 h before stimulation. After 34 h of stimulation, cells were pulsed for 14 h with 0,05 mCi/well [methyl-^{3}H]-thymidine (Perkin Elmer, Hamburg, Germany), harvested and counts per minute were measured. Proliferation rate after stimulation was expressed as factor of [^{3}H]-thymidine incorporation with respect to the unstimulated cells or after inhibition as percentage to ConA stimulated cells.

Flow Cytometry Analyses

Staining of 5×10^5 cells per well was performed with anti-bovine cluster of differentiation (CD) 4 (mouse IgG1 monoclonal, Bio-Rad AbD Serotec, Puchheim, Germany; 1:100), anti-bovine CD8 (mouse IgG2a monoclonal, Bio-Rad AbD Serotec; 1:50) and FITC-conjugated anti-bovine IgM (Bio-Rad AbD Serotec, Puchheim, Germany; 1:50) antibodies, diluted in staining buffer (1% BSA + 0,001% NaN_3 in PBS). Respective secondary antibodies anti-mouse IgG1 FITC and anti-mouse IgG2a FITC (both Santa Cruz Biotechnology; Heidelberg, Germany 1:200) were added. All antibodies were incubated for 30 min on ice. Cells were washed with staining buffer between primary and secondary antibody staining steps (200 × g, 4°C, 1 min). For all stainings with secondary antibodies respective isotype controls were used. Cells were fixed in 1% PFA diluted in staining buffer and stored at 4°C until analysis. On FACS Canto II, measurement of cells was performed with FACS Diva Software (BD Biosciences). Lymphocytes were gated according to forward scatter (cell size) and side scatter (intercellular granularity) properties of cells. Per staining, between 5×10^3 and 1×10^4 cells were measured. Further analysis of flow cytometry data was performed using open source Flowing Software 2.5.1 http://flowingsoftware.btk.fi/ (Perttu Terho, Turku Centre for Biotechnology, Finland).

Differential Proteome Analyses

PBL (2.2×10^7 cells) of two PregSure BVD vaccinated control cows and two BNP dams were stimulated with PWM and ConA (5 µg/ml) for 48 h. For LC-MS/MS analysis, peptides were separated on a reversed chromatography column (75 µm ID × 15 cm, Acclaim PepMAP 100 C18. 100Å/size, LC Packings, Thermo Fisher Scientific, Bremen, Germany) and the analysis

Abbreviations: BanLec, Banana lectin; BNP, Bovine neonatal pancytopenia; ConA, Concanavalin A; CD, Cluster of differentiation; ID, Immundeviant phenotype; IFNγ, Interferon gamma; IL, Interleukin; JAK, Janus kinase; MHC I, major histocompatibility complex class I; PBL, peripheral blood lymphocytes; PWM, Pokeweed Mitogen; STAT, Signal transducer and activator of transcription; Tfh, follicular T helper cells; Th, T helper cells; Tr, regulatory T helper cells.

was conducted with an Ultimate 3000 nano-HPLC system (Dionex, Idstein, Germany). Nano-HPLC was connected in a linear quadruple ion trap-Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Up to 10 most intense ions were selected for fragmentation on the linear ion trap using collision induced dissociation at a target value of 100 ions and subsequently dynamically excluded for 30 s. Elevated spectra were imported into Progenesis software (version 2.5). After comparison and normalization, spectra were exported as Mascot Generic files and searched against the Ensembl bovine database (version 80) with Mascot (Matrix Science, version 2.4.1). Peptide assignment was reimported to Progenesis Software. All unique peptides allocated to a protein were considered for quantification. Only proteins quantified with at least two peptides were included for further analysis.

For hierarchical cluster analyses of normalized protein abundances, arithmetic means of two respective vaccinated control and BNP samples were generated and proteins with similar expression patterns were clustered with Perseus software algorithm (version 1.6.1.1; Computational Systems Biochemistry, Martinsried, Germany) (25). Proteins with a ratio of at least 2-fold in normalized abundance between control and BNP samples were defined as differentially.

Western Blots

For protein expression analyses with western blots, PBL were first lysed in lysis buffer (9 M Urea, 2 M Thiourea, 65 mM Dithioerythritol, 4% CHAPS). Proteins were then separated by SDS-PAGE on 8% gels (7 µg protein/slot) and blotted semidry onto 8.5 × 6 cm PVDF membranes (GE Healthcare, Freiburg, Germany) and blocked with 4% BSA (1 h). Blots were incubated overnight with respective primary antibodies: rabbit anti-pSTAT1 Tyr701, rabbit anti-pSTAT3 Tyr705 (Cell Signaling, Darmstadt, Germany, 1:500) or mouse anti-beta actin (Sigma, Taufkirchen, Germany, 1:5000). As secondary antibodies, either HRP-coupled goat anti-rabbit IgG (H+L) antibody (Cell Signaling, Darmstadt, Germany, 1:5000) or goat anti-mouse IgG (H+L) antibody (Sigma-Aldrich, Taufkirchen, Germany, 1:5000) were used (1 h). Signals were detected by enhanced chemiluminescence on X-ray film (SUPER-2000G ortho, Fuji; Christiansen, Planegg, Germany). Films were scanned on a transmission scanner and densitometric quantification of Western blot signals was performed using ImageJ software (open source: http://imagej.nih.gov/ij/). Abundances of pSTAT1 and pSTAT3 were subsequently normalized to beta actin.

Health Parameters

Milk production and health parameters (observed by veterinarians) of 100 non-PregSure BVD vaccinated cattle from one dairy farm were analyzed for 38 months retrospectively. Results were then correlated to immune response data of *in vitro* assays. Seventy-three control cows with low proliferation rate after ConA stimulation and 27 ID cows, which showed a BNP-like hyperproliferation to ConA, were included in this study. Ten health parameters [daily milk yield, average lactation performance (300 days), milk structure (lactose, fat, urea, somatic cell count), fertility parameters (amounts of inseminations, calving-to-conception intervals, medicinal induction of oestrus, ovarian cysts)] and 6 diseases [diseases of musculoskeletal system, claws, digestive tract, respiratory system, and metabolic disorders (ketosis, hypocalcemia)] were analyzed. All parameters were recorded and listed by the same veterinarians and the statistical analysis was performed using the odds ratio and chi-square distribution.

Statistical Analyses

Data were analyzed in Prism software (GraphPad, version 5.04) with Kolmogorow-Smirnow (KS) test. If KS test was significant ($p < 0.05$; normal distribution), Student's t-test was used for statistical analysis, if KS test was not significant ($p > 0.05$; no normal distribution) statistics were performed using Mann-Whitney test. For statistical analysis of health parameters, we used odds ratio and chi-square distribution. Differences were considered statistically significant with the following p-values: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Lymphocytes of BNP Dams Show Hyperproliferation After Polyclonal Stimulation *in vitro*

After *in vitro* stimulation (48 h) with T and B cell mitogen PWM (26) and T cell mitogen ConA (27), a clearly divergent reaction of lymphocytes from PregSure BVD vaccinated cows became evident. Cells from cows known to produce alloreactive BNP antibodies after vaccination (BNP

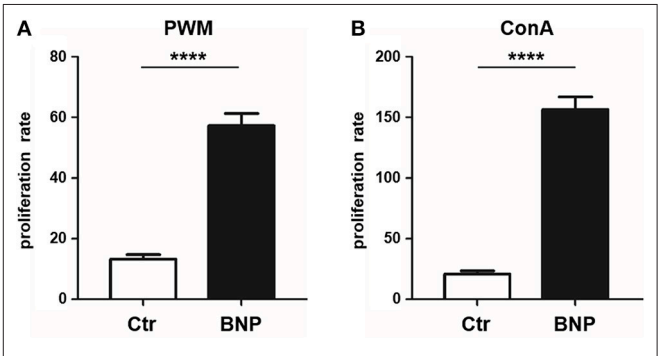


FIGURE 1 | Hyperproliferation of BNP lymphocytes after polyclonal stimulation *in vitro*. **(A)** Lymphocytes from BNP cows (black bars, $n = 5$, technical replicates $n = 98$) proliferated 4.5 times stronger ($^{****}p < 0.0001$ vs. control) after PWM stimulation than lymphocytes from control dams (white bars, $n = 6$, technical replicates $n = 53$). **(B)** Increased proliferation rate (8-fold; $^{****}p < 0.0001$ vs. control) of lymphocytes from BNP dams (black bars, $n = 5$, technical replicates $n = 86$) compared to control dams (white bars, $n = 6$, technical replicates $n = 52$) after *in vitro* stimulation with ConA. Proliferation rate shown in mean ± SD. Mann-Whitney test was used.

lymphocytes) proliferated significantly stronger (4.5-fold) than cells from vaccinated control dams after PWM stimulation (**Figure 1A**, BNP to Ctr, $^{****}p < 0.0001$) and ConA stimulation (8-fold stronger; **Figure 1B**, BNP to Ctr, $^{****}p < 0.0001$). Thus, *in vitro* proliferation assays revealed a highly significant hyperproliferation of BNP lymphocytes demonstrating an

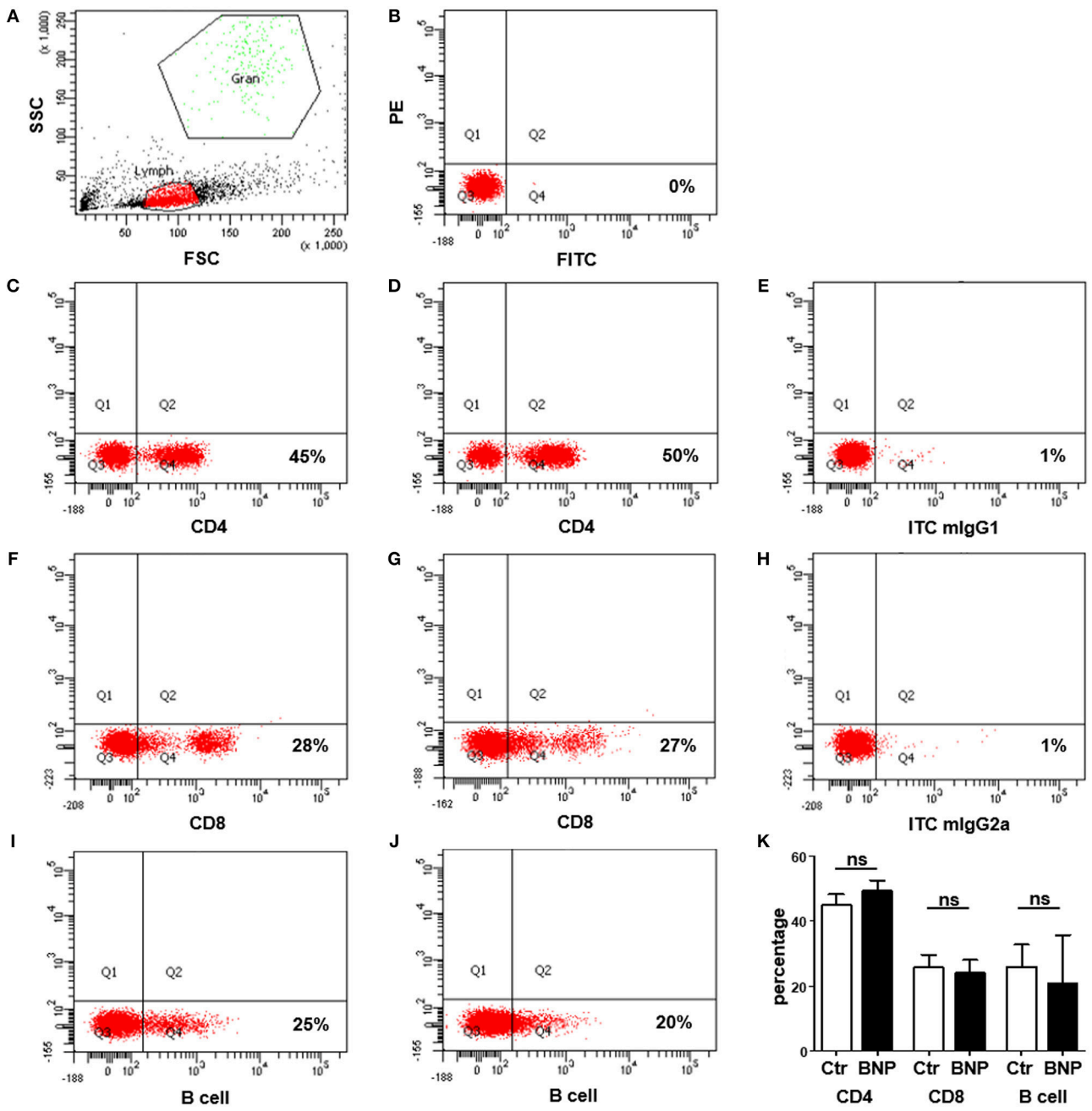
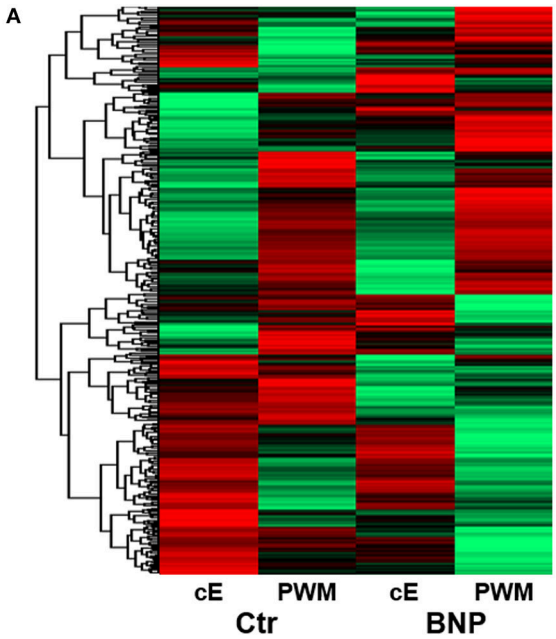
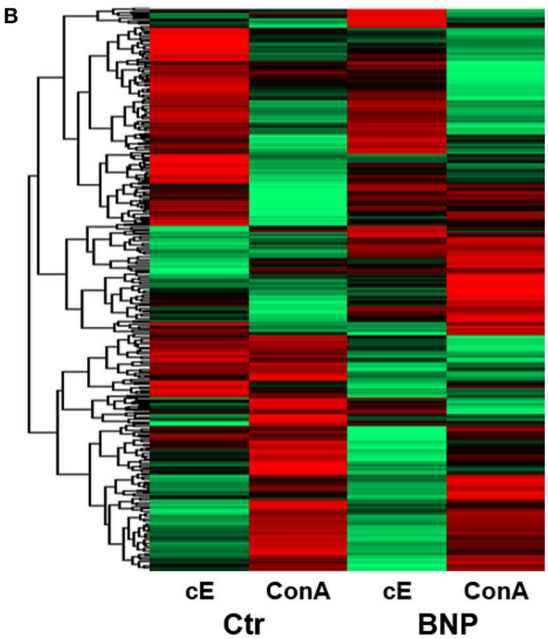


FIGURE 2 | No differences in distribution of lymphocyte subsets between control and BNP dams. **(A–J)** Representative dot plots of flow cytometry analyses from lymphocytes of control and BNP cows. **(A)** Identification of lymphocytes (Lymph) based upon properties of forward (FSC) and sideward scatter (SSC). The population to be measured is shown in red (lymphocytes). **(B)** Unstained cells in Q3 to determine position of quadrants for gating. **(C–J)** Respective staining shown in Q4. **(C)** Expression of CD4 in control lymphocytes (45%). **(D)** Expression of CD4 in BNP lymphocytes (50%). **(E)** Respective isotype control staining for CD4 antibody (mIgG1, 1%). **(F)** Expression of CD8 in control lymphocytes (28%). **(G)** Expression of CD8 in BNP lymphocytes (27%). **(H)** Respective isotype control staining for CD8 antibody (mIgG2a, 1%). **(I)** Expression of B cell marker IgM in control lymphocytes (25%). **(J)** Expression of B cell marker IgM in BNP lymphocytes (20%). **(K)** Flow cytometry analyses of lymphocyte subset distribution revealed no significant differences (ns) between control (white bars; $n = 4$) and BNP cells (black bars; $n = 3$). Percentage of subset is shown in mean ± SD. For statistical analyses Mann-Whitney test was used.





C

PWM			
Ensembl accession	Protein	Enrichment factor	
		Ctr	BNP
ENSBTAP00000010351	STAT1	1.3	0.8
ENSBTAP00000028687	STAT3	0.6	2.1
ENSBTAP00000047905	STAT4	1.0	1.0
ENSBTAP00000034715	STAT5A	0.5	2.0
ENSBTAP00000012497	STAT5B	0.7	1.4

D

ConA			
Ensembl accession	Protein	Enrichment factor	
		Ctr	BNP
ENSBTAP00000010351	STAT1	15.2	0.7
ENSBTAP00000005749	STAT2	0.7	1.5
ENSBTAP00000028687	STAT3	0.6	2.6
ENSBTAP00000047905	STAT4	0.8	1.3
ENSBTAP00000034715	STAT5A	1,2	1.3
ENSBTAP00000012497	STAT5B	0.4	1.9
ENSBTAP00000008311	STAT6	3.2	1.0

FIGURE 3 | Lymphocyte proteomes reveal regulation of differential expression of proteins between normal and deviant immune responders to PregSure BVD vaccination. Differential proteome analyses of PBL from control (Ctr, $n = 2$, shown as arithmetic means) and BNP ($n = 2$, shown as arithmetic means) cows already revealed differences in protein expression patterns in constitutive expression (cE) of proteins, that shifted even stronger after **(A)** PWM and **(B)** ConA stimulation (48 h). Highly abundant proteins are presented in green and low abundant proteins in red. **(C/D)** In-depth analysis of part of the proteome relating to regulation of immune cells revealed some differentially (factor ≥ 2.0) expressed STAT proteins in bovine PBL after polyclonal activation with PWM **(C)** or ConA **(D)**. STAT3 and STAT5A were selectively upregulated in BNP lymphocytes **(C)** compared to controls due to PWM. After ConA stimulation, controls significantly upregulated STAT1 and STAT6 **(D)**, whereas in PBL of BNP cases STAT3 was enhanced during immune response **(D)**.

increased reaction to polyclonal immune stimulation in these cows.

No Significant Differences in Lymphocyte Subsets Were Detectable

To exclude differences in lymphocyte subset percentages between control and BNP dams as a possible cause for the differential responses toward polyclonal immune stimulation, we analyzed the distribution of $CD4^+$, $CD8^+$, and B cells in control and BNP PBL. There were no significant differences in proportional composition of subpopulations between PregSure BVD vaccinated control and BNP animals (**Figure 2**).

Lymphocyte Proteomes Reveal Differential Protein Regulation Between Normal And Deviant Immune Responders to PregSure BVD Vaccination

To investigate if observed deviant reactions to *in vitro* stimulation originated from differences in immune cell regulation, we executed a differential proteomics experiment with PWM and ConA treated lymphocytes as well as unstimulated cells. Overall, we detected 5,471 proteins. Hierarchical cluster analysis of complete proteomes already visualized fundamental differences in protein abundances between PregSure BVD vaccinated control and BNP cows in constitutive protein

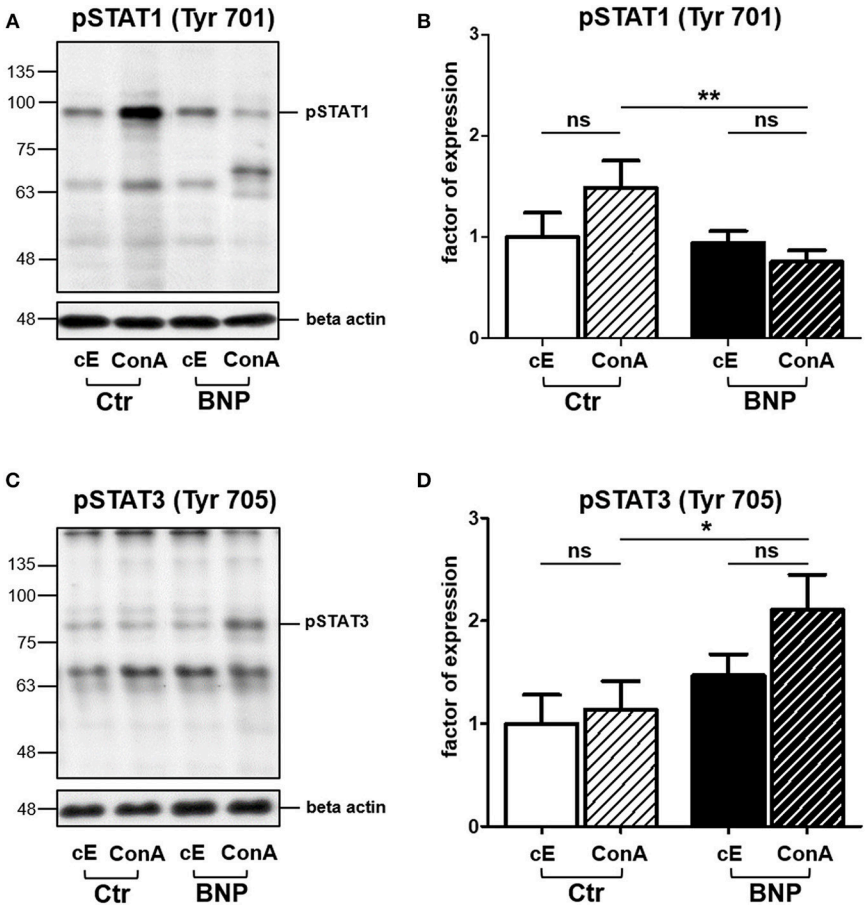


FIGURE 4 | In PBL of control and BNP cows, different transcription factors were activated after ConA stimulation *in vitro*. **(A)** Representative western blot of constitutive (cE) pSTAT1 Tyr701 expression and after stimulation with ConA (ConA) in control PBL (Ctr, $n = 1$) and BNP PBL (BNP, $n = 1$). **(B)** Lymphocytes of vaccinated control cows (white-black striped bars, $n = 5$) phosphorylated STAT1 Tyr701 significantly stronger than BNP lymphocytes (black-white striped bars, $n = 5$) after ConA stimulation (**$p < 0.01$). **(C)** Representative western blot of constitutive (cE) pSTAT3 Tyr705 expression and after stimulation with ConA (ConA) in control PBL (Ctr, $n = 1$) and BNP PBL (BNP, $n = 1$). **(D)** BNP lymphocytes (black-white striped bars, $n = 5$) phosphorylated STAT3 Tyr705 comparatively stronger than vaccinated control lymphocytes (white-black striped bars, $n = 5$) in response to ConA stimulation (*$p < 0.05$). **(A/C)** Phosphorylated STAT1 Tyr701 and pSTAT3 Tyr705 signals were normalized to beta-actin and quantified using Image-J software. Protein expression is shown in mean ± SD. For statistical analyses Student's *t* test was performed.

levels (cE, **Figures 3A,B**). Differences in protein expression were also confirmed after immune stimulation (PWM and ConA, **Figures 3A,B**, hierarchical clustering of all identified proteins). These proteome analyses therefore revealed substantial quantitative differences on protein level.

Immune Stimulation Leads to Different Usage of STAT Pathways in PBL of Control and BNP Cows

Next, we used our dataset to perform more in-depth analyses on part of the proteome involved in transcription pathways of immune cell regulation. To identify possible differences in master transcription factor expression, we compared lymphocytes from vaccinated control and BNP cows after polyclonal *in vitro* stimulation. Signal transducers and activators of transcription (STAT) induce different T helper (Th) responses in mice and man (24) as master transcription factors. Therefor we analyzed all identified STATs from both proteomic approaches and characterized usage of different STAT pathways as a response to immune stimulation in both cow groups. After polyclonal activation of lymphocytes with PWM, STAT3 and STAT5A were selectively upregulated at least 2-fold in BNP lymphocytes (**Figure 3C**). ConA stimulation of control cells, however, caused upregulation of STAT1 and STAT6 expression levels (**Figure 3D**), whereas BNP PBL showed enhanced STAT3 in immune response (**Figure 3D**). Thus, control and BNP PBL upregulated different master transcription factors as response to stimulation pointing to usage of different immune pathways.

Lymphocytes of Control and BNP Cows Regulate Different STATs in Response to ConA Stimulation *in vitro*

Since we decided to further analyze the differential activation of STATs through T cell mitogen ConA, we determined

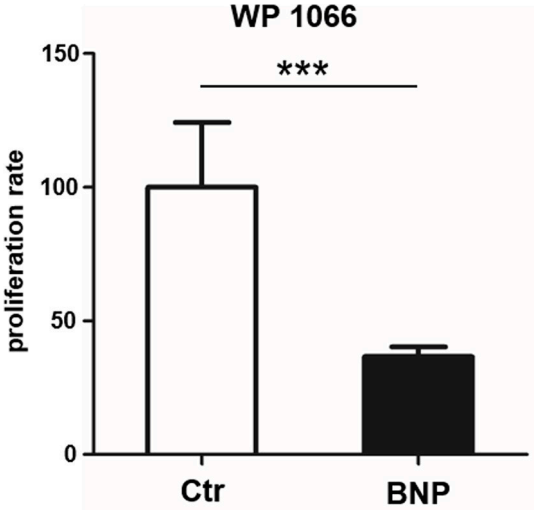


FIGURE 5 | STAT3 inhibitor WP1066 selectively inhibits hyperproliferation of lymphocytes from BNP cows. STAT3 inhibitor did not inhibit proliferation of control PBL (white bar, $n = 4$), but significantly inhibited ConA stimulated BNP cells (black bars, $n = 4$, $^{***}p < 0.001$). Percentage of inhibition rate shown in mean ± SD. Student's *t*-test was used for statistical analyses.

phosphorylation of STAT1 and STAT3 in response to immune stimulation. In lymphocytes of vaccinated controls, phosphorylation of STAT1 Tyr701 significantly increased after *in vitro* stimulation with ConA compared to BNP PBL, where STAT1 Tyr701 phosphorylation decreased after ConA stimulation (**Figures 4A,B**, Ctr to BNP after ConA stimulation, $^{**}p < 0.01$). In contrast, lymphocytes of BNP dams phosphorylated STAT3 Tyr705 comparatively stronger than PBL of vaccinated control cows after ConA (**Figures 4C,D**, BNP to Ctr after ConA stimulation, $^{*}p < 0.05$). Thus, these experiments ascertained a qualitative difference in immune reactions between the cow groups to a T cell stimulus.

Inhibition of STAT3 in PBL of BNP Cows Abolishes Hyperproliferation as Reaction to Polyclonal Immune Stimulation

Since BNP lymphocytes responded to polyclonal stimulation with hyperproliferation and activation of STAT3, we next tested if inhibition of STAT3 reversed respective hyperproliferation. WP1066 (STAT3 inhibitor) significantly reduced proliferation of BNP lymphocytes after ConA stimulation (**Figure 5**, inhibition of BNP compared to Ctr, $^{***}p < 0.001$), but not of control lymphocytes. This verified the importance of STAT3-pathway for the hyperproliferation in BNP PBL after polyclonal T cell stimulation.

Immune Deviant Phenotype Is Also Detectable in Unvaccinated Cows

Since we first detected the deviant immune phenotype in BNP cows, we next tested cows that were never vaccinated with PregSure BVD in order to clarify whether the deviant phenotype was induced through vaccination or if it occurs naturally in a certain subset of cows. Therefore, we examined response of lymphocytes to different polyclonal stimuli in a large group of PregSure BVD unvaccinated cows. To exclude differences caused by environmental factors, cows all came from the same farm. In *in vitro* proliferation assays with T cell mitogen ConA we observed that 22% of the unvaccinated cows reacted similar to BNP dams by showing a hyperproliferative reaction to ConA (immune deviant (ID) phenotype; **Figure 6A**, reaction difference ID to controls or BNP to controls, $^{****}p < 0.0001$). Lymphocytes from ID cows with a hyperproliferative immune phenotype did not show significant differences to lymphocytes of BNP cows in these assays (**Figure 6A**, reaction difference BNP to ID). These data confirmed the natural occurrence of this ID phenotype in cattle and gave stronger evidence for genetic predisposition instead of vaccine-induced appearance.

Lymphocytes of ID Cows Also React to BanLec and IL-2 With Hyperproliferation

In order to further characterize the pathway targeted by the polyclonal activators PWM and ConA we tested additional mitogens. The T cell mitogen BanLec (28), which activates human T cells through the IL-2 pathway (29, 30), led to similar hyperproliferation of PBL from BNP dams as observed with ConA (**Figure 6A**, reaction difference factor: 5.0, BNP to controls, $^{****}p < 0.0001$). PBL of ID cows responded to BanLec stimulation with a similar immune response intensity as BNP cows (**Figure 6B**, reaction difference factor: 3.0, ID compared to controls, $^{****}p < 0.0001$). The reaction of lymphocytes showed no significant differences between ID and BNP cows. Since the results with ConA and BanLec pointed to a response via IL-2 pathway, we next tested stimulation with purified bovine IL-2 only. After IL-2 stimulation, lymphocytes of ID cows also reacted excessively (**Figure 6C**, reaction difference factor: 3.1, ID to controls, $^{****}p < 0.0001$) and did not show significant differences to BNP lymphocytes (**Figure 6C**, reaction difference factor: 3.7, BNP to controls, $^{****}p < 0.0001$).

IL-2, but Not IFNγ or IL-4 Promote the Different Immune Response in ID Cows

Finally, we tested further signature cytokines for different Th immune responses, bovine Interferon gamma (IFNγ; Th1) and Interleukin 4 (IL-4; Th2), to analyze differentiation of T helper subsets. We detected no significant differences between lymphocytes of vaccinated and unvaccinated controls and BNP cases after IFNγ or IL-4 stimulation *in vitro* (**Figures 6D,E**). This proves a crucial role for IL-2 in the deviant immune responses, but not for IFNγ or IL-4.

Immune Deviant Phenotype in Cows Correlates With Significantly Increased Incidence of Mastitis

In this study, the proliferative phenotype of controls and ID cows could be repeatedly tested during an overall observation period of 38 months. All of the sampled immune deviant cows showed hyperproliferative reaction to ConA at least three

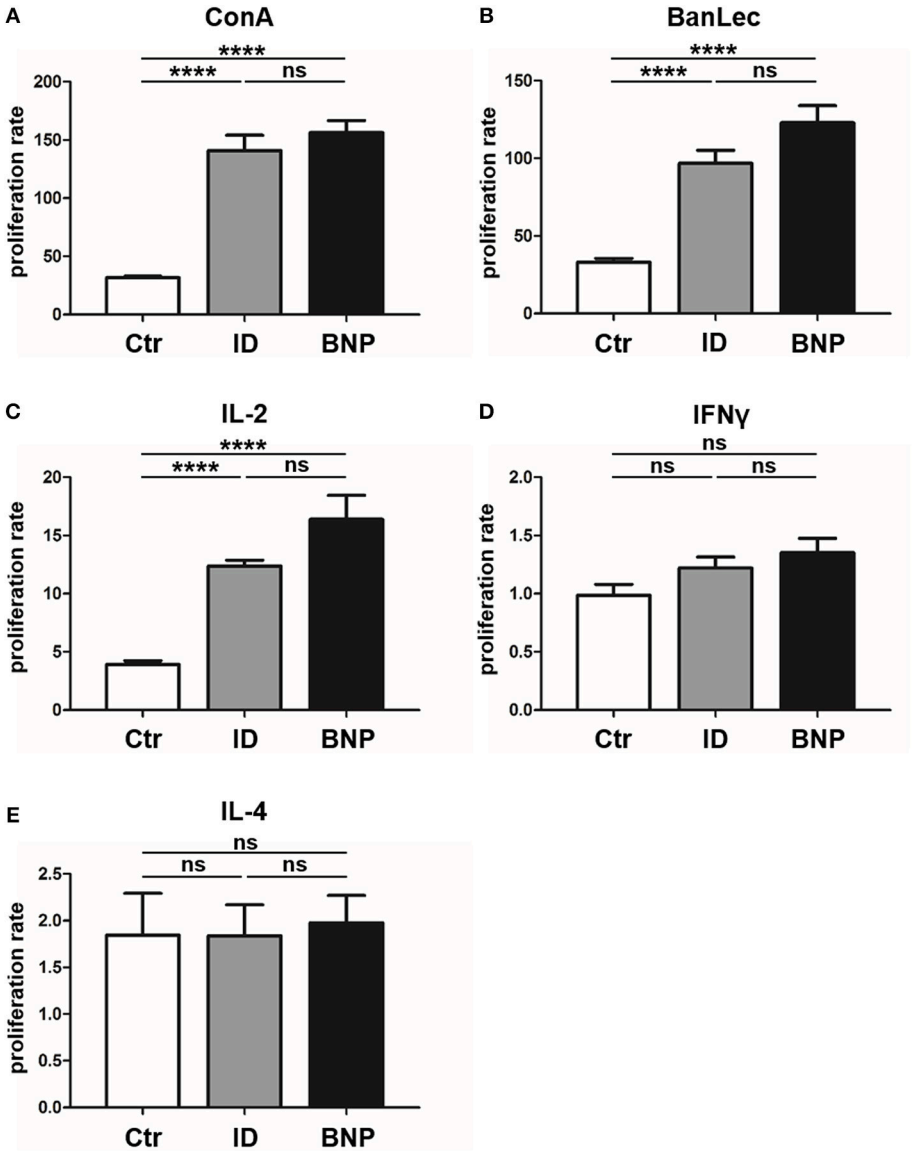


FIGURE 6 | Immune deviant phenotype also detectable in unvaccinated cows. **(A)** In *in vitro* proliferation assays, 22% of unvaccinated cows showed a BNP-like hyperproliferative reaction to ConA [****$p < 0.0001$ vs. Ctr (white bar, $n = 121$)]. Lymphocytes from ID cows (gray bars, $n = 27$) with a hyperproliferative immune phenotype showed no significant (ns) differences to lymphocytes of BNP cows (black bars, $n = 4$, technical replicates n =86). **(B)** After BanLec stimulation, ID lymphocytes reacted hyperproliferative in comparison to control lymphocytes (****$p < 0.0001$) and the proliferation rates of these ID lymphocytes did not show significant differences (ns) compared to BNP lymphocytes. **(C)** After IL-2 stimulation, lymphocytes of ID cows also reacted excessively (****$p < 0.0001$ vs. Ctr) just like lymphocytes from BNP donors (ns). **(D/E)** After bovine IFNγ **(D)** and IL-4 **(E)** stimulation, no significant differences between lymphocytes of unvaccinated and BNP cows were determined (ns). Proliferation rate shown in mean ± SD. Student's *t*-test was used.

times. Overall, two-thirds of the ID animals consistently showed significant differences in their immune response compared to the unvaccinated control cows (**Figure 7A**). Since we wanted to know if this newly discovered immune deviant phenotype related to impaired health parameters in respective cows, we retrospectively analyzed milk production, health parameters and different diseases of control and ID cows from the same farm never vaccinated with PregSure BVD. From a total of 16 parameters tested, there was no difference in 15 parameters. However, mastitis incidence was significantly different between both cow groups. We detected that ID cows suffered from mastitis twice as often as control cows (**Figure 7B**, *$p < 0.05$). At the moment of blood sampling, all cows were clinically healthy and showed no signs of mastitis which could potentially influence adverse effects to immune stimulation. Thus, this clearly indicates that the hyperproliferative phenotype of ID cows has clinical relevance apart from the response to the BVD vaccine.

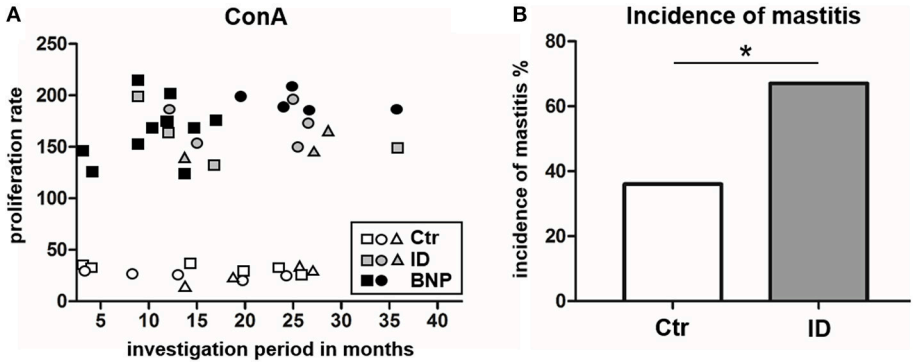


FIGURE 7 | Proliferation response types remain consistent and ID have a significantly increased incidence of mastitis. **(A)** The hyperproliferative phenotype of BNP cows (black) and ID cows (gray) as well as the low proliferation of control cows (white) were consistently detectable over the entire observation period (38 months). Three representative control cows and ID cows are shown and two BNP cows, white symbols = controls, gray symbols = ID, black symbols = BNP. **(B)** ID cows (gray, $n = 27$) suffered twice as often (difference in incidence $^*p < 0.05$) from mastitis than the control cows (white, $n = 73$). Incidence of mastitis shown in percent. Odds ratio and chi-square distribution was used for statistical analyses.

DISCUSSION

BNP was the unwanted result of a fatal immune response to vaccination with PregSure BVD (8, 9). Five to ten percentage of vaccinated cattle produced disease inducing antibodies which were transferred via colostrum, killing 90% of receiving calves, regardless of kin (15, 31). It is still unclear, why a certain subgroup of cows responded differently to the vaccination. We hypothesize that the origin of this altered immune response lies in the existence of a naturally occurring deviant immune phenotype. In this study, we could effectively confirm a quantitative difference in immune cell activation of BNP PBL through hyperproliferative responses *in vitro* (**Figures 1A,B**). A shift in lymphocyte subpopulations ($CD4^+$, $CD8^+$, B cells) as a potential reason for these differential responses toward polyclonal immune stimulation could be excluded (**Figure 2**). The hierarchical clustering of identified lymphocyte proteins from our shotgun proteomics experiment substantiated a general quantitative difference in constitutive expression of proteins in PBL of controls and BNP dams (cE, **Figures 3A,B**). After immune stimulation, these differences increased even further (PWM/ConA, **Figures 3A,B**). In order to perform more in-depth investigations of potential functional differences, we subsequently focused on the analysis of master transcription factors of immune cell regulation from our dataset. As a result, we detected several differentially expressed STATs. STATs initiate the differentiation of various Th cell subsets (24) as first-response master regulators and provide lineage specificity by promoting the differentiation of a given Th cell subset while opposing the differentiation to alternative Th cell subsets (32). This makes them especially interesting to us, since expression of different STATs in controls and BNP specimen indicates divergent immune response pathways in these groups. In control lymphocytes we identified STAT1 with increased expression (**Figure 3D**) and significantly higher activation (**Figures 4A,B**) after T cell stimulation with ConA. In mice and man, STAT1 is important for the differentiation of naive $CD4^+$ T cells to Th1 cells (33) or to type 1 regulatory (Tr) T cells (34). From these findings, we conclude that after immune stimulation, bovine control lymphocytes use the STAT1 pathway and we hypothesize that control cows react with a Th1 or Tr immune response in polyclonal stimulation assays. The effect of STAT1 inhibition could not be tested, since we found no STAT1 specific inhibitor that was commercially available. In contrast to controls, PBL proteome of BNP dams showed higher expression levels of STAT3 (**Figure 3D**), with increased phosphorylation (**Figures 4C,D**) in BNP lymphocytes after immune stimulation. STAT3 could therefore be a possible master regulator for the deviant immune response in BNP dams. In mouse and man, STAT3 induces the development of follicular Th (Tfh), Th17, and Th22 cells (32, 35, 36). Our findings proved a STAT3 pathway dependent immune response of BNP lymphocytes after polyclonal stimulation, but so far it is unknown which Th subsets exactly are regulated by STAT3 in cows. The dependence of the hyperproliferative phenotype of immune deviant PBL on STAT3 was shown by inhibition of respective factor using WP 1,066, a cell permeable tyrphostin analog which blocks the STAT3 pathway through inhibition of Janus kinase 2 (JAK2) protein tyrosine kinase (37). As a result, we observed a significantly reduced proliferation to 37% in BNP PBL, but no effect in control PBL (**Figure 5**). Our findings therefore show, that the deviant immune response of BNP donors is associated with STAT3/JAK2 pathway. Further studies will be needed to clarify whether immune cells from control cows differentiate to Th1 or Tr subsets and if BNP donor cows develop Tfh, Th17, or Th22 cells after immune stimulation. Hyper-reactivity of BNP PBL and the STAT3 driven differentiation of Tfh cells after immune stimulation are supported by findings of other research groups, describing higher alloantibody levels in BNP cows compared to PregSure BVD vaccinated control dams (22). This group also hypothesized, that differences between BNP and control dams are likely due to genes controlling the

quantitative alloantibody response after vaccination (22). To find evidence for a genetic predisposition of this immune deviant BNP phenotype in cows (22, 23), we additionally analyzed the immune phenotypes of 121 non-PregSure BVD vaccinated cows. In our study, 22% of cows (ID cows) showed a BNP-like hyperproliferative response to T cell stimulation *in vitro* (ID, **Figures 6A,B**). This percentage fits to the findings of Benedictus et al., who hypothesized a heritability of 19% in dams for the development of BNP in the calf (22). This proves the existence of an immune deviant phenotype among a certain subpopulation of cattle, which is not triggered by vaccination with PregSure BVD but occurs naturally. The immune deviant response described here in these ID cows clearly depends on IL-2 (**Figure 6C**), which we could prove through hyperproliferative response of BNP and ID lymphocytes to stimulation with BanLec (**Figure 6B**), a mitogen that activates human T cells (28, 30) via the IL-2 pathway (29). For bovine T cells, the specific impact of IL-2 itself was not described so far. In man and mice, however, IL-2 has pleiotropic autocrine or paracrine functions that regulate proliferation of T cells (38). It also plays a key role in promoting development, homeostasis and function of regulatory T cells though IL-2/STAT5 signals (39) and balances expression of different STATs in Th cells of mice (40). Therefore, the association of IL-2 with the immune deviant response in cows is a very interesting finding which merits further in-depth investigations in future studies. To determine whether the newly detected deviant immune phenotype is of clinical relevance, we analyzed 10 clinical parameters and 6 diseases in 100 cows from the sampled dairy farm and compared incidence between controls ($n = 73$) and ID animals ($n = 27$). Interestingly, we found a significantly increased mastitis incidence in ID cows compared to controls (**Figure 7B**). This highly important correlation points to an altered immune reaction of ID cows to classical mastitis pathogens, facilitating mastitis onset in these animals.

In conclusion, we could prove the existence of an immune deviant phenotype in 22% of cattle, regardless of the vaccination status. The ID phenotype shows altered reactions to immune stimulation identical to BNP dams, such as hyperproliferation of lymphocytes, STAT3/JAK2 regulated pathway and association with IL-2. These features potentially triggered alloantigen-production after maternal PregSure BVD vaccination, causing BNP. The correlation of the ID phenotype with high mastitis incidence (**Figure 7B**) underlines its clinical relevance.

AUTHOR CONTRIBUTIONS

CD conceived and designed the experiments. KL, KK, and BH performed the experiments. KL, KK, SH, SN, AS, and CD analyzed the data. KL, KK, and CD wrote the manuscript. All authors critically read the manuscript and approved the final version to be published.

FUNDING

This work was supported by the H. Wilhelm-Schaumann-Stiftung (to KL and KK), the Bayerische Tierseuchenkasse and the Federal Ministry for Economic Affairs and Energy (BMWi), grant Aif 18388 (to CD).

ACKNOWLEDGMENTS

The authors thank Sven Reese for statistical analyses and Roxane Degroote for critical discussions. The manuscript was released as a pre-print at https://www.biorxiv.org/content/early/2018/05/13/311316 (41).

REFERENCES

1. Orenstein WA, Ahmed R. Simply put: vaccination saves lives. *Proc Natl Acad Sci USA*. (2017) 114:4031–3. doi: 10.1073/pnas.1704507114
2. Rasmussen SA, Watson AK, Kennedy ED, Broder KR, Jamieson DJ. Vaccines and pregnancy: past, present, and future. *Semin Fetal Neonatal Med*. (2014) 19:161–9. doi: 10.1016/j.siny.2013.11.014
3. Kim YI, Song JH, Kwon BE, Kim HN, Seo MD, Park K, et al. Pros and cons of VP1-specific maternal IgG for the protection of Enterovirus 71 infection. *Vaccine* (2015) 33:6604–10. doi: 10.1016/j.vaccine.2015.10.103
4. Rabinovitz BC, Gerhardt E, Tironi Farinati C, Abdala A, Galarza R, Vilte DA, et al. Vaccination of pregnant cows with EspA, EspB, gamma-intimin, and Shiga toxin 2 proteins from *Escherichia coli* O157:H7 induces high levels of specific colostral antibodies that are transferred to newborn calves. *J Dairy Sci*. (2012) 95:3318–26. doi: 10.3168/jds.2011-5093
5. Crouch CF, Oliver S, Francis MJ. Serological, colostral and milk responses of cows vaccinated with a single dose of a combined vaccine against rotavirus, coronavirus and *Escherichia coli* F5 (K99). *Vet Rec*. (2001) 149:105–8. doi: 10.1136/vr.149.4.105
6. Benedictus L, Bell CR. The risks of using allogeneic cell lines for vaccine production: the example of Bovine Neonatal Pancytopenia. *Expert Rev Vaccines* (2017) 16:65–71. doi: 10.1080/14760584.2017.1249859
7. Sauter-Louis C, Carlin A, Friedrich A, Assad A, Reichmann F, Rademacher G, et al. Case control study to investigate risk factors for bovine neonatal pancytopenia (BNP) in young calves in southern Germany. *Prev Vet Med*. (2012) 105:49–58. doi: 10.1016/j.prevetmed.2012.02.008
8. Bastian M, Holsteg M, Hanke-Robinson H, Duchow K, Cussler K. Bovine neonatal pancytopenia: is this alloimmune syndrome caused by vaccine-induced alloreactive antibodies? *Vaccine* (2011) 29:5267–75. doi: 10.1016/j.vaccine.2011.05.012
9. Reichmann F, Pfitzner A, Rademacher G, Schwedinger E, Cussler K, Sauter-Louis CM. Incidence of bovine neonatal pancytopenia in 243 farms in Germany. *BMC Vet Res*. (2016) 12:220. doi: 10.1186/s12917-016-0857-7
10. Penny CD, Bell C, Morrison L, Howie F, Willoughby K. Pancytopenia and haemorrhage in young beef calves. *Vet Rec*. (2009) 164:762. doi: 10.1136/vr.164.24.762
11. Jones BA, Sauter-Louis C, Henning J, Stoll A, Nielen M, Van Schaik G, et al. Calf-level factors associated with bovine neonatal pancytopenia–a multi-country case-control study. *PloS ONE* (2013) 8:e80619. doi: 10.1371/journal.pone.0080619
12. Kasonta R, Sauter-Louis C, Holsteg M, Duchow K, Cussler K, Bastian M. Effect of the vaccination scheme on PregSure(R) BVD induced alloreactivity and the incidence of bovine neonatal pancytopenia. *Vaccine* (2012) 30:6649–55. doi: 10.1016/j.vaccine.2012.08.069
13. Euler KN, Hauck SM, Ueffing M, Deeg CA. Bovine neonatal pancytopenia–comparative proteomic characterization of two BVD vaccines and the producer cell surface proteome (MDBK). *BMC Vet Res*. (2013) 9:18. doi: 10.1186/1746-6148-9-18

14. Benedictus L, Rutten VP, Koets AP. Pregnancy boosts vaccine-induced bovine neonatal pancytopenia-associated alloantibodies. *Vaccine* (2016) 34:1002–5. doi: 10.1016/j.vaccine.2016.01.013
15. Friedrich A, Buttner M, Rademacher G, Klee W, Weber BK, Muller M, et al. Ingestion of colostrum from specific cows induces Bovine Neonatal Pancytopenia (BNP) in some calves. *BMC Vet Res*. (2011) 7:10. doi: 10.1186/1746-6148-7-10
16. Laming E, Melzi E, Scholes SF, Connelly M, Bell CR, Ballingall KT, et al. Demonstration of early functional compromise of bone marrow derived hematopoietic progenitor cells during bovine neonatal pancytopenia through *in vitro* culture of bone marrow biopsies. *BMC Res Notes* (2012) 5:599. doi: 10.1186/1756-0500-5-599
17. Deutskens F, Lamp B, Riedel CM, Wentz E, Lochnit G, Doll K, et al. Vaccine-induced antibodies linked to bovine neonatal pancytopenia (BNP) recognize cattle major histocompatibility complex class I (MHC I). *Vet Res*. (2011) 42:97. doi: 10.1186/1297-9716-42-97
18. Foucras G, Corbiere F, Tasca C, Pichereaux C, Caubet C, Trumel C, et al. Alloantibodies against MHC class I: a novel mechanism of neonatal pancytopenia linked to vaccination. *J Immunol*. (2011) 187:6564–70. doi: 10.4049/jimmunol.1102533
19. Bell CR, MacHugh ND, Connelley TK, Degnan K, Morrison WI. Haematopoietic depletion in vaccine-induced neonatal pancytopenia depends on both the titre and specificity of alloantibody and levels of MHC I expression. *Vaccine* (2015) 33:3488–96. doi: 10.1016/j.vaccine.2015.05.069
20. Benedictus L, Luteijn RD, Otten H, Lebbink RJ, van Kooten PJ, Wiertz EJ, et al. Pathogenicity of bovine neonatal pancytopenia-associated vaccine-induced alloantibodies correlates with major histocompatibility complex class I expression. *Sci Rep*. (2015) 5:12748. doi: 10.1038/srep12748
21. Demasius W, Weikard R, Hadlich F, Buitkamp J, Kuhn C. A novel RNAseq-assisted method for MHC class I genotyping in a non-model species applied to a lethal vaccination-induced alloimmune disease. *BMC Genomics* (2016) 17:365. doi: 10.1186/s12864-016-2688-0
22. Benedictus L, Otten HG, van Schaik G, van Ginkel WG, Heuven HC, Nielen M, et al. Bovine neonatal pancytopenia is a heritable trait of the dam rather than the calf and correlates with the magnitude of vaccine induced maternal alloantibodies not the MHC haplotype. *Vet Res*. (2014) 45:129. doi: 10.1186/s13567-014-0129-0
23. Demasius W, Weikard R, Kromik A, Wolf C, Muller K, Kuhn C. Bovine Neonatal Pancytopenia (BNP): novel insights into the incidence, vaccination-associated epidemiological factors and a potential genetic predisposition for clinical and subclinical cases. *Res Vet Sci*. (2014) 96:537–42. doi: 10.1016/j.rvsc.2014.03.019
24. Krappmann K, Weikard R, Gerst S, Wolf C, Kuhn C. A genetic predisposition for bovine neonatal pancytopenia is not due to mutations in coagulation factor XI. *Vet J*. (2011) 190:225–9. doi: 10.1016/j.tvjl.2010.10.007
25. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* (2016) 13:731–40. doi: 10.1038/nmeth.3901
26. Mellstedt H. *In vitro* activation of human T and B lymphocytes by pokeweed mitogen. *Clin Exp Immunol*. (1975) 19:75–82.
27. Perrin PJ, Davis TA, Smoot DS, Abe R, June CH, Lee KP. Mitogenic stimulation of T cells reveals differing contributions for B7-1 (CD80) and B7-2 (CD86) costimulation. *Immunology* (1997) 90:534–42. doi: 10.1046/j.1365-2567.04.00215.x
28. Singh SS, Devi SK, Ng TB. Banana lectin: a brief review. *Molecules* (2014) 19:18817–27. doi: 10.3390/molecules191118817
29. Cheung AH, Wong JH, Ng TB. *Musa acuminata* (Del Monte banana) lectin is a fructose-binding lectin with cytokine-inducing activity. *Phytomedicine* (2009) 16:594–600. doi: 10.1016/j.phymed.2008.12.016
30. Koshte VL, van Dijk W, van der Stelt ME, Aalberse RC. Isolation and characterization of BanLec-I, a mannoside-binding lectin from *Musa paradisiac* (banana). *Biochem J*. (1990) 272:721–6. doi: 10.1042/bj2720721
31. Pardon B, Stuyven E, Stuyvaert S, Hostens M, Dewulf J, Goddeeris BM, et al. Sera from dams of calves with bovine neonatal pancytopenia contain alloimmune antibodies directed against calf leukocytes. *Vet Immunol Immunopathol*. (2011) 141:293–300. doi: 10.1016/j.vetimm.2011.02.017
32. Tripathi SK, Lahesmaa R. Transcriptional and epigenetic regulation of T-helper lineage specification. *Immunol Rev*. (2014) 261:62–83. doi: 10.1111/imr.12204
33. Kanhere A, Hertweck A, Bhatia U, Gokmen MR, Perucha E, Jackson I, et al. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nat Commun*. (2012) 3:1268. doi: 10.1038/ncomms2260
34. Sadreev II, Chen MZQ, Umezawa Y, Biktashev VN, Kemper C, Salakhieva DV, et al. The competitive nature of signal transducer and activator of transcription complex formation drives phenotype switching of T cells. *Immunology* (2018) 153:488–501. doi: 10.1111/imm.12851
35. Nalbant A, Eskier D. Genes associated with T helper 17 cell differentiation and function. *Front Biosci*. (2016) 8:427–35. doi: 10.2741/e777
36. Alshekaili J, Chand R, Lee CE, Corley S, Kwong K, Papa I, et al. STAT3 regulates cytotoxicity of human $CD57^{+}$ $CD4^{+}$ T cells in blood and lymphoid follicles. *Sci Rep*. (2018) 8:3529. doi: 10.1038/s41598-018-21389-8
37. Ferrajoli A, Faderl S, Van Q, Koch P, Harris D, Liu Z, et al. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. *Cancer Res*. (2007) 67:11291–9. doi: 10.1158/0008-5472.CAN-07-0593
38. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol*. (2012) 12:180–90. doi: 10.1038/nri3156
39. Mahmud SA, Manlove LS, Farrar MA. Interleukin-2 and STAT5 in regulatory T cell development and function. *JAKSTAT* (2013) 2:e23154. doi: 10.4161/jkst.23154
40. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol*. (2011) 12:247–54. doi: 10.1038/ni.1995
41. Lutterberg K, Kleinwort KJH, Hobmaier BF, Hauck SM, Nueske S, Scholz AM, et al. A functional different immune capacity in cattle is associated with higher mastitis incidence. *bioRxiv*. (2018) doi: 10.1101/311316

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer VR and handling Editor declared their shared affiliation at the time of review.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership