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MECHANISMS OF IMMUNE ENHANCEMENT BY BENEFICIAL MICROBES AND PROBIOTICS

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SUMMARY

This review explores the abilities of beneficial microbes including probiotics to stimulate mucosal and systemic immunity, so that global vaccination strategies may be enhanced. Beneficial microbes secrete microbial factors and express cell surface features that stimulate different types of immune cells to alter their gene expression programs and produce different sets of cytokines and immune mediators. Microbes can affect the B lymphocyte program, and the production of different antibody subclasses due to class switching. Immune responses to antigenic challenges as a result of vaccination may be stimulated by B lymphocyte-promoting signals that result from microbial stimulation. Effector and regulatory T lymphocyte programs may be modulated by microbial effects on different signalling pathways. Beyond adaptive immunity, beneficial microbes may stimulate signalling pathways in intestinal epithelial cells, macrophages, and dendritic cells of the innate immune system. Beneficial microbes including probiotics may "prime" the immune system and supplement nutritional approaches so that infants and young children in the developing world are vaccine-ready. If these strategies can be combined, success rates for diverse vaccines may effectively increase in resource-poor regions of the world.

INTRODUCTION – IMMUNITY IN THE ERA OF THE MICROBIOME

The human body consists of a vast ecosystem that includes more bacterial cells than human cells, by an estimated difference that exceeds an order of magnitude. Commensal microbes have co-evolved with animals, including *Homo sapiens*, for thousands of years, and functions encoded by microbial genomes may supplement the functions encoded in the human genome. The human genome contains an estimated 25,000 genes with many genes of unknown function and genes that encode multiple proteins via alternative splicing or posttranslational processing. The rich functional repertoire of the human genome is exceeded by the metagenome, which is estimated to contain a gene content that is more than 100-fold greater in terms of gene number, with

an estimated gene pool exceeding 3 million genes. This aggregate metagenome, or microbiome, encodes for diverse metabolic pathways and signals that may have a profound impact on mammalian physiology and immunity.

In this manuscript, we will describe the impact of probiotics and beneficial microbes on the mammalian immune system, with an emphasis on mechanisms of immune enhancement or stimulation by beneficial microbes. Probiotics are defined as viable microbes that, when ingested in adequate amounts, confer some benefit to the host (*FAO/WHO*, 2001). The benefits are vaguely defined, and these benefits may include stimulation or modulation of host immunity. In this review, we will use beneficial microbes as a broader term that refers to any microbe with a benefit to the host that has been published in any single study. Beneficial microbes include probiotics as a subgroup, and this subgroup includes organisms that have been vetted in published clinical trials and meta-analyses in terms of benefits to human health.

Our hope is that beneficial microbes can facilitate the development of a robust immune system that may protect animals from various pathogens, radiation, and diverse biochemical challenges. Microbes may stimulate the development and differentiation of effector T lymphocytes, thereby enhancing populations of helper and cytotoxic T cells. Regulatory T cell populations may also be expanded in number as a result of microbial stimulation, resulting in inhibitory effects on cell mediated immunity and cytokine responses. Such responses may serve to quench immune responses, enabling the host to avert immunopathology as a result of overzealous immune responses. Conversely, commensal microbes may suppress the functions of regulatory T cells, thereby promoting more robust immune responses when hosts are challenged. B lymphocytes produce pathogen-specific antibodies, and the differentiation of antibody-secreting cells can be stimulated by beneficial microbes. Innate immunity, including dendritic cells and macrophages, may be affected so that subpopulations of these cells may "tilt" immune responses towards inflammation or more effective neutralization of pathogens.

The central question of this Old Herborn University seminar is whether vaccine challenges can be more effective with respect to protection if the host is exposed to the optimal combination of beneficial microbes. This review describes multiple mechanisms of immune enhancement (Figures 1 and 2), and the final section will attempt to point the way forward regarding strategies for harvesting the power of the microbiome and antigenic diversity of these communities to stimulate immunity and efficacy of vaccination programs for global health.

B LYMPHOCYTES AND ANTIBODY RESPONSES

Probiotics and beneficial microbes may stimulate humoral immunity by stimulating the production of mucosal and systemic antibodies. Microbes may promote differentiation of B lymphocytes and class switching, and such stimulation may serve to "prime" or prepare the immune system for subsequent pathogen or vaccine challenges. Intestinal microbes may strictly promote mucosal immunity, and such immune enhancement may be sufficient

to enhance enteric or mucosal vaccination strategies.

The consumption of probiotics during pregnancy may stimulate production of antibodies in the mother and consequently serve to transfer passive immunity to the infant via breast milk. In human studies, oral consumption of either *L. rhamnosus* or *B. lactis* during pregnancy stimulated the production of IgA in human breast milk at one week and 3 months post-partum (*Prescott* et al., 2008). The consumption of these probiotic strains resulted in the elevation of cord blood interferon-gamma levels in neonates, and these results indicate that stimulation of immunity in mothers may be effectively linked to enhanced systemic immunity in newborns. The production of mucosal IgA may be enhanced by signals derived from intestinal epithelial cells such as APRIL, BAFF, or TGF-β. Human enterocytes produce APRIL (a proliferation inducing ligand) in response to microbial signals from commensal bacteria such as *Lactobacillus plantarum* or *Bacillus subtilis*. APRIL mediates class-switch recombination in B lymphocytes to IgA_2 (*He* et al., 2007), and this antibody subclass is known to promote mucosal protection. Our recent work in an outbred, new-born mouse model suggests that probiotic *Lactobacillus reuteri* stimulates pathogen-specific mucosal IgA responses (G. Preidis, unpublished data). Whereas mucosal rotavirus-specific IgA antibodies are elevated in the presence of a single probiotic strain, systemic rotavirus-specific IgA responses do not seem to be affected.

Several studies with different vaccine challenges have documented the potential of probiotics to serve as "adjuvants" or to function as enhancers of vaccination. The delivery of systemic vaccines in parallel with probiotics may be enhanced by the ability of probiotics to stimulate antigen-specific IgG responses in peripheral blood. Oral or mucosal vaccination challenges with whole organisms or recombinant subunits have also demonstrated enhancement of antigen-specific IgA or IgG responses when probiotics are co-administered. New genetically engineered vaccines that are based on commensal microbes as "delivery vectors" may contain immunostimulatory or adjuvant properties that serve to boost vaccine responses (*Van Huynegem* et al., 2009).

T LYMPHOCYTES AND CELL-MEDIATED IMMUNITY

Beneficial microbes stimulate the proliferation of effector T lymphocytes globally or in response to specific antigens. In the presence of specific antigens, probiotics can stimulate proliferation of antigen-specific T lymphocytes. Probiotic species are known to promote anti-apoptotic signalling pathways and suppression of caspases in T lymphocytes and other immune cells. Lamina propria or intra-epithelial lymphocyte populations may be enhanced *in vivo*, and these immunostimulatory effects have been documented in mouse models (*Ivanov* et al., 2009; *Mileti* et al., 2009). Probiotic strategies may stimulate antigen-presenting cell function with subsequent effects on effector T cell stimulation. Effects on signalling pathways in macrophages and dendritic cells will be described later in this review, and several studies have documented stimulation of dendritic cell function by probiotics. Dendritic cells treated by probiotics will subsequently drive effector T lymphocyte proliferation and function in response to specific antigens (*Baba* et al., 2009; *Mileti* et al., 2009).

Regulatory T lymphocytes may suppress the functions of effector T cells, and the functions of Treg populations may be enhanced by probiotics and beneficial microbes. Diverse microbes such as *B. lactis* W51, *L. acidophilus* W55, and *L. plantarum* W62 induce $FOXP3^+$ Treg cell differentiation, and $FOXP3^+$ Treg cells demonstrate a suppressive phenotype that is contact-dependent with T effector cells (*Izcue* et al., 2009). TGF-β-expressing regulatory T cells were induced by a probiotics cocktail (VSL#3), and these cell subpopulations were associated with protection against colitis (*Di Giacinto* et al., 2005). Conversely, probiotics may inhibit the functions of regulatory T cells, thereby promoting more robust immune responses to pathogen or vaccine challenges. Three of six probiotic strains, *L. acidophilus* NCFM and *B. bifidum* (2 strains), suppressed Treg activity in a contact-dependent manner by modulation of spleen-derived APCs. Splenic entero-antigen-presenting cells (APCs) were exposed to individual probiotic strains and used to stimulate $CD4^+CD25^-$ proliferative T cells in the presence of absence of Treg cells (*Schmidt* et al., 2010). The proliferation of $CD4^+CD25^-$ cells was effectively enhanced by probiotic-mediated suppression of Treg function.

IMMUNE SIGNALLING IN INTESTINAL EPITHELIAL CELLS

Some probiotics stimulate NFκB activation, and consequently these microbes promote immunity and increase cytokine secretion (Figure 1). The commensal anaerobe *Bacteroides vulgatus* activates NFκB in intestinal epithelial cells via TLR4 signalling, interleukin-1 receptor associated kinase-1 (IRAK1) degradation, and RelA phosphorylation. The end-result is enhanced transcriptional activity of NFκB secondary to increased DNA binding capacity. The presence of peripheral blood mononuclear cells counteracts the effects on intestinal epithelial cells, resulting in suppression of NFκB activation (*Haller* et al., 2002), and these results indicate that different cell types can modulate signalling pathways in response to microbial agonists. The cytokine interleukin-6 (IL-6) has important roles in the promotion of innate and adaptive immune responses. For example, *Bifidobacterium lactis* BB12 increased IL-6 secretion by transient induction of RelA. RelA is the p65 sub-unit of NFκB that is the active component responsible for transcriptional activation of multiple cytokine genes. B. *lactis* BB12 also stimulates p38 MAP kinase by phosporylation, and both RelA and p38 MAP kinase are necessary for induction of IL-6. Stimulation of IL-6 is dependent on the Toll-like receptors, specifically TLR2 (*Ruiz* et al., 2005), and TLR2 has also been implicated in suppression of IL-6 production using the porcine IPEC-J2 line (*Liu* et al., 2010).

Microbial signals may also modulate the activity of hormone receptors that may result in attenuation of intestinal inflammation. The nuclear hormone receptor, PPARγ, is one such target that may contribute to cycling of transcription factors such as RelA in and out of the nucleus (Figure 1). The commensal organism *Bacteroides thetaiotamicron* may diminish secretion of the chemokine interleukin-8 (IL-8) by promoting nuclear export of RelA through a PPARγ dependent pathway (*Kelly* et al., 2004). Other microbes,

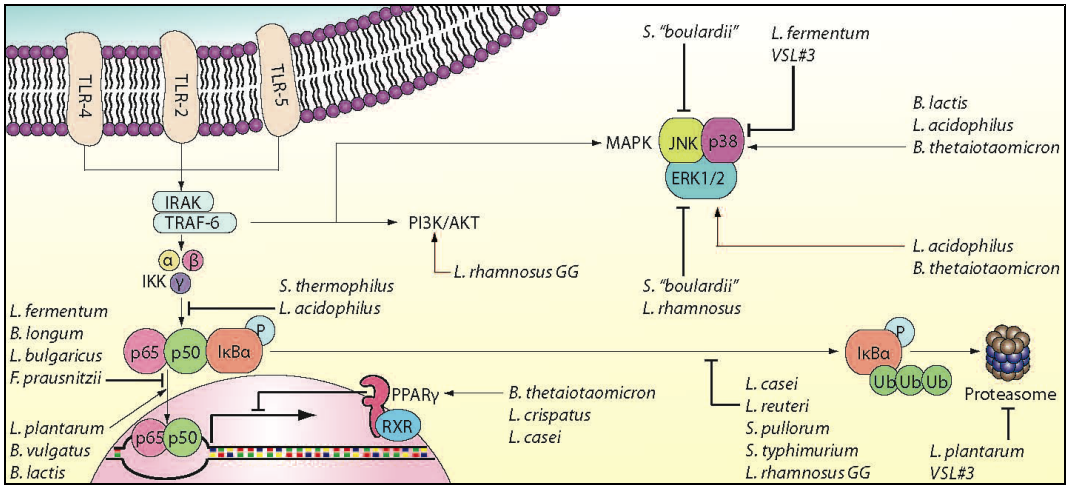

Figure 1: Probiotics modulate key signalling pathways in intestinal epithelial cells. Various probiotics prevent NFκB activation by inhibiting IκBα phosphorylation, ubiquitination, proteasomal degradation, or translocation of NFκB into the nucleus (suppression is indicated by a block sign "⊣"). Probiotics can also enhance RelA export from the nucleus via PPARγ. Other probiotics increase NFκB activation through enhanced translocation into the nucleus (activation is indicated by an arrow sign "→"). Apoptosis of intestinal epithelial cells can be prevented by probiotic modulation of the PI3K/ Akt pathway. Probiotic-induced changes in phosphorylation levels of p38, JNK, and ERK1/2 MAPKs can affect cytokine secretion and apoptosis. ERK, extracellular signal-regulated kinases; IκBα, inhibitor of NFκB α; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; P, phosphorylation; PPARγ, peroxisome proliferator activated receptor-γ; RXR, retinoid X receptor; TLR, Toll-like receptor; Ub, ubiquitin. [Reprinted from: Thomas, C.M. and Versalovic, J.: Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbe 1, 1-16 (2010)].

such as the lactobacilli, *Lactobacillus crispatus* and *Lactobacillus casei*, also enhance signalling via PPAR-γ. In addition to inhibition of cytokines, modulation of PPARγ by *Enterococcus faecalis* may also stimulate production of regulatory cytokines such as interleukin-10 (IL-10) (*Are* et al., 2008). *E. faecalis* is a commensal bacterium that colonizes the human intestine early in infancy. So, microbes may stimulate immune signalling pathways that promote immune tolerance and reduce inflammation. Conversely, suppression of signalling pathways such as NFκB or MAP kinases may alleviate disease phenotypes such as colitis, depending on the genetic background of the host.

One published study examined the effects of oral ingestion of one probiotic strain on mucosal signalling pathways in the proximal small intestine. In this study, gene expression profiles were gathered from samples of duodenal mucosa in human volunteers 6 hours after oral ingestion of *Lactobacillus plantarum* strain WCFS1 (*van Baarlen* et al., 2009). This strain yielded different gene expression profiles depending on the growth phase of the organism. Stationary phase *L. plantarum* increased expression of several genes involved in NFκB signalling, whereas midlog phase *L. plantarum* modulated genes involved in the cell cycle and cell proliferation such as MYC and cyclin D1. The central lesson of this study is that the physiologic state of microbes affects the production of microbial signals that may differentially modulate mammalian signalling pathways.

In summary, probiotics and beneficial microbes may modulate different signalling pathways involved in innate or adaptive immune responses (Figure 1). Microbial signals may activate or suppress NFκB signalling, and activation of NFκB signalling may stimulate cytokine production and enhance immune "readiness." NFκB signalling may be regulated at the level of the inhibitor IκB, entry of NFκB sub-units into the nucleus, or extrusion of sub-units from the nucleus via PPARγ-dependent pathways. Microbes may also modulate MAP kinases such as JNK or p38, resulting in immune activation. Populations of microbes may produce complex combinations of counteracting signals so the net effect on mammalian physiology may depend on relative quantities and the physiologic status of complex microbial communities.

IMMUNE SIGNALLING IN MACROPHAGES AND DENDRITIC CELLS

Innate immunity may be enhanced by beneficial microbes as a consequence of the stimulation of pattern recognition receptors and various signalling pathways. Prior studies showed that microbial stimulation of Toll-like receptors was important for maintenance of homeostasis in the intestine, and microbial eradication or lack of MyD88 signalling increased the vulnerability of the host to chemical challenge (*Madara*, 2004; *Rakoff-Nahoum* et al., 2004).

Some probiotic strains can stimulate innate immunity by activating NFκB or STAT signalling pathways in macrophages (Figure 2). STATs are cytoplasmic proteins that may become activated by cytokine or antigen signals, resulting in functional transcription factors after nuclear translocation. *Lactobacillus crispatus* induced the production of pro-inflammatory TNF and interleukin-1β (IL-1β) following the activation of NFκB in the human myeloid cell line THP-1 (*Klebanoff* et al., 1999). The established probiotic strain, *Lactobacillus rhamnosus* GG (ATCC 53103), induced DNA binding by STAT1 and STAT3, resulting in enhanced immune signalling in human PBMCs (*Miettinen* et al., 2000). Different *Lactobacillus* species may counteract each other, so that the net effect on dendritic cell populations may depend on relative quantities or potencies of microbial signals. For example, *L. casei* strain CHCC3139 induces production of IL-12, IL-6, and TNF by dendritic cells, but *L. reuteri* DSM 12246 counteracts this effect and suppresses the production of these cytokines in the presence of *L. casei* CHCC3139 (*Christensen* et al., 2002).

MICROBIAL SIGNALS THAT TRIGGER IMMUNE STIMULATION

Complex microbial communities in the intestine may secrete or present diverse signals that serve to enhance immune responses in the mammalian host. Germ-free animals have poorly developed mucosal immune systems with a relative paucity of lymphoid tissue (*Brandztaeg*, 2009). Individual microbial species or microbe-derived molecules with defined immunostimulatory activities have been challenging to isolate from this complex assemblage of microorganisms, but recent studies highlight exciting new findings. From hundreds of possible microbial species, a single organism could be identified

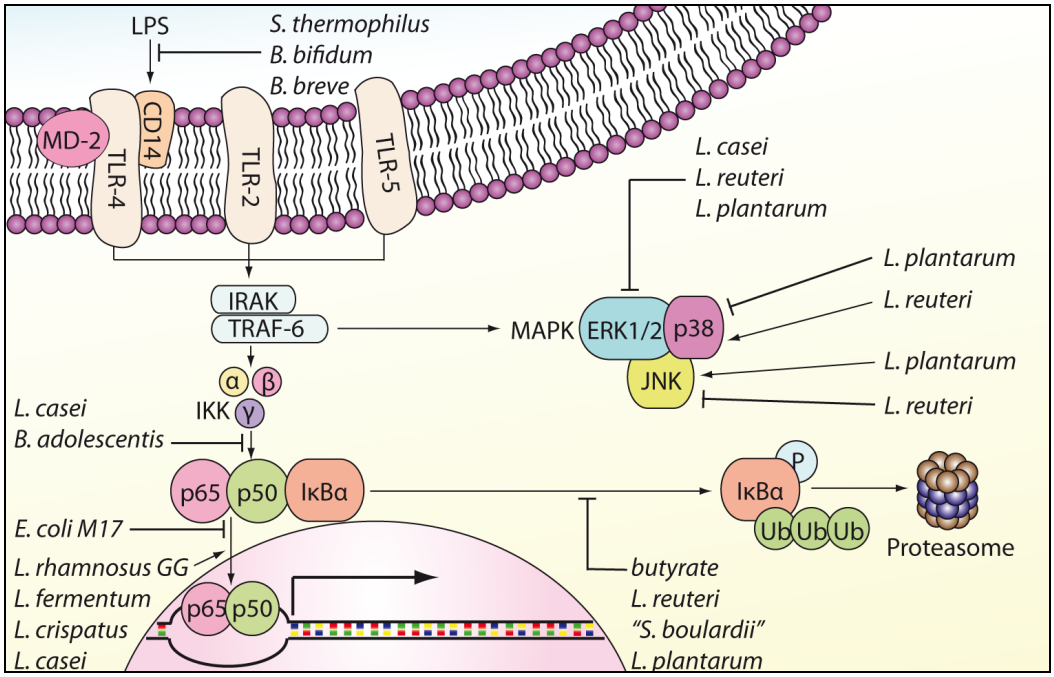

Figure 2: Probiotics modulate inflammatory signalling pathways in macrophages. Select probiotics can block binding of LPS to the CD14 receptor, interfering with LPS signal transduction. Various probiotics prevent activation of NFκB by decreasing phosphorylation or ubiquitination of IκBα or blocking NFκB translocation into the nucleus (suppression is indicated by a block sign "⊣"). NFκB activation is enhanced by other probiotics via increased nuclear translocation of transcriptionally active NFκB sub-units (activation is indicated by an arrow sign "→"). MAPK proteins p38, JNK and ERK1/2 are also targets of probiotic modulation in macrophages. ERK, extracellular signal-regulated kinases; IκBα, inhibitor of NFκBα; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MD-2, myeloid differentiation 2; P, phosphorylation; TLR, Toll-like receptor; Ub, ubiquitin. [Reprinted from: Thomas, C.M. and Versalovic, J.: Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbe 1, 1-16 (2010)].

with potent effects on mucosal immunity. A fascinating combination of studies by two research groups demonstrated the requirement of a specific commensal intestinal microbe for effective induction of Th17 effector cell populations in the mammalian intestine (*Gaboriau-Routhiau* et al., 2009; *Ivanov* et al., 2009). This uncultured organism was identified as *Candidatus Arthromitus*, or Segmented Filamentous Bacterium (SFB), by high density micro-array studies with the PhyloChip (*Ivanov* et al., 2009). Mouse colonies that were defective in this missing microbial ingredient lacked robust populations of Th17 lymphocyte populations, and these cell populations normally produce cytokines such as IL-17 or IL-22 that contribute to protection of the host from bacterial and fungal infections.

A mutant strain of *L. plantarum* NCIMB8826, defective in D-alanylation of cell surface lipoteichoic acids, induced production of the regulatory cytokine IL-10 while suppressing production of pro-inflammatory IL-12 (*Grangette* et al., 2005). These findings suggest that cell wall components such as lipoteichoic acids may play an important role in immunomodulation by

probiotics, and the alanyl component of lipoteichoic acids may serve to stimulate immunity or oppose immunosuppressive features of the microbiome. Consistent with this concept, lipoteichoic acids from *L. casei* YIT 9029 and *Lactobacillus fermentum* YIT 0-159 activated NFκB signalling and induced murine TNF production by mouse macrophages (*Matsuguchi* et al., 2003. Alanylation of lipoteichoic acids correlates with upregulation of IL-12p40 by mammalian cells when treated with *Lactobacillus plantarum* L-137 (*Hirose* et al., 2010), and conversely, IL-10 induction in mouse peritoneal macrophages by *L. plantarum* depends on ERK activation (*Kaji* et al., 2010). Presumably, differences in structures of lipoteichoic acids such as patterns of amino acid or glycosyl modifications may affect relative propensities to stimulate or suppress mammalian immune signalling pathways.

In addition to cell surface components, bacterial nucleic acids may also be released from beneficial microbes and stimulate immune responses. CpG oligodeoxynucleotides (ODN) derived from commensal bacteria upregulated immune responses via Toll-like receptor signalling pathways. CpG ODN from *Streptococcus thermophilus* induced upregulation of IL-33 (*Shimosato* et al., 2010), and CpG-rich sequences from different *Bifidobacterium* species stimulated production of MCP-1 and TNF by murine macrophages (*Menard* et al., 2010). Stimulation of murine MCP-1 and TNF by RAW 264.7 cells is mediated by enhanced TLR9 signalling. While CpG ODN have potent immunostimulatory effects in mouse models, results in human vaccination models have been disappointing. Bacterial DNA derived from *Lactobacillus rhamnosus* GG or *Bifidobacterium longum* suppressed chemokine production and NFκB signalling in polarized human intestinal epithelial cells. The addition of DNA to cultured human intestinal epithelial cells suppressed TNF-induced NFκB activation and NFκB-mediated IL-8 production via inhibition of IκBα degradation and p38 phosphorylation (*Ghadimi* et al, 2010a). Probiotic-derived DNA sequences may have different effects in human cells, versus mouse cells, despite the fact that effects in both mammalian systems depend on TLR9 signalling.

VACCINATION AUGMENTATION STRATEGIES

Probiotics may stimulate Th1 responses by enhancement of interferon-γ production from peripheral blood mononuclear cells (PBMCs) and human monocyte-derived macrophages (HMDMs), and beneficial microbes can suppress production of Th2 cytokines such as IL4 and IL13 (*Ghadimi* et al., 2010b). Co-treatment with *Mycobacterium tuberculosis* antigen with either *L. rhamnosus* LGG or *B bifidum* resulted in the stimulation of IFN-γ and NO production, resulting in greater IFN-γ / IL4 and IFN-γ / IL13 ratios. Autophagy biomarkers such as Beclin-1 and LC3-I were induced by treatment of PBMCs with *M. tuberculosis* antigen and either LGG or *B bifidum*. In this model, the presence of a vaccine-related antigen and probiotics stimulated autophagy and an associated Th1 response; these results suggest that probiotics may effectively augment vaccination strategies for *M. tuberculosis* and other microbial pathogens.

Probiotics can stimulate immune responses to pathogens and augment vaccination strategies via enhanced mucosal and systemic immunity. Probiotics enhance protection by the influenza vaccine (*Namba* et al., 2010; *Olivares* et al., 2007). The ingestion of human breast milk-derived *L. fermentum* enhanced the production of antigen-specific IgA following intramuscular influenza vaccination and reduced the incidence of influenza infection in the probiotics group (*Olivares* et al., 2007). *Lactococcus lactis* engineered to produce pneumococcal protective protein A induced effective protection against *Streptococcus pneumoniae* infection in mice via nasal vaccination (*Vintini* et al., 2010).

SUMMARY AND FUTURE DIRECTIONS

In summary, beneficial microbes including probiotics may serve as potent stimulators of mucosal and systemic immunity. As microbial communities have co-evolved with the immune systems of mammals for thousands of years, it is reasonable to suggest that microbes have played an important role in the development of immunity in the context of human individuals and entire populations. Mankind should exploit the fruits of human microbiome research and mucosal immunology to effectively couple vaccination strategies with probiotics and commensal microbiology.

New vaccine strategies may include combinations of micronutrients, recombinant vaccines, and probiotics to enhance the success rates of mucosal vaccination strategies in the developing world. In the context of undernutrition, novel approaches may be necessary to deliver efficacies comparable to the success stories in the developed world. Firstly, nutrition should be considered as part of the overall strategy for improving efficacy of vaccines in the developing world. The delivery of micronutrients and adequate nutritional support enables each child to fully develop immunity and responsiveness to vaccine challenges. Breastfeeding is a primary source of nutrition in infancy, in addition to its role in maternal:infant bonding. The quality of the breast milk is dependent on maternal nutrition so that the mother's diet becomes an important consideration for any comprehensive disease prevention strategy during infancy and early childhood. Supplementation of the maternal diet with probiotics, prebiotics, and other nutrients may maximize the production of complex saccharides in human breast milk and facilitate the establishment of a "beneficial breast milk microbiome". Bifidobacteria and other species are considered to be part of human breast milk in healthy, lactating women, and these breast milk-associated microbes may lay the foundation for the human intestinal microbiome.

The presence of complex microbial communities on mucosal surfaces early in life promotes the development and differentiation of a robust immune system. The combination of adequate nutritional support and a probiotics/prebiotics strategy will provide the "substratum" for mucosal immunity to flourish in children. Vaccine challenges with the proper mucosal adjuvant(s) will be poised to succeed if delivered on a solid foundation of nutrition and a rich microbiome. Enteric vaccines may be re-engineered to combine the best of both worlds by creating recombinant vaccines within probiotic strains as delivery vectors. Such recombinant

vaccines could merge the microbial-derived immunostimulatory signals and adjuvant-like properties of probiotics with the specific antigenic challenge. New research tools such as "humanized" mouse models, or mice with a human-derived microbiome and a human-like immune system, may enhance research in mucosal vaccinology and combination strategies with probiotics. New tools for clinical research may include micro-volume assays for different antibody subclasses and T lymphocyte function, and new protein arrays that can provide more complete assessment of immunity in the field. Our hope is that new research tools, when combined with nutritional support and microbiome-reshaping strategies for vaccine delivery, will point the way towards improved success rates with vaccine strategies for enteric and systemic infections in the developing world.

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