



ENHANCEMENT OF HUMAN HEALTH WITH *LACTOBACILLUS REUTERI* *A Probiotic, Immunobiotic and Immunoprobiotic*

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INTRODUCTION

Elie Metchnikoff was awarded the Nobel Prize for Physiology and Medicine in 1908 for the discovery of what today is known as innate or cell mediated immunity. In the previous year, Metchnikoff's musings on gastrointestinal microbes and their role in human health were published. In this work, entitled *The Prolongation of Life: Optimistic Studies* (1), he wrote that:

'A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honorable place.'

Thus the 'Probiotic Concept' was proclaimed, and the term 'probiotic' (from the Greek meaning 'for life') eventually assigned to Metchnikoff's honorable 'lactic bacilli'. Definitions of the term probiotic have evolved over the years, from Parker's 1974 version (2): '*Organisms and substances which contribute to intestinal microbial balance,*' to Fuller's 1989 redefinition (3): '*A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance,*' to today's consensus definition: Live commensal microbes administered orally in adequate amounts able to confer health effects on the host by improving its intestinal microbial balance. In each case, the concept proposes that consumption of live probiotics will

improve one's health by achieving a well balanced intestinal microbiota. It is one thing to define a concept, another to understand it. Only recently have answers begun to emerge to questions such as: What specific health benefits are probiotics able to confer, and by what molecular processes are they able to confer these benefits? What constitutes an intestinal microbial balance, and how might such a balance confer health benefits?

This report addresses these and other questions through the prism of information obtained during twenty years of intensive research on a unique probiotic species - *Lactobacillus reuteri*. It is one of only few enterolactobacillus species whose natural ecosystem is the vertebrate gastrointestinal (GI) tract, including humans. Host specific strains of *L. reuteri* have been isolated and tested for probiotic safety and efficacy when administered orally to their respective hosts. Safety and probiotic efficacy were experimentally demonstrated in each of a variety of hosts, and a

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summary of these experiments will be discussed under the heading: *L. reuteri* - A Unique Probiotic.

During the past five years, evidence has been obtained that *L. reuteri*'s probiotic efficacy stems from its ability to beneficially modulate the host's immune responses. Clancy (4) proposed that probiotics possessing such immunomodulatory activities be referred to as immunobiotics rather than as probiotics. Accordingly, information in this report on *L. reuteri*'s immunomodulatory effects will be discussed under the heading: *L. reuteri*: A Unique Immunobiotic. The third section of this report is entitled: **Why Should a Healthy Person Consume Immunobiotics?** The author will address this question based on in situ human studies - a first report of its kind - by Valeur *et al* (5). The reader will note that another term - immunoprobiotic - is used in this report's title. The reason for this term will become evident in the fourth and final section of this report entitled: *L. reuteri*: A Unique Immunobiotic.

***L. reuteri*: A Unique Probiotic**

A team of American and Swedish microbiologists commenced studies on *L. reuteri* in the mid-1980s. Interest in this species as a potential reservoir of probiotic strains was based on (a) discovery that *L. reuteri* is unique among enterolactobacilli in its ability to produce reuterin (6), a potent antimicrobial agent, (b) reports that this species is indigenous in human and animal GI tracts (7), and (c) reports that *L. reuteri* together with *L. gasseri* are the predominant indigenous *Lactobacillus* species in human infants and adults (8). In addition, the unique ability of *L. reuteri*, to secrete reuterin on agar colony plates, provided a specific and sensitive method for distinguishing

and accurately enumerating their presence in complex biological tissues, including gut tissues and fecal samples. This method was used for isolating, enumerating, and selecting host-specific *L. reuteri* strains from humans and animals, including mice, rats, chickens, turkeys, pigs, cats, dogs, horses and ostriches. This ability to specifically enumerate *L. reuteri* cells in animal GI tracts was a key factor in providing a quantitative base for probiotic efficacy experiments using a variety of animal model systems. All animals to be tested for probiotic efficacy, whether purchased as laboratory stocks or obtained from commercial farms, were first examined for presence and numbers of indigenous *L. reuteri* in their gut and feces. If high numbers were found, the animals were deemed unsuitable inasmuch as comparative *L. reuteri*-deficient controls were therefore unavailable. On the other hand, those shown to contain negligible levels (i.e. < 200 colony forming units per g tissue) of *L. reuteri* cells in their gut, served as suitable controls to be compared to their *L. reuteri*-treated counterparts when confronted with a serious biological, chemical, and/or environmental challenges. Human clinical trials were conducted in a similar manner by comparing untreated subjects, which exhibited negligible levels of fecal *L. reuteri*, to their *L. reuteri*-treated counterparts.

Host-specific strains of *L. reuteri* exhibit broad-spectrum probiotic efficacy in a broad spectrum of hosts and meet all probiotic requirements.

A thorough review of studies on *L. reuteri*'s probiotic safety and efficacy was published in 2000 (5).

It was shown that host-specific strains of *L. reuteri* provided their hosts with significant probiotic protection from an assortment of challenges. The results shown in *Table 1* were obtained from laboratory animal models studies and large-scale field trials with poultry and swine. The results of numerous human clinical trials were published in an earlier issue of this journal (9). In addition to proof of their safety and efficacy in enhancing their host's health and well being, host-specific *L. reuteri* strains were shown to meet all requirements demanded of a probiotic (*Table 2*). These studies contributed significantly to validation of the probiotic concept (10), and the following conclusions were drawn:

- *L. reuteri* is a symbiotic bacterial species well adapted to colonize human and animal GI tracts, with hosts' spanning the vertebrate phylogenetic spectrum from avian to mammalian species, including humans.

Table 1 Administrations of viable host-specific strains of *Lactobacillus reuteri* confer significant protection from a broad-spectrum of diseases in a broad spectrum of animal models

Animal model	Disease/Syndrome	<i>L. reuteri</i> treatment effect
Mouse	<i>Salmonella typhimurium</i> infection	Decreased mortalities; moderation of gut inflammation
Mouse (TCR- α)*	<i>Cryptosporidium parvum</i> infection	Diminished hyperplasia and cecal inflammatory lesions
Mouse (bg/bg-nu/nu)*	<i>Candida albicans</i> infection	Systemic candidiasis reduced
Mouse (retrovirus)*	<i>Cryptosporidium parvum</i> infection	Reduced <i>C. parvum</i> oocytes in gut
Mouse	Fat diet-induced hyperlipidemia	Normal serum lipids restored
Rat	Acetic acid-induced colitis	Prophylactic protection; decreased bacterial gut translocation
Rat	Methotrexate-induced enterocolitis	Reduced enterocolitis; decreased bacterial gut translocation
Rat	Liver resection	Decreased bacterial gut translocation
Pigs (colostrum dep)*	<i>Cryptosporidium parvum</i> infection	Decreased diarrhea
Pigs	w/o growth promoting antibiotics	Growth as good or better than w/ antibiotics
Pigs	Fat diet-induced hyperlipidemia	Normal serum lipids restored
Chickens	<i>Salmonella</i> & <i>E. coli</i> infections	Decreased mortalities; moderation of gut inflammation
Chickens	Protein deficiency, growth depression	Enhanced growth
Turkeys	<i>Salmonella</i> & <i>E. coli</i> infections	Decreased mortalities; moderation of gut inflammation
Turkeys	Environmental/commercial stressors	Decreased mortalities; improved body weight & feed conv.

* (TCR- α)= T cell receptor- α deficient mice; (bg/bg-nu/nu) = athymic mice; (retrovirus) = LP-BM5 leukemia virus immunosuppressed mice; (colostrum dep.) = colostrum-deprived piglets; feed conv = feed conversion factor

Table 2 *Lactobacillus reuteri* meets all requirements for use as a human probiotic

Requirements	<i>L. reuteri</i> compliance
Species/strains identified?	Yes, physiologically and genetically.
Physiology, genetics studied?	Yes, including sequenced genome.
Indigenous to host?	Yes, indigenous to humans and animals.
Safety determined?	Yes, w/ adults, children, & HIV+ adults.
Efficacy established?	Yes, animal models, human clinical trials.
Antimicrobial activities	Yes, reuterin, reutericyclin, bacteriocins.
Published studies?	Yes, > 120 peer reviewed publications
Mode(s) of action evaluated?	Yes, Multiple modes of action indicated.
Commercially produced?	Yes, w/ patents, insured OTC viability as capsules, tablets, drops, straws, powders, yoghurts, milks, drinks, lozenges, gums

- *L. reuteri* is unique among probiotic microorganisms in its ability to produce and secrete a metabolic intermediate (reuterin) capable of antagonizing pathogens.
- Selected strains of *L. reuteri* confer broad-spectrum protection from various diseases in an equally broad-spectrum of hosts. Included in this regard is protection from: (a) certain viral, bacteria, fungal, and protozoan diseases, (b) certain chemically-induced and environmental stressor-induced diseases, and (c) hypercholesterolemia associated with a high fat diet.
- Metchnikoff's probiotic concept was fully validated, based on evidence of *L. reuteri*'s unique

broad-spectrum probiotic efficacy established in well-controlled laboratory experiments, field trials with animals, and clinical trials with human subjects.

- *L. reuteri* exhibits biotherapeutic effects as well as prophylactic protection.
- *L. reuteri* can be grown on a commercial scale, and methods have been developed to preserve its viability for extended periods of time, allowing for the production of *L. reuteri*-contain-

ing functional foods and supplements for human and animal applications.

It should be noted that prior to undertaking these safety and efficacy studies, the *L. reuteri* research teams adopted strict standards and demanded statistical evaluations in determining probiotic efficacy in animal model studies as well as human clinical trials. Probiotic efficacy was defined as the ability of pure, viable, orally administered cultures of host-specific strains of *L. reuteri* to significantly and consistently improve their host's health and well-being when compared to placebo-controlled counterparts by (a) preventing (i.e. functioning as a prophylactic agent), and/or (b) moderating (i.e. functioning as a biotherapeutic agent) the negative consequences of diseases to which the host was susceptible and/or with which the host was experimentally challenged.

In the earlier years of probiotic research it was paradigmatic that probiotic efficacy, if demonstrated, was the consequence of successful microbe vs microbe interactions in which the probiotic competitively excluded or otherwise inhibited growth of pathogens. Occasionally, such competitive exclusion effects were seen in *L. reuteri*-treated subjects. Its health-enhancing efficacy, however, was more consistently associated with its ability to down-regulate intestinal inflammations (Figs. 1,2). Furthermore, one could not easily explain *L. reuteri*'s ability to down-regulate inflammations caused by chemical insults (e.g., methotrexate-induced enteritis; 11, 12) as being a microbe vs microbe interaction. It soon became apparent that *L. reuteri*-host rather than *L-reuteri*-pathogen interactions were involved. The following observations confirmed this view

(10). First, morphometric analyses of ileal tissues obtained from perfectly healthy young control vs *L. reuteri*-treated chicks showed that treated birds exhibited statistically larger crypts and villi in the ileal sections of the intestine (Fig. 3).

Secondly, *L. reuteri* alone, in an otherwise germ-free mouse, stimulated normal development of gut villi (Fig. 4). Thirdly, *L. reuteri* administrations significantly increased the CD4+/CD8+ T cells ratio in ileal tissues of both normal, healthy chicks as well as in *Salmonella typhimurium* infected and cold-stressed chicks (Fig. 5).

Figure 1 Lactobacillus reuteri down-regulates Cryptosporidium parvum-induced cecal inflammation in TCR- α -deficient gnotobiotic mice

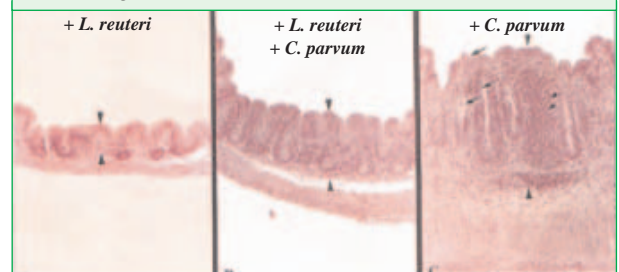


Figure 2 Lactobacillus reuteri and Gentamicin down-regulate ileal inflammation in young chicks orally challenged at hatch with Salmonella typhimurium

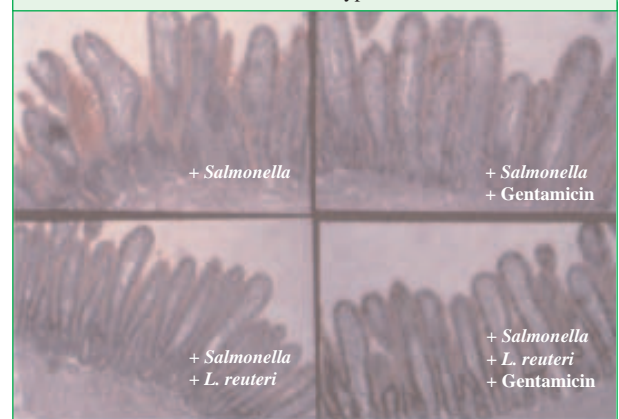


Figure 3 Healthy Lactobacillus reuteri-treated chicks have deeper ileal crypts and longer villi than untreated controls

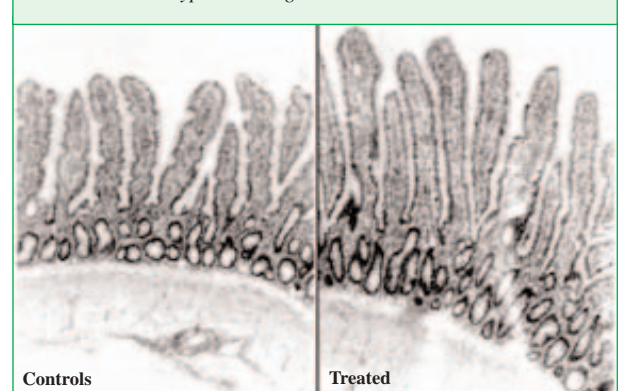


Figure 4 Mouse-specific strain of *Lactobacillus reuteri* stimulates normal ileal villi development in otherwise germ-free mice



It became clear that *L. reuteri* was interacting directly with the host's tissues, and that such interactions occurred even in perfectly healthy animals. A paradigm shift had taken place in the field of probiotics, and new quests were undertaken to better understand the nature of beneficial microbe-host interactions. Rapid advances along these lines are being made, and studies on *L. reuteri*-host interactions have contributed greatly to this advance.

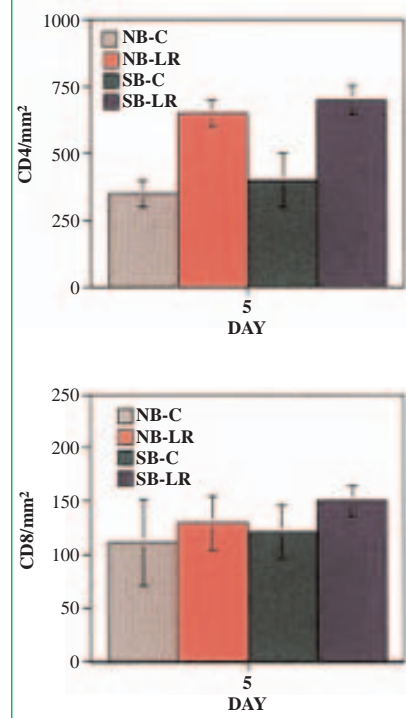
L. reuteri: A Unique Immunobiotic

Clancy (4) proposed "that the term 'probiotic' had served its useful generic function of drawing attention to 'health promoting' bacteria at mucosal surfaces', but 'to continue to use the term 'probiotic' for those bacteria which promote health by 'restoring mucosal T cell balance' would appear outmoded, and potentially confusing when communicating about immune-regulating bacterial species.' He suggested a new term - immunobiotics - to identify 'those bacteria that promote health through activation of the mucosal immune apparatus', and that 'recognition of bacteria that promote mucosal T cell function as immunobiotics moves probiotic biology forward by focusing on a mechanism of outcome, i.e., immunomodulation at distant mucosal sites.' It does not, however, diminish the need to attend longstanding concerns regarding the biology and accreditation of these bacteria as health enhancing agents.' The previous section of this report focused on the biology and accreditation concerning *L. reuteri* strains - as probiotics. The following section will focus on recent studies confirming *L. reuteri* strains - as immunobiotics.

L. reuteri immunomodulation of dendritic cells (DCs)

L. reuteri's immunomodulatory activities have been recorded and reviewed in considerable detail else-

Figure 5 *Lactobacillus reuteri* stimulates production/recruitment of CD4+ T cells (but not CD8+ T cells) in the ileal region of both healthy un-stressed (normal Brooded) and Salmonella-stressed (Stress Brooded) 5 day-old chicks



where (13). Only the 'distilled essence' of *L. reuteri*'s immunobiotic activities are presented in this report.

The first of these was reported by Christensen *et al* (14) who showed that probiotic lactobacilli exerted their immunomodulatory effects by modulating the Th1/Th2/Th3/Tr1/Treg-promoting capacity of dendritic cells (DCs). They showed that when murine DCs were exposed to co-cultures of different *Lactobacillus* species, including *L.*

reuteri, they were differentially modulated for production of cytokines IL-6, IL-10, IL-12, and TNF- α , and for up-regulation of MHC class II and CD86 surface markers in a concentration dependent manner. All lactobacilli up-regulated surface MHC class II and CD86 markers - indicative of DC maturation.

Particularly notable in these studies was that *L. reuteri* (strain 12246) was a poor IL-12 inducer, but when in co-culture with *L. johnsonii* or *L. casei*, it differentially inhibited production of the pro-inflammatory cytokine signals IL-12, IL-6 and TNF- α , which were stimulated by the latter two species. IL-10 production remained unaltered under these conditions. These findings led to their conclusions that '*L. reuteri* may contribute to an environmental modulation of the intestinal dendritic cell generation favoring tolerance toward antigens bearing no 'danger signal' while at the same time keeping intact the capacity to respond against pathogens recognized via a danger signal like LPS.' And that '*L. reuteri* might be a potential fine-targeted treatment effective for downregulating production of IL-12 and TNF- α (and IL-6) while inducing the anti-inflammatory IL-10, thus representing an alternative therapeutic approach to counter-balance the pro-inflammatory intestinal cytokine milieu.' And thus 'the potential exists for Th1/Th2/Th3-driving capacities of the gut to be modulated according to composition of gut microflora, including ingested probiotics.'

Smits *et al* (15) extended these observations and showed that *L. reuteri* has the ability to prime DCs to stimulate T regulatory (TR) cell production.

They used three different

Lactobacillus species co-cultured in vitro with human monocyte-derived DCs. Two of the lactobacilli, a human *L. reuteri* strain (ATCC 53609) and *L. casei*, but not an *L. plantarum* strain, primed these DCs to stimulate development of TR cells. These TR cells were shown to produce increased levels of IL-10 and were able to inhibit proliferation of bystander T cells in an IL-10-dependent fashion.

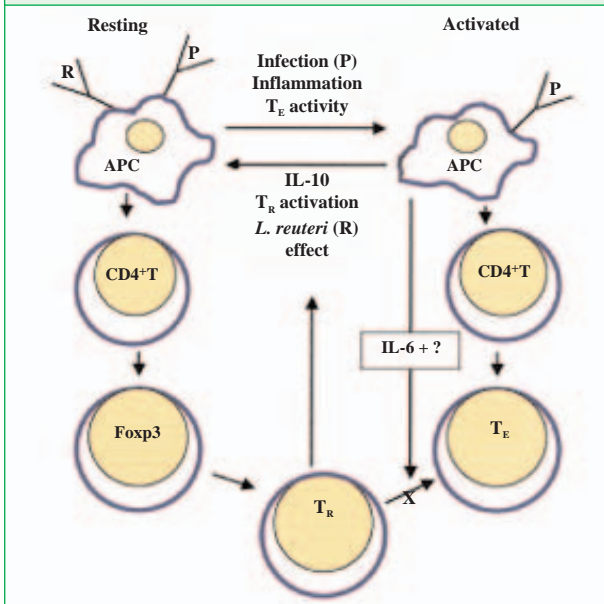
These studies on *L. reuteri*-DC interactions, viewed in connection with ground-breaking studies by Hori *et al* (16) and Pasare and Medzhitov (17), have provided valuable insights into one of *L. reuteri*'s immunobiotic modes of action.

A model to this effect, based on one constructed by Powrie and Maloy (18), is presented in Figure 6.

Depicted in this model is an equilibrium between resting DCs and activated DCs, both capable, through their Toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs), of sampling the mucosal 'state of affairs' including luminal antigens. The activation state of these DCs (and other antigen presenting cells, APCs) determines the type and magnitude of the CD4+ T cell response. Recognition of luminal and/or infiltrated pathogens (or other 'danger signals') activates the DCs/APCs (a) to produce IL-6 and another TLR-induced factor which together override the suppressive effects of TR cells, thereby allowing efficient generation of T effector (TE) cells capable of eradicating pathogenic threats. In the absence of pathogens (or other 'danger signals') the DCs/APCs acquire their resting state. Based on the study by Christensen *et al* (14) and Smits *et al* (15), DC-TLR recognition of immunobiotic microbes such as *L. reuteri* promotes an equilibrium shift favoring the resting DC state even in presence of pathogens as depicted in Figure 6.

Powrie and Maloy (18) among others view TR cells as an integral component of the immune response. These cells primarily appear to fine tune protective antimicrobial immunity in order to minimize harmful immune pathology. And, that future studies on factors controlling the development and activation of TR cells should enable us to shift the equilibrium either toward TR cell activity (to treat autoimmune diseases and to enhance survival of organ transplants), or away from TR cell activity (to boost vaccination and tumor rejection). The present author and colleagues propose that immunobiotic strains of *L. reuteri* shift the equilibrium toward TR cell

Figure 6 Proposed role for *Lactobacillus reuteri* in promoting development of CD4⁺CD25⁺ T regulatory (T_R) cells and maintenance of intestinal immune homeostasis



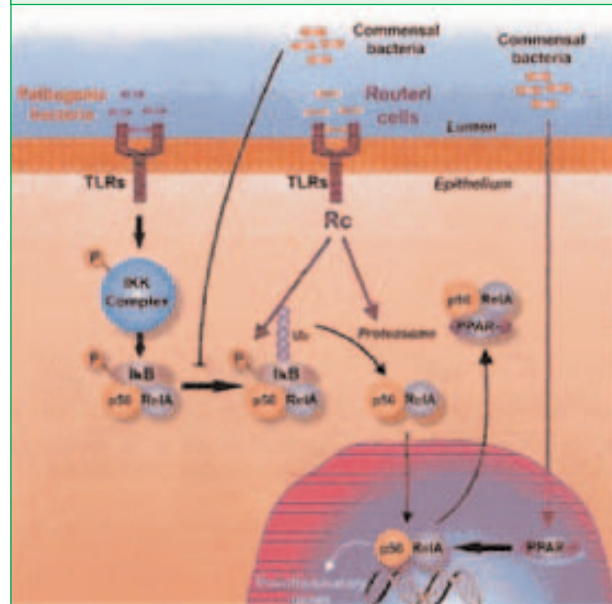
activity thereby accounting, at least in part, for their beneficial effects on the host's health. Described below is evidence that *L. reuteri*'s promotion of TR cell development and activity may occur in concert with yet another immunobiotic mode of action - one involving intestinal epithelial cells as described below.

***L. reuteri* immunomodulates intestinal epithelial cell (IEC) activities**

Ma *et al* (19) recently showed that *L. reuteri* cells also interact directly with IECs. Human IEC lines T84 and HT29 were cultured in the presence a viable human strain of *L. reuteri* (ATCC 23272) and analyzed for production of nerve growth factor (NGF), IL-10, and TNF- α - induced IL-8. The results showed that only live *L. reuteri* cells up-regulated NGF in both cell lines while inhibiting synthesis of IL-8 as well as IL-8 synthesis induced either by TNF- α or by *Salmonella enterica* (serovar *typhimurium*). The importance of an immunobiotic's viability on gut mucosal stimulation was stressed also by Galdeano and Perdigon (20).

Ma *et al* (19) showed that pre-incubation and adherence of live *L. reuteri* cells was also required for these effects. Convincing evidence was obtained showing the ability of these adhered *L. reuteri* cells to inhibit translocation of NF- κ B to the nucleus of the IECs occurred by preventing degradation of the I κ B complex. A model illustrating *L. reuteri*'s ability to prevent degradation of I κ B and thereby translocation to the IEC nucleus is presented in Figure 7.

Figure 7 *Lactobacillus reuteri* cells down-regulate intestinal epithelial cell inflammatory responses by preventing NK- κ B access to the nucleus



The *L. reuteri* effect shown in this figure was superimposed on an illustration by Beg (21) depicting a similar inhibition of I κ B degradation by a non-virulent *Salmonella* strain (22). In this case, the degradation was prevented by the ability of the non-pathogenic *Salmonella* to inhibit the ubiquitination of the I κ B complex that is induced by pathogens. This inhibition prevents I κ B proteosomal degradation and thereby NF- κ B translocation to the nucleus.

Whether *L. reuteri* ATCC 23272 inhibits the ubiquitination and/or the subsequent proteosomal degradation is not clear at this time. Also shown in this model is another unique anti-inflammatory mechanism induced by the commensal microbe, *Bacteroides thetaiotamicron* (23). This species is believed to antagonize the pro-inflammatory transcription factor, NF- κ B, in pathogen-challenged Caco-2 cells by triggering association of the Rel A subunit of NF- κ B with the nuclear hormone receptor and transcription factor PPAR- γ (peroxisome proliferator activated receptor- γ).

Kelly *et al* (23) propose that 'This newly formed complex is rapidly exported from the nucleus, thus attenuating expression of NF- κ B-regulated inflammatory genes.' *L. reuteri* cells appear to do so by denying NF- κ B access to the IEC nucleus (19).

Fate of L. reuteri cells in IL-10 deficient mice

Madsen *et al* (24) showed that IL-10 deficient mice have a normal colon at two weeks of age, but by four weeks develop a mild spontaneous colitis that worsens until it plateaus at eight weeks.

At the two week onset, deficient mice, relative to controls, show a significant increase both in colonic mucosal adherent and translocated aerobic bacteria, indicating that a primary alteration occurred in bacterial

colonization of the gut. At this age, the mice also show a significant decrease in the total population of protective colonic *Lactobacillus* species, commensurate with development of colitis. 'Speciation experiments showed that at two weeks of age, the control mice were colonized predominately by *L. reuteri*; whereas, the IL-10-gene-deficient mice were predominately colonized by *L. johnsonii*' (24).

Observing these profound microbial alterations, they asked if inoculating the deficient mice at one week of age with an *L. reuteri* strain isolated from healthy control mice would prevent these alterations and development of colitis. An *L. reuteri* inoculation was administered and (a) the total *Lactobacillus* population increased - an increase primarily accounted for as *L. reuteri* cells, (b) the colonic mucosal adherent and translocated aerobic bacteria were normalized, and (c) the development of the colitis was attenuated.

Pena *et al* (25) discovered similar changes in IL-10-deficient C57BL/6 mice when compared to control Swiss Webster, nitric oxide synthetase-deficient C57BL/6 mice. From 20 control mice, without colitis, six *Lactobacillus* species were recovered, 72% of them being either *L. reuteri* or *L. murinus*. In contrast, only *L. johnsonii* was isolated from fourteen IL-10 deficient mice.

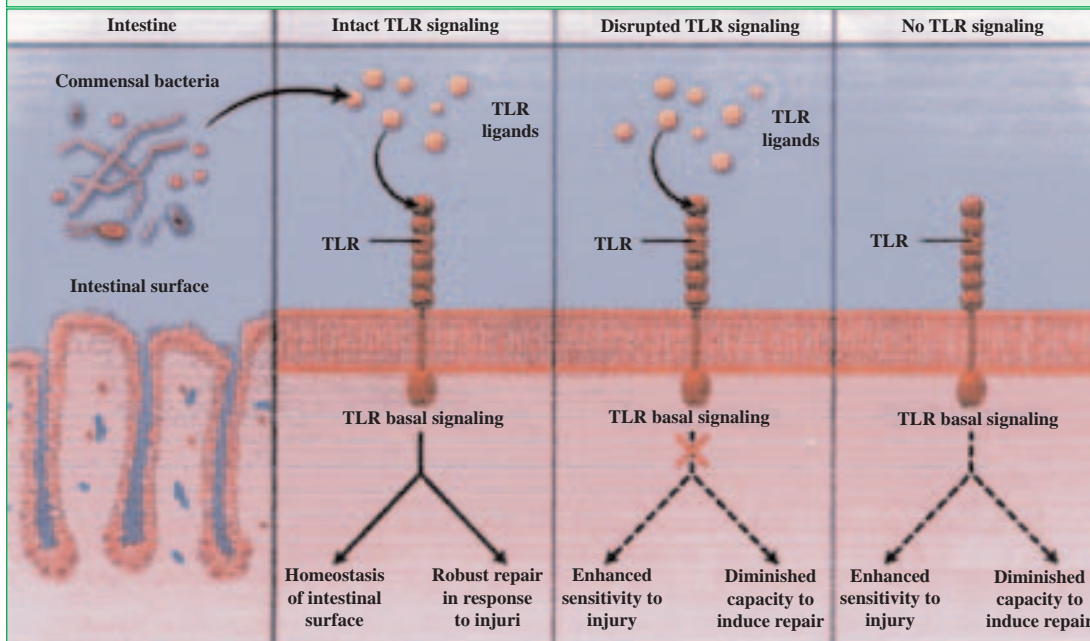
Furthermore, when representative lactobacilli from both sets of mice were tested for their ability to inhibit TNF- α production by LPS-activated murine RAW 264.7 macrophages, the strains recovered from the mice without colitis displayed TNF- α inhibitory properties, while none of the *L. johnsonii* isolates from the IL-10-deficient mice exhibited this effect. This and the Madsen *et al* (24) study have provided an interesting new perspective on *L. reuteri*'s role in protecting gut tissues from immune pathology. They also raised interesting questions concerning the molecular processes that underpin and regulate microbe-host interactions. In particular, how does a host's genetic deficiency in IL-10 production cause specific species and strain alterations in the gut microbiota - a consequence of which is a loss of immunobiotic protection and onset of inflammatory pathologies?

Requirement for gut microbiota - TLR interactions in human and animal health

Perhaps answers to these questions will be forthcoming as important advances are being made in understanding host-microbe interactions. The following seminal discovery is a case in point.

Although not directed to the nature of probiotic/immunobiotic microbe-host interactions *per se*, recently published studies by Nakoff-Naholm *et al* (26) provide valuable new insights on the evolution of host-microbial interactions. They showed that '*commensal bacteria are recognized by TLRs under normal steady state conditions, and this interaction plays a crucial role in the maintenance of intestinal immune homeostasis.*'

An analysis of their findings by Madara (27) concludes that '*exposure of the intestinal surface to commensal-derived TLR ligands and the resulting activation of the TLR pathway are required for full health.*' As illustrated in *Figure 8*, this recent study by Rakoff-Naholm and colleagues provides (26) new insights into the fundamental nature of microbe-host interactions, namely, that commensal bacteria interact with the intestinal surface and, to some degree, trigger TLR signaling - an interaction

Figure 8 Role of Toll-like receptor (TLR)-microbe interactions in maintenance of intestinal homeostasis

that is actually required to maintain the architectural integrity of the intestinal surface. 'Thus, it seems that the epithelium and resident immune cells do not simply tolerate commensal bacteria but are dependent on them' (27).

Based on (a) evidence that gut microbe- TLR interactions are required for full health, (b) evidence showing TLR involvement in *L. reuteri*-IEC interactions, and (c) evidence showing TLR involvement in *L. reuteri*- DCs/APCs interactions, *it is proposed that immune functions of IELs and resident gut APCs/DCs may be dependent on immunobiotics such as L. reuteri for maintenance of host intestinal immune homeostasis and prevention of inflammatory damage.*

Why Should a Healthy Person Consume Immunobiotics?

It was noted earlier in this report that the first evidence obtained by our laboratories concerning an effect of orally administered *L. reuteri* on the host per se was an increased CD4⁺/CD8⁺ T cell ratio observed in the chick ileum. Valeur *et al* (5) recently extended this observation to include the human ileum. They obtained direct *in situ* evidence in human subjects showing (a) distribution of individual human-specific *L. reuteri* ATCC 55730 cells throughout the human gut and (b) apparent involvement of *L. reuteri* ATCC 55730 in the recruitment and/or proliferation of CD4⁺ T cells specifi-

cally in the ileal regions of the intestines.

They concluded that '*L. reuteri* administrations elicited a recruitment of CD4⁺ T-helper cells to the human epithelium. This recruitment may be one factor in explaining the probiotic effect of this *L. reuteri* strain in man.'

Based on the Smits *et al* (15) findings, it is tempting to presume that this population is enriched with TR cells, but this remains to be determined. Nonetheless, and to the best of the author's knowledge, the evidence provided by Valeur *et al* (5) shows for the first time a beneficial effect of administering an immunobiotic such as *L. reuteri* strain ATCC 55730/SD2112 to healthy individuals.

The beneficial effect being that: the presence in sufficient numbers of immunobiotic agents throughout the GI tract perpetually primes the host's immunoresponsive cells to maintain a state of intestinal immune homeostasis.

Another benefit for healthy individuals is *L. reuteri*'s ability to function as an adjuvant for vaccinations (10).

Vaccinations have been shown to be significantly more effective when administered to *L. reuteri*-colonized animals in comparison to their untreated controls (13). The adjuvant effect of *L. reuteri* is awaiting clinical evaluations with human subjects.

***L. reuteri*: A Unique Immunoprobiotic**

The author accepts the consensual definition of a probiotic that is widely used today and agrees with Clancy's (4) proposal that the term immunobiotic better defines those strains shown to confer beneficial immunomodulatory effects on recipient hosts.

Based on the above cited studies, all *L. reuteri* strains evaluated to date can be classified as immunobiotics.

On the other hand, what if an immunobiotic species/strain is able to confer significant benefits to its hosts, but some of those benefits are not the result of immunomodulatory activities? The author proposes another term - *immunoprobiotic* - for such microbes (Table 3).

L. reuteri strains are offered as the prototypic immunoprobiotics based on the following information:

- ***L. reuteri* strains (including the human strain ATCC 55730/ SD2112) are unique among lactobacilli in their ability to synthesize vitamin B₁₂ (cyanocobalamin).** This finding was anticipated inasmuch as the author and colleagues had previously reported on *L. reuteri*'s ability to convert glycerol to reuterin - a B₁₂-requiring dehydrase reaction (10).

The human *L. reuteri* genome has been sequenced confirming presence of the genes required to encode vitamin B₁₂. Concerning B₁₂ synthesis by *L. reuteri*, Taranto *et al* (28) concluded that: '*the finding of a LAB (lactic acid bacteria) strain able to produce cobalamin would be of remarkable importance for the food industry and in medical and veterinary fields. To our knowledge this is the first report of cobalamin biosynthesis by lactic acid bacteria (LAB).*'

However, it is unknown at the present time if probiotic administrations of *L. reuteri* contribute significantly to the host's vitamin B₁₂ needs.

- ***L. reuteri* strains synthesize unique prebiotics**
It would be of considerable interest and enormous commercial value if already proven efficacious immunobiotic *Lactobacillus* species/strains were found also to produce prebiotic oligosaccharides. *L. reuteri* (strain LB 121), among others, was found to produce unique glucan and fructan exopolysaccharides (29-34). It remains to be seen if these prebiotics are produced under *in vivo* conditions.
- ***L. reuteri* strains produce conjugated linoleic acid (CLA)**
CLA is a mixture of positional (e.g., 7,9; 9,11; 10,12; 11,13) and geometric (cis or trans) isomers

Table 3 As new information accumulates, new definitions emerge

Probiotics (general definition):

Live commensal microbes administered orally in adequate amounts able to confer health effects on the host by improving its intestinal microbial balance.

Immunobiotics (Clancy, 2003):

Probiotics shown to confer these health benefits by beneficial modulation of the host's mucosal immune responses.

Immunoprobiotics (Dobrogosz, 2005):

Immunobiotics which confer additional health benefits, such as, production of prebiotics, production of vitamin B₁₂, production of conjugated linoleic acid, etc.

of octadecadienoic acid - a microbial product recognized as a cancer inhibitor (35), an antioxidant (36), an anti-cholesterolemic agent (37), a body fat reducing agent (38), an atherosclerosis inhibitor (39), a modulator of the immune system, and a growth promoting factor (40). Pariza *et al* (41) found *L. reuteri* to be one of the CLA-producing bacterial species in the conventional rat. It is now well documented that a variety of anaerobic bacteria produce CLA, including a number of lactic acid bacteria, and lactobacilli, including *L. reuteri* (42). Lee *et al* (42) examined various physiological parameters affecting CLA production from linoleic acid by *L. reuteri*, and selected *L. reuteri* as the species of choice for CLA production because it was among only a few species that produced not only the cis-9 trans-11 isomer of CLA but also the trans-10, cis-12 isomer - both considered to be the biologically active isomers (43). Lee *et al* (43) using *L. reuteri* immobilized on silica gel to develop a highly effective and efficient method for large scale isolation and manufacture of CLA from linoleic acid.

Jenkins and Courtney (44) found that *L. reuteri* membranes contain 14-18% linoleic acid under normal growth conditions. This fatty acid was not detected in other lactobacilli analyzed. In fact, no other lactobacilli have been reported to contain linoleic acid in their membrane phospholipids. However, although both of their *L. reuteri* strains contained membrane linoleic acid, only one of them was able to convert this acid to CLA. It remains to be determined if production of linoleic acid and CLA by *L. reuteri* strains is a signature characteristics of this species.

- ***L. reuteri* strains exhibit anti-hypercholesterolemic activity**

L. reuteri strains have been shown to exhibit anti-hypercholesterolemic effects when orally administered to animals (pigs and mice) fed a high fat diet (10).

This effect derives from *L. reuteri*'s ability to produce and secrete the enzyme bile salts dehydrogenase into the gut digesta, resulting in deconjugation and excretion of bile, thus preventing the bile from being recycled back into the liver. In addition, *L. reuteri* strains were shown to neutralize the potential toxic/mutagenic effects believed associated with the deconjugated bile salts (10).

- ***L. reuteri* strains induce mucus binding factors when grown in the presence of mucin**

It has been shown that *L. reuteri* strains exhibit enhanced mucus-binding, and enhanced colonization of the GI tract, when grown in the presence of mucin (45). This binding function obtains from their ability to synthesize a cell surface protein, encoded by a cloned and sequenced gene designated Mub (46).

SUMMARY AND CONCLUSIONS

The ideas Elie Metchnikoff's planted over a hundred years ago and which eventually emerged as the probiotic concept are being harvested today as health-enhancing probiotic products.

The disbeliefs and skepticisms concerning the concept and the health-enhancing effects of these products are being replaced by insights into the molecular processes underlying their protective mode(s) of action.

Credibility issues concerning both concept and products have been contravened, and significant new contributions to human and animal health are forthcoming.

In all likelihood, the near future will witness accelerated scientific research and commercial development of prospective probiotics.

Those exhibiting beneficial immunomodulatory activities will be submitted for clinical scrutiny also at an accelerated rate.

Present interest in enterolactobacilli as a source for health-enhancing immunobiotics can be attributed to (a) emergence of sound clinical evidence attesting efficacy of established immunobiotic strains, such as *L. reuteri* ATCC 55730, and (b) a growing body of evidence that such strains play indispens-

able roles in promoting and maintaining gastrointestinal health.

L. reuteri, a unique species from which to isolate host-specific probiotic strains, is now considered a unique source for isolation of host-specific immunobiotic strains. Perhaps eventually this species will be considered a unique source for isolation of host-specific immunoprobiotic strains.

Whatever term will eventually apply, evidence has been obtained concerning their immunobiotic mode of action.

It is proposed that presence of in sufficient numbers in a host's GI tract is recognized by certain Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) present on dendritic cells (DCs)/antigen presenting cells (APCs) and intestinal epithelial cells (IECs).

This recognition promotes conversion of activated DCs/APCs to a resting state which in turn promotes conversion of naïve CD4⁺ T cells into T regulatory cells (TR cells) which in turn appear to fine tune protective immunity in order to minimize immune pathology. This involvement of *L. reuteri* strains in prevention of destructive inflammation and maintenance of intestinal homeostasis is believed to account, at least in part, for its proven clinical efficacy.

This conclusion obtains from (a) application of Ocham's razor to the seminal studies presented in this report, (b) direct in situ evidence obtained by Valeur *et al* (27) establishing a clear nexus between *L. reuteri* micro-colonies distributed through the human GI tract and their beneficial immunobiotic influences on these mucosal tissues, and (c) sound clinical evidence of *L. reuteri*'s biotherapeutic and prophylactic efficacy.

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