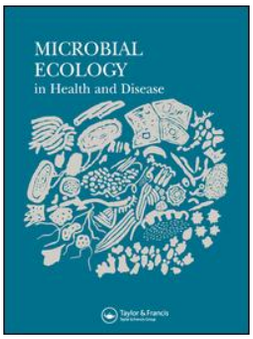

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Validation of the Probiotic Concept: *Lactobacillus reuteri* Confers Broad-spectrum Protection against Disease in Humans and Animals

Ivan A. Casas[1] and Walter J. Dobrogosz[2]

[1]BioGaia Biologics Inc. Raleigh, North Carolina, USA; [2]Department of Microbiology, North Carolina State University, Raleigh, North Carolina, USA

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Nobel Laureate Elie Metchnikoff formulated the Probiotic Concept approximately 100 years ago. He proposed that consumption of certain 'lactic bacilli' would enhance one's health and well being by maximizing health-promoting activities of the gastrointestinal microbiota and minimizing their potentially harmful effects. It has taken almost all these intervening years to discover specific strains of 'lactic bacilli' able to accomplish these 'probiotic' tasks. And only very recently has an entire species, *Lactobacillus reuteri*, been shown to possess probiotic efficacy. *L. reuteri* is the only *Lactobacillus* species reported to inhabit the gastrointestinal tract of all vertebrates and mammals, ranging from birds to humans, and with whom it is believed to have established a symbiotic relationship. In this review the authors have attempted to compile all available information reported to date concerning *L. reuteri* and the ability of host-specific strains to protect their respective hosts from an assortment of diseases induced by biological agents (bacteria, viruses, fungi, and protozoans), certain chemical agents (methotrexate, acetic acid), or environmental stressors (cold-stress). This information is based on laboratory experiments, field trials with animals, and clinical trials with human subjects. It has been concluded that discovery of *L. reuteri's* broad-spectrum probiotic efficacy in a broad-spectrum of hosts has (a) fully validated Metchnikoff's Probiotic Concept, and (b) resulted in development of new bioprotective and biotherapeutic applications for improving human and animal health.

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"Dare to be Naïve."
(R. Buckminster Fuller)

I. Introduction. In its most simplistic form, the 'probiotic concept' holds that consumption of certain viable microbial cultures as dietary supplements will improve a human or animal host's health and well being by improving its intestinal microbial balance (1). Envisioned almost 100 years ago by Nobel Laureate Metchnikoff, this concept remains controversial even today for many reasons, some based on theoretical grounds, others on practical considerations. Freter (2), for example, contends that probiotic "preparations containing a single or few types of bacteria are limited by ecological necessity." This argument appears supported by Mead and Impey (3) who showed that as many as 48 strains of bacteria were required to produce a protective effect against colonization of the avian gut by salmonellae. Along these same lines, Hentges (4) states that: "In the restoration of colonization resistance in the gastrointestinal (GI) tract, it is improbable—that probiotic measures designed to alter the ecology of the intestinal luminal contents will be successful."

Tannock (5) proposed that the 'ideal probiotic' should possess the following characteristics. It should persist for a long time in the GI tract and produce substances that inhibit gastrointestinal pathogens or stimulate immunity so as to increase the host's resistance to intestinal infections. It should contribute to the host's nutrition by synthesizing essential nutrients and/or by digesting dietary substances (e.g., lactose) that the host may be physiologically ill-equipped to utilize. It should be amenable to large-scale commercial production, be safe and devoid of characteristics that could compromise the host's health, and it should exhibit stability in all the above characteristics. He theorized, however, that attempts to isolate such an ideal probiotic strain would most likely fail, and that an alternative stratagem would be to derive such a strain by genetic manipulation.

On a somewhat more optimistic note, Havenaar, et al. (6) argued that it may be possible to isolate an 'ideal probiotic', but only if proper screening methods are used to identify the above listed 'ideal' traits. They suggested that *in vivo* efficacy testing be conducted only on strains thus selected to possess these 'ideal' traits. Barrow (7) on the other hand expressed concern about the high degree of variability observed when *in vivo* probiotic efficacy tests are conducted. How can probiotic efficacy of any strain be determined if the tests used to determine efficacy are unreliable owing to this high degree of variability? He attributes this variability, among other factors, to poor characterization of the strains used in the past and/or poor understanding of the microecology of the GI tract, positing that efficacy evaluations conducted using strains lacking either 'ideal' traits or host specificity are not likely to succeed in any event. And furthermore, that in too many instances, the occasional 'positive' results obtained are often over-optimistically, naively, and/or uncritically inter-

preted, serving "only to create a mystique of probiosis without an adequate rational assessment of its true value."

Perhaps most damaging to the 'probiotic concept' over the years has been the lamentable degree of commercial exploitation of the concept in the marketplace. Gilliland and Speck (8) reported, among numerous other examples of misuse, that both human and animal probiotic products labeled as containing *Lactobacillus acidophilus* often do not contain any viable *Lactobacillus* species, let alone the advertised *L. acidophilus*. Furthermore, the study of probiotics has languished too long under the burden of shallowness supported more by anecdotes, abstracts of unpublished findings, and commercial testimonies than by sound scientific analyses. This state of affairs has contributed to the skepticism that can be found today in some scientific, biomedical, and commercial circles. Little wonder that articles appear in contemporary literature with titles such as: Probiotics: Fact or Fiction? (9), Are Probiotics a Confidence Trick? (10), Probiotics, Prebiotics or 'Conbiotics'? (11), and, Probiotic bacteria: myth or reality (12).

The authors of this review entered the field of probiotics approximately 15 years ago fully aware that it was besieged by these seemingly insurmountable theoretical and practical obstacles. We knew that skepticism about probiotics was rampant, and we were skeptical ourselves as to whether or not any meaningful outcome would emerge from our endeavors. Our venture was considered by colleagues to be naïve at best, but, perhaps naively, we were heartened by R. Buckminster Fuller's admonition: "Dare to be Naïve" (13). The purpose of this review article is to record the laboratory experiments, field trials with animals, and clinical trials with human subjects that, in our opinion, have validated the probiotic concept in its most simplistic form. Namely, that a single 'lactic bacillus' species is able to confer to its human and/or animal host probiotic protection from certain diseases.

In this review the authors have focused almost exclusively on *L. reuteri* (hereafter referred to simply as Reuteri). References to urogenital tract lactobacilli have been excluded, as are references to probiotic fungal strains (e.g., *Saccharomyces boulardii*), streptococci, Gram-negative enterobacteria, or spore-forming bacilli. We defer to others to provide information on other intestinal lactobacilli whose probiotic efficacy has been subjected to scientific and clinical examination. These include: *L. rhamnosus* strain GG distributed by Valio in Finland, *L. acidophilus* strain NCFM by Rhodia (USA), *L. casei* strain Shirota by Yakult (Japan), and *L. casei* strain CRL431 by Chr. Hansen (USA) (14). The rationale for our singular focus on Reuteri stems from two considerations. First, it is apparent that a worldwide interest in the 'probiotic concept' has entered an 'exponential' phase of growth (Figure 1). To thoroughly review information on even those few strains of lactobacilli for which credible efficacy has been demonstrated would be more appropriately presented in book rather than review format. An extensive review along these latter lines was recently published (14a). Secondly, and of foremost consideration in this regard, is the fact that Reuteri is unique among probiotic cultures in that **the entire species has been shown to exhibit 'probiotic efficacy'**. Reuteri cultures isolated from various hosts, ranging phylogenetically from avians to humans, have been shown to exhibit probiotic efficacy when administered to those hosts. To date, no other 'lactic bacillus' has accomplished this task or so convincingly validated Metchnikoff's probiotic concept.

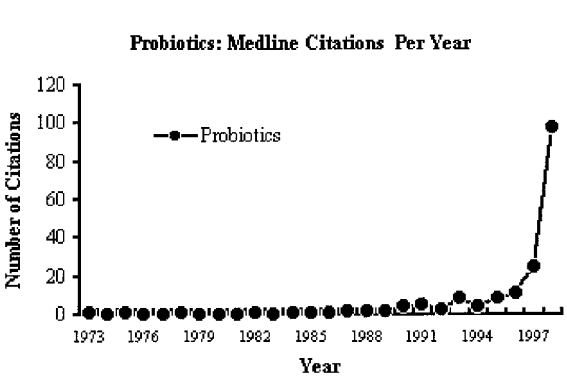

Fig. 1. Probiotic citations (per year) on MEDLINE database (1973 to 1998).

Probiotic Efficacy Defined. The term probiotic efficacy will be used throughout this report. The authors use this term to denote the demonstrated ability of a pure, viable culture of a well-identified microbial species (such as Reuteri), administered orally to human or animal hosts, to significantly and consistently improve the health and well-being of that host 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 that host is susceptible.

The etiology of diseases amenable to probiotic treatment may or may not be well defined. They may be induced, for example, by known microbial pathogens such as the bacterium *Salmonella typhimurium*, the protozoan *Cryptosporidium parvum*, the fungus *Candida albicans*, or by viruses such as rotavirus. Or, they may be instigated by chemical challenges, e.g., induction of gastroenteritis in an animal model by the drug methotrexate. Or, the disease may be the consequence of genetic aberrations discernable using 'gene knockout' animal model systems. Or, the disease may have ill-defined or unknown causes. A disease known as avian growth depression (AGD) exemplifies this situation. AGD occurs when chickens and turkeys are grown under intensive animal production conditions in which multifactorial stressors (crowding, sub-optimal temperatures, dust, litter dampness, etc.) cause deaths and assorted morbidities, including growth depression. A probiotic is deemed efficacious when it can be proven to

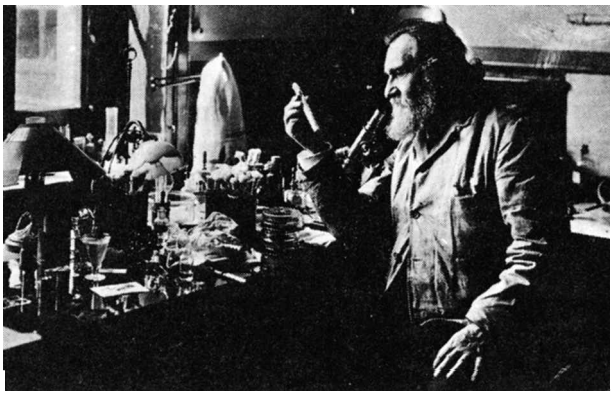

Fig. 2. Elie Metchnikoff (1845–1916) in his laboratory. Founder of the Probiotic Concept. Metchnikoff received the Nobel Prize for Medicine and Physiology in 1908 (shared with Paul Erlich) for his discovery of cell-mediated immunity. (Reprinted from Microbiology: Fundamentals and Applications, Macmillan Publishing Company NY, with permission of the publisher.)

prevent and/or moderate the deleterious consequences of a disease. This proof must be based on sound scientific evidence determined to be statistically significant. Probiotic efficacy cannot be claimed based only on evidence that a particular culture is able to establish itself in a host's GI tract. It must be proven to be safe and to enhance the host's health and well being as described.

Our Reuteri probiotic studies commenced in the mid-1980s stemming from discovery that this species produced and secreted a unique, non-bacteriocin, antimicrobial substance, termed reuterin. Research on this species continues today at an accelerated pace, and has been joined by laboratories around the world. Considerable information is now available concerning Reuteri's taxonomic status, habitats, general physiology, cell surface properties, plasmid biology, production of antimicrobial substances, its genetic character, and most importantly, its safety and efficacy as a probiotic for human and animal use. The purpose of this review is to summarize as briefly as possible all information published to date concerning this particular enterolactobacillus species. From the beginning, these studies focused on obtaining answers to the following four questions:

First, can scientifically sound, statistically significant evidence be obtained showing that Reuteri has probiotic efficacy?

Secondly, can this beneficial effect be demonstrated under 'real world' conditions, that is, in field trials with commercially grown animals, in animal model systems, and/or in clinical trials with human subjects?

Thirdly, if 'real world' probiotic efficacy is demonstrated, can Reuteri be produced on a large scale so as to be commercially available to enhance human and animal health?

And fourthly, what is the mechanism(s) underlying Reuteri's health-enhancing effects?

Both human and animal studies will be reported in this review, together with preliminary attempts to understand Reuteri's underlying mode of action. The reader is directed to an earlier review focused on Reuteri's efficacy as a probiotic for agriculturally important animals, particularly chickens and turkeys (15) and to an earlier overview of Reuteri's role in human and animal health (16). The reader is also referred to a treatise by Falk, et al. (17) concerning use of gnotobiotic animals in research aimed at understanding the 'cross talk' that occurs between gastrointestinal microbiota and their host's gastrointestinal tissues.

II. Developmental Stages in the Science of Probiotics. The Science of Probiotics is believed to have commenced approximately a hundred years ago (18–20) based on Elie Metchnikoff's (Figure 2) statement 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" (19).

Our understanding and expectations concerning the scientific and practical aspects of probiotics have undergone many changes since then (1, 21–24). And, if there is a single consensus to be gleaned from these recent studies and writings, it is that the field of probiotics has entered into a fourth stage of development.

The first stage began near the turn of this century when it witnessed Metchnikoff's formulation of the probiotic concept and prescience of a functional role of diets in human health (19). In addition to his recommendation to "absorb large quantities of microbes", Metchnikoff proposed that:

> "systematic investigations should be made on the relation of intestinal microbes to precocious old age, and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body."

Later, during the 1920s and 1930s, a second developmental stage ensued. It was based primarily on the findings of Rettger and colleagues (25, 26) who conducted systematic investigations and initiated human clinical trials in which intestinal isolates of *L. acidophilus* were used rather than the 'Bulgarian bacillus' yogurt strains which had been shown not to survive passage through the intestine. But it was not until after the World War II, and particularly in the 1950s, that a resurgent interest in gastrointestinal microbiology marked entry into a third stage of development. It was shown, for example, that certain peroral antibiotic treatments resulted in an increased growth of chickens, and that germfree animals not only maintained good health but in many instances outlived their non-germfree counterparts. These findings clearly confirmed Metchnikoff's contention that certain gut microorganisms can adversely affect a host's health and life span.

Additionally, it was shown that an indigenous microbiota provides the host with a mucosa-associated shield

protecting it from a variety of infectious diseases. Seminal findings along these lines by Bohnhoff, et al. (27), Freter (2, 28), and others showed that oral antibiotic administrations rendered experimental mice more susceptible to infections with *Salmonella*, *Shigella*, and *Vibrio* spp. Other researchers demonstrated that antibiotic treatments induced pseudomembraneous colitis caused by *Clostridium difficile* (29), and that administration of fecal suspensions could successfully treat such antibiotic-associated diarrheas. Later, Nurmi and Rantala (30) demonstrated the protective role of the gastrointestinal microbiota, showing that competitive exclusion (CE) of *Salmonella* from the gut of chickens could be achieved by early oral administrations of cecal extracts obtained from healthy adults. CE attributable to the gut microbes was subsequently confirmed for other *Salmonella* spp. (31, 35), and other enteropathogens such as *Escherichia coli* (32), *Campylobacter* (33, 34), *Clostridium* (36), and *Yersinia enterocolitica* (36). Impey, et al. (37) showed a similar protective effect following administration of 48 selected gut microbes. Collins and Carter (38) used germfree animals to provide one of the most convincing studies showing the protective effect of the gastrointestinal microbiota. They showed that whereas a germfree guinea pig was killed by as few as 10 *Salmonella enteritidis* cells inoculated per os, 10^9 cells were required to kill a guinea pig possessing its conventional microbiota.

The field of study generally known as Gastrointestinal Microbiology acknowledges both these positive and negative roles for that heterologous organ otherwise known as the gastrointestinal microbiota, and it seeks to understand the processes underlying these roles. Probiotic research is a sub-discipline of this field whose goals are embodied in its definition. According to Havenaar and Huis in't Veld (6) probiotics are defined as: "a mono- or mixed culture of live microorganisms which, applied to animal or man, beneficially affect the host by improving the properties of the indigenous gastrointestinal microbiota, but restricted to products which (a) contain live microorganisms, (e.g., as freeze-dried cells or in a fresh or fermented product), (b) improve the health and well-being of man or animals (including growth promotion of animals), and (c) can have their effect on all host mucosal surfaces, including the mouth and gastrointestinal tract (e.g., applied in food, pill, or capsule form), the upper respiratory tract (e.g., applied as an aerosol), or in the urogenital tract (local application)." Clearly, this field of study has remained faithful to Metchnikoff's recommendation "to absorb large quantities of microbes." But which microbes?

The probiotic concept appears to have entered a fourth stage of development-a stage in which researchers, practitioners, and the marketplace will henceforth reject uncritical appraisals of probiotics and accept only rational, scientifically sound assessments. This view is prevalent in recently published compendia on this matter (1, 21–24). Havenaar and Huis in't Veld (6) among others have set goals and criteria for assessing probiotic efficacy. They propose that: "If we want to get rid of the mysticism surrounding probiotics, fundamental research is necessary to collect information on how probiotics act. This involves development of adequate methods to quantify, localize, and identify the changes in the intestinal microflora, to establish basic criteria for the selection of bacterial strains, and to perform well-controlled animal experiments, field trials, and studies in humans. The mechanism by which probiotics exert their action must be the subject of future research". Following is evidence of Reuteri's contributions to demystification and validation of the probiotic concept.

III. Reuteri: From Species Obscurity to Prototypic Probiotic. Until recently, Reuteri existed in obscurity, misclassified as *Lactobacillus fermentum*. It was only in 1970s that suspicion of its misclassification emerged. Kandler, et al. (39) showed that lactobacilli previously identified by Reuter (40) and Lerche and Reuter (41) as *Lactobacillus fermentum* biotype II were in fact clearly distinguishable from other biotypes of this species based on several phenotypical and genetic characteristics. They proposed that *L. fermentum* biotype II be given distinct species status as *Lactobacillus reuteri* (named after Gerhard Reuter) and that Reuteri strain DSM 20016 (isolated from humans) be designated the type strain. This proposal was accepted, and since 1980 Reuteri has been classified as a distinct species in the genus *Lactobacillus* (42). It cannot be definitively distinguished from *L. fermentum* by simple physiological tests. Determinations of mole% G + C, diamino acid of the peptidoglycan, or electrophoretic mobility of LDH clearly separate the two species. Reuteri cells are slightly irregular, bent rods with rounded ends, generally 0.7–1.0× 2.0–3.0 μm in size, occurring singly, in pairs and in small clusters with generally good growth at 45°C and produce ammonia from arginine (42). The reader is referred to a comprehensive report by Axelsson (43) reviewing newer methods now available for identification and classification of Reuteri and the many other species in this complex and commercially important genus.

The authors and colleagues became interested some years ago in an enterolactobacillus culture isolated from pig intestine that manifested a novel antimicrobial activity. The antimicrobial agent was isolated, purified and characterized, and the culture manifesting this activity was subsequently identified as Reuteri (44) based on its physiological properties and DNA/DNA hybridization analysis. Today, Reuteri strains are identified based on (a) phenotype profiles, using the API 50 CH kit for lactobacilli (bioMerieux, Inc, Hazelwood, MO, USA), (b) their ability to produce the antimicrobial substance designated reuterin, and (c) PCR amplification (as described by Versalovic, et al. (45) of (i) a 1.5 kb DNA fragment corresponding to a DEAD-box helicase specific to Reuteri with primer pair S4 (5' ATTCC AATGG TTCTT GAGGG 3') and R4 (5' CCTTC CACGG CGAA TAAGC 3'), and (ii) a 0.9 kb

fragment from the reuterin synthesis gene (glycerol dehydratase) specific to Reuteri with primer pair DHAB1 (5' AACTA CGATA ACATG TTTGC 3') and DHAB7 (5' CCTTC TTCTT CAATT CCGGC A 3'). These species-specific primers were developed by Stephan Roos (Dept. microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden). Both primers have been tested in a wide variety of lactobacilli, and only Reuteri DNA has been shown to amplify these genes (Casas and Abad, unpublished data). It is expected that additional genetypic characterizations of Reuteri will be used in the future as described elsewhere (45a).

A. Natural Habitats: Is Reuteri a Universal Enterolactobacillus?. Reuteri has been isolated from a variety of food products including: meats and milk products (40, 41), lamb rennet paste, sheep milk and Pecorino Romano cheese (46), sour dough sponge (47, 48), fermented rice noodles (49), and fermented cane molasses (50). The species' primary habitat, however, appears to be the GI tract of humans and animals. Lerche and Reuter (41) reported finding what is now Reuteri in high frequency in human stool samples and contents of the proximal segments of bowels obtained from autopsy subjects. Oral intake experiments (40) using Reuteri demonstrated this species' ability to survive gut passage and to grow in the human bowel. Its propagative phase lasted only 3–6 days following a single administration. Survivability in gut passage was demonstrated for other hetero- and homofermentative lactobacilli as well.

Reuteri has been isolated directly from the GI tract or feces of humans (39–42, 51), chickens (52), pigs (53, 54), lambs (46), rodents (55), and minks (56). It has been suggested that Reuteri may be a unique, 'universal' enterolactobacillus (Table 1). Of the 18 species of enterolactobacilli isolated and identified by Mitsuoka (57) from humans, pigs, chickens, cattle, dogs, mice, rats, and hamsters, only Reuteri had the distinction of being a "major component of *Lactobacillus* species" found in all these hosts. Mitsuoka and colleagues (57a) subsequently reported Reuteri to be almost the only heterofermentative *Lactobacillus* species found in the intestines of their human subjects. They reported that essentially all gas-forming lactobacilli isolated from feces of healthy adults were identified as Reuteri, the exception being 3 cellobiose-positive heterofermenters. They also reported that of 305 *Lactobacillus* isolates obtained from the feces of 40 healthy adults, the homofermentative lactobacilli most frequently found in these samples were biovars of *L. acidophilus* (subsequently identified as strains of *L. gasseri* and *L. crispatus*), followed by biovars of *L. casei*, and *L. salivarius*.

In another study on human lactobacilli, Reuteri was isolated from different parts of the human intestine. It was shown to be among only 5 of 19 *Lactobacillus* strains capable of re-colonizing the adult human intestinal mucosa (51). Human colonization was host-specific for humans but not for rat Reuteri strains (55). Conversely, it was observed that among 6 different *Lactobacillus* strains

Table 1

Distribution of Lactobacillus species in human and animal intestines

Species	Humans	Pigs	Chickens	Cattle	Dogs	Mice	Rats	Hamsters
L. acidophilus group[b]								
L. acidophilus (A-1)	?					?	?	
L. amylovorus (A-3)		M	?	+				
L. crispatus (A-2)	M		M					
L. gallinarum (A-4)			M					
L. gasseri (B-1)	M			+				
L. johnsonii (B-2)	+	+	M					
L. murinus/animalis		?	?	M	M	M	+	
L. intestinalis						M	M	
L. salivarius	M	M	M					
L. agilis		+	+					
L. ruminis	+			M				
L. vitulinis	+							
L. hamsteri								M
L. aviarius			+					
L. casei	+							
L. plantarum	+							
L. brevis	+							
L. reuteri	M	M	M	M	M	M	M	M

[a]Symbols: M = Major component of *Lactobacillus* species; + = occasionally recovered; ? = questionable.
[b]DNA homology group by Johnson et al (ref. (57b)). Table reprinted from reference 57 with permission of publisher.

tested, only a rat strain of Reuteri was able to effectively colonize rat mucosa (55). The present authors recently obtained information adding further credence to the importance of host specificity (58). Three sets of germfree mice were used in the following experiment. Using germfree isolators, one set remained germ free, the other two sets were monocontaminated with a mouse strain of Reuteri and a human strain of Reuteri, respectively. Excellent and stable colonization by these strains was observed for up to 45 days as determined by fecal analyses. At that time (i.e., at 45 days), the monocolonized mice were given cecal contents obtained from Schaedler's cocktail-associated mice. Fecal samples continued to be analyzed for total lactobacilli and Reuteri. It was found that whereas the mouse-specific strain of Reuteri persisted for at least 30 days, the human Reuteri strain appears to have been rapidly replaced by the Schaedler lactobacilli. Based on this and other indications of host specificity among Reuteri strains, the authors have chosen to use only host-specific strains of Reuteri when conducting laboratory experiments, field trials, or clinical trials.

The authors have isolated Reuteri (identified using the parameters described above) from a variety of hosts. Included in our collection are strains obtained from humans (feces, mothers' milk, and vagina), pigs, chickens, turkeys, ostriches, mice, rats, hamsters, gerbils, cattle, horses, monkeys, and doves. We have noted that these strains exhibit varied and distinguishable colony characteristics (16), a trait not reported for other enterolactobacilli isolated from their respective host animals.

B. Horizontal Transfer of Reuteri to Newborn Animals

Comparative analyses of Reuteri and other lactobacilli recovered from frozen gut tissues obtained from two groups of piglets provided insights concerning how sows transfer Reuteri to their offspring (53). One piglet group had been allowed to suckle their mothers, the other group was colostrum-deprived. They were taken from their mothers immediately after birth and reared on an artificial diet in a clean environment. Whereas all suckling piglets were rapidly colonized with Reuteri and had high numbers of lactobacilli in all regions of their stomach and proximal GI tract, piglets isolated from their mothers at birth and fed the colostrum-deprived diet were less likely to be colonized. An examination of samples obtained from sows milk and nipple swabs revealed presence of Reuteri indicating association of the mother's mammary duct/milk with lactobacilli and other microorganisms apparently destined to become components of the newborn's gut microbiota.

An extensive study involving healthy infants and their mothers is presently underway (conducted under the auspices of the Department of Obstetrics, Tampere University hospital, Tampere, Finland) to determine how Reuteri accesses the human GI tract. Frozen samples of colostrum, breast milk, mothers' stools, mothers' vaginal swabs, infant meconium and subsequent stools are being analyzed for Reuteri and other lactobacilli. Results obtained to date (60) show that approximately 50% of the infants with Reuteri in their GI tract were born to mothers with Reuteri in their breast milk, vagina, and/or feces. This correlation supports the hypothesis that Reuteri is transmitted from mother to infant during birth and the nursing process. However, this hypothesis will be deemed correct only if the Reuteri strains isolated from the mothers and their off spring are determined to be identical by DNA identification. These identification analyses are in progress.

How do non-mammals, such as avian species, access Reuteri or other gut microbiota? How do they access Reuteri when hatched and grown in the absence of their mothers or other adults as occurs in the commercial poultry industry? Simply put, could probiotic administrations of Reuteri compensate poultry flocks for consequences linked to the absence of mother hens? An answer to the first question was obtained by showing that commercially grown turkeys had a substantial number of lactobacilli in their ceca at day 3 post-hatch (ranging from 9×10^{8} to 1.5×10^{10} cfu per g cecal contents). However, Reuteri could be found in only approximately 20% of these turkeys and in only approximately 10% of chickens tested under comparable conditions (61). Probiotic applications of Reuteri consistently resulted in 80 to 100% colonization. These findings provided the experimental basis for all our subsequent 'mother hen' studies; namely, to determine if probiotic treatments using host-specific Reuteri improved a flock's health and performance (i.e., decreased flock deaths, increased body weights, and increased feed efficiency) in direct comparison to the untreated control birds. These studies yielded two important findings. First, it was shown (see below for more details) that Reuteri-treated birds exhibited improved health and commercial performance when compared to the Reuteri-deficient controls (61–63), and secondly, Reuteri's probiotic efficacy was demonstrated even though all birds used in these studies. i.e., both control and experimental birds, had high numbers of other lactobacilli in their GI tract. While these other lactobacilli may contribute to a healthy gut ecosystem and ultimately to the host's health, their contributions were unable to obscure Reuteri's probiotic effect.

C. Effect on Microflora-Associated Characteristics (MACs) in the Rat. It is well known that the chemical composition of a host's lumen/fecal contents is determined by combined metabolic activities of the host intestinal tissues and resident microbiota. And, that much can be learned concerning the 'cross talk' that occurs between the host and its GI microbiota by analyzing the composition of lumen/fecal contents using gnotobiotic approaches (17). Chemical changes attributable only to the host's metabolic

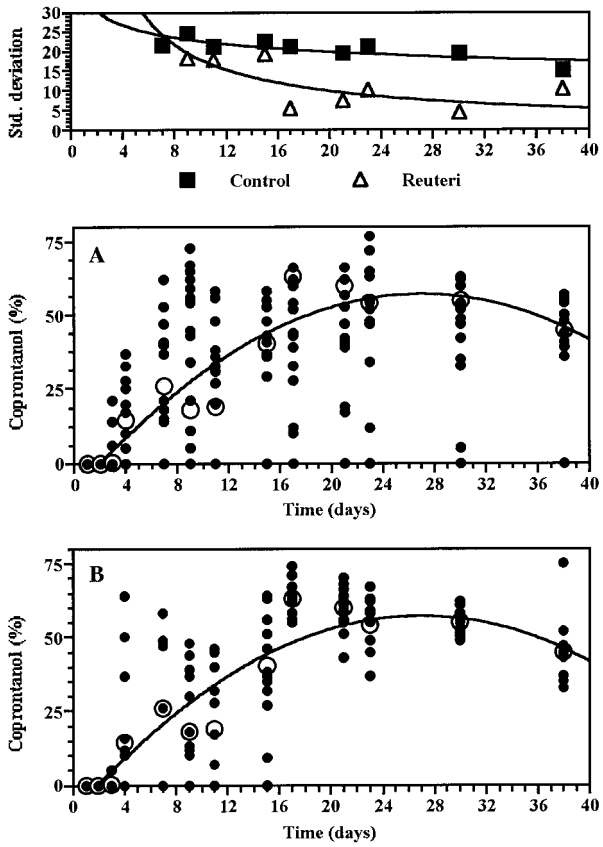

Fig. 3. Effect of *Lactobacillus reuteri* priming on conversion of cholesterol to coprostanol during conventionalization of gnotobiotic rats. Shown in this figure is the percent conversion of cholesterol to coprostanol during the experimental period in each (n = 14) of the control rats (graph A), and in each (n = 14) of the Reuteri-pre-colonized rats (graph B). Conventionalization was initiated at day 0 in both groups as described in the text. Statistical comparisons of the standard deviations (shown at the top of Graphs A and B) showed that although both groups of rats acquired the ability to convert cholesterol to coprostanol, the Reuteri-treated rats did so in a significantly more uniform manner (significantly lower standard deviation) by the second week post-conventionalization

activities can be determined by analyzing lumen/fecal material obtained from germfree animals. These are referred to as germfree animal characteristics, or GACs. Changes in these GACs attributable to intestinal microbial activities can be measured and recorded as microflora-associated characteristics, or MACs. Experiments along these lines were recently initiated using germfree rats to determine what effect, if any, Reuteri colonization had on MACs in these animals as measured by changes in their fecal chemistry. These studies were conducted in collaboration with Elisabeth Norin at the Karolinska Institute (Stockholm, Sweden), and although still in a preliminary stage, a number of interesting observations have been recorded (64).

These experiments aimed to address the following question. Does oral administration of Reuteri to rats (i.e., in a Reuteri mono-colonized rat) influence the subsequent development of MACs, in either a quantitative or qualitative manner, as the animals become conventionalized with a normal microbiota? Germfree rats were monocolonized with rat-specific Reuteri for 10 days, and a control group was kept germfree (GF). All animals were taken from the isolators, placed in individual cages kept in an ordinary animal facility and allowed to establish their 'normal' intestinal microbiota in two ways. Two groups (Groups A and C) were moved to a conventional animal room. The other two groups, (B and D) were allowed social contact with conventional animals nightly for one week. Fecal samples were taken daily during the first week, thereafter 2 times per week for 3 weeks. The last samples were collected 1 week after all animals were given an enema of homogenated fresh feces from 3 rats raised under conven-

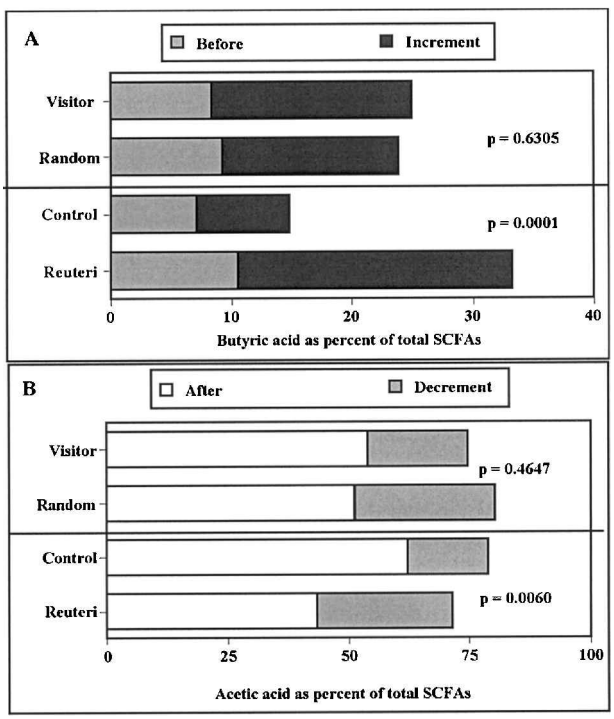

Fig. 4. Effect of *Lactobacillus reuteri* priming on production of butyric and acetic short chain fatty acids (SCFAs) as a function of conventionalization in gnotobiotic rats. This experiment was designed to evaluate Reuteri's influence on production of SCFAs in the rat GI tract. Two factors were considered in this study: First, the effect of conventionalization method (visitor vs. random conventionalization), and secondly, the effect of Reuteri-priming (germ-free controls rats vs. Reuteri pre-colonized rats). Graph A shows the influence of these factors on butyric acid production before and after administration of the enema; Graph B shows the influence of these factors on acetic acid production before and after administration of the enema to obtain full conventionalization. Whereas the method of conventionalization was shown to have no significant effect on production of either of these SCFAs, pre-colonization with Reuteri resulted in enhanced production of butyric acid concomitant with decreased production of acetic acid.

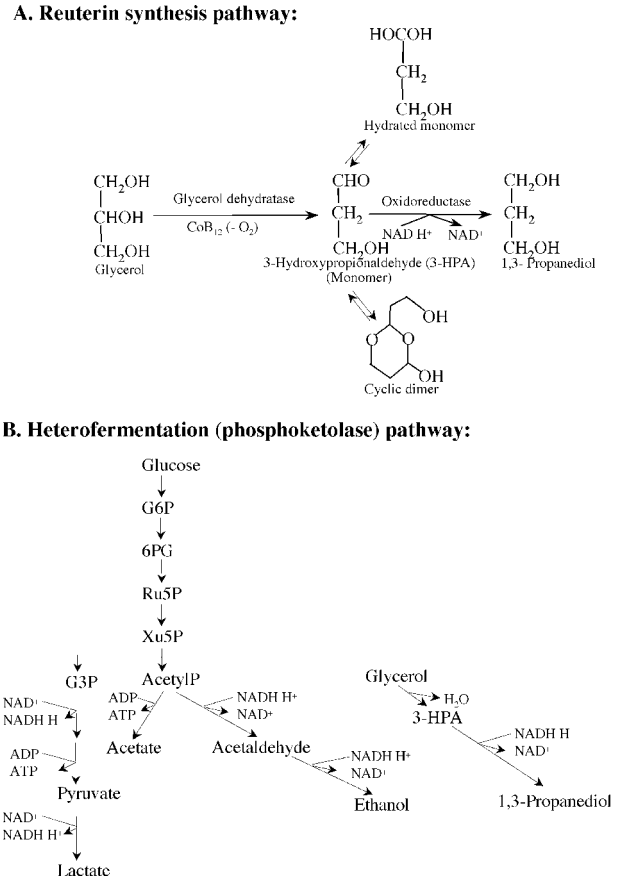

Fig. 5. Pathways for production of reuterin (A) and heterofermentation of glucose by *Lactobacillus reuteri* (B).

tional conditions. This was done to insure that the animals acquired a complete microbiota. The fecal analyses for MACs included: (a) degradation of fecal tryptic activity, (b) degradation of β-aspartylglycine, (c) degradation of mucin, (d) conversion of cholesterol to coprostanol, (e) conversion of bilirubin to urobilinogen, and (f) production of short chain fatty acids (SCFA).

The results showed that early Reuteri colonization had no observable effect on degradation of mucin, β-aspartylglycine, or conversion of bilirubin to urobilinogen, but it had influence on other MACs. In fact, these Reuteri-primed animals were conventionalized quicker than the controls (65–67). The two major differences were: first, the control rats established their ability to degrade fecal tryptic activity and to convert cholesterol to coprostanol within 30 to 36 days. The Reuteri-primed rats, on the other hand, established activities in a more modulated manner within 15 days as seen in Figure 3. Secondly, Reuteri priming significantly influenced SCFA production after conventionalized by the full flora. In comparison to the respective controls, the Reuteri-associated animals exhibited a significant increase in propionic (data not shown) and butyric acid production, concomitant with a significant decrease in acetic acid production (Figure 4). Given the importance of butyric acid in maintenance of healthy colonic tissue (68), this influence of Reuteri may be an important clue concerning Reuteri's probiotic efficacy.

It has been suggested that the MAC/GAC characterizations of gut microbiotic activities can be further subdivided to distinguish those MACs associated with pathogenic activities as PACs, i.e., pathogen-associated characteristics (Tor Savidge, personal communication). If this PAC designation for gut pathogenic activities becomes accepted in the gnotobiotic lexicon, the authors suggest it would then be appropriate to likewise identify symbiont-associated characteristics as SACs. The above-mentioned effects of pre-colonization of the rat gut with Reuteri and its associated beneficial consequences could in the future be classified as SACs.

IV. Methods Developed for Administering Reuteri to Animals. Our laboratories developed four methods for administering Reuteri to poultry, some of which have been used for administering probiotics to other animals as well. First, an in ovo inoculation method was developed in which approximately 10^4 to 10^6 colony forming units (cfu) of Reuteri are injected into the egg air cell at embryonic day 18 for chicks or embryonic day 24 to 27 for poults. This method yields hatchlings whose ceca are pre-colonized with Reuteri. Repeated tests showed these inoculations had no detrimental effect on either hatchability or livability, although higher inoculation levels (e.g., 10^7 to 10^8 cfu per egg) had a negative effect on livability in turkeys (62). Second, Reuteri can be administered as an aerosol spray (69). Freshly prepared or lyophilized cells, suspended in buffer or diluted culture medium at a concentration of ca. 10^8 cfu per ml, are sprayed on birds during hatching. Cecal colonization was demonstrated following this treatment, and a commercial form of this spray (GAIAspray®) was developed and used in commercial field trials (69, 70). Third, excellent colonization was obtained when the animal's first feed is supplemented with approximately 10^5 to 10^6 cfu Reuteri per g of feed. A commercial feed-supplement preparation (GAIAfeed®) was developed containing viable Reuteri cells vectored on particles of compressed whey (i.e., a lactose-based prebiotic) (69, 70). When GAIAfeed® was used as the source of Reuteri, it was admixed with the mash or pelleted feed at a 2% (w/w) concentration and included in the diet for 11 days in chickens and at least 3 but up to 6 weeks in turkeys depending on the duration of the trial. The lactose component of this formulation (used also as placebo in early trials) was shown to have no significant probiotic effect on either livability or growth depression in poults or chicks. It was shown however, to function as a 'probiotic enhancer or prebiotic' by enhancing the ability of Reuteri to antagonize the *Salmonella* population in birds challenged with this enteropathogen (15). Freeze-dried or frozen Reuteri preparations are administered to rodents in measured amounts (i.e., known cfu per g vector) either by oral gavage or

addition to their drinking water. Fourthly, unless the water source is chlorinated (levels > 5 ppm) Reuteri can be administered to poultry through their drinking water.

V. Reuteri Physiology. Lactobacilli fall into one of three groups based on the type of metabolic pathway used to ferment carbohydrates. The obligatively homofermentative group (e.g., *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*) possess a fructose diphosphate (FDP) aldolase pathway dictating a glycolytic conversion of sugars primarily into lactic acid. The facultatively heterofermentative group (e.g., *L. casei*, *L. curvatus*, *L. plantarum*, *L. sake*, *L. rhamnosus*) can use either this FDP aldolase pathway to ferment certain sugars, or they can induce the phosphoketolase pathway to ferment other sugars. The obligate heterofermentative group (e.g., *L. brevis*, *L. buchneri*, *L. fermentum*, *L. reuteri*) has only the phosphoketolase-based option. The author's defer to Axelsson (71) for a recent, in-depth review of the general physiological properties of lactic acid bacteria, including Reuteri.

A. Production and Secretion of Reuterin. Reuteri's unique ability to synthesize and secrete the antimicrobial agent reuterin is relatively well understood (72, 73). Reuterin was shown to be an intermediary metabolite involved in two-step pathway by which glycerol is first dehydrated to form reuterin some of which is then reduced to 1,3-propanediol (Figure 5A). Reuterin was isolated, purified, and identified using nuclear magnetic resonance, mass spectrometry, and infrared analyses, followed by chemical synthesis and re-confirmatory analyses. It was shown to be an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (74). Concentrations in the range of 15 to 30 μg per ml inhibit growth of Gram-negative and Gram-positive bacteria, yeasts, fungi, and protozoa. Concentrations 4 to 5 times higher are required to kill lactic acid bacteria, including Reuteri itself.

A coenzyme B_{12}-dependent glycerol dehydratase, which catalyzes the conversion of glycerol into reuterin, was purified and characterized (75), as was an NAD^+-dependent oxidoreductase, responsible for reducing reuterin to 1,3-propanediol (76). These two enzymes allow Reuteri to use glycerol as an alternative hydrogen acceptor during carbohydrate co-fermentation, thereby providing greater ATP yields per mole of substrate utilized, increased growth rates, and higher biomass yields than obtained in the absence of glycerol (76). This auxiliary pathway (Figure 5B) has been demonstrated in a few other bacterial species, e.g., *Klebsiella pneumoniae* (77). However, 3-HPA is produced by non-Reuteri species only as a transient metabolite that is immediately reduced to 1,3-propanediol. Reuteri appears unique in its ability to produce more 3-HPA than required to satisfy its bioenergetic needs. The excess is secreted, imparting potent antimicrobial activity to the surrounding microenvironment.

El-Ziney, et al. (78) recently obtained additional information concerning cultural conditions that influence reuterin production. They confirmed our studies showing that reuterin production is repressed by glucose during co-fermentation of glucose and glycerol (74–76). They reported that chemostat cultures of Reuteri containing limited, non-repressing levels of glucose produce reuterin continuously in concentrations sufficient for possible industrial production and use as a biopreservative. Subsequently, El-Ziney and Debevere (79) showed that reuterin can be used effectively to reduce the number of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in UHT milk and cottage cheese (79) and meats (80). Addition of 3% salt to cottage cheese enhanced the lethal effect of reuterin. For example, it diminished the initial population of *L. monocytogenes* by 4.5 log cycles in 3 days at 7 degrees C. In meats, the addition of 5% lactic acid significantly enhanced the decontamination rate of these pathogens. Use of the Reuteri/reuterin technology to reduce the number of potential pathogens in food products may eventually become a reality (81, 82).

Reuterin production *in vitro* occurs under conditions of pH and Eh similar to those found in the small and large intestines (73). It may be produced in the more distal anaerobic regions of the gut where sufficient amounts of glycerol become available as a product of luminal microbial fermentations, digestion of luminal fats, sloughed mucus and desquamated epithelial cells, and intestinal clearing of endogenous plasma glycerol. Although epithelial receptors for glycerol have been identified in the distal small intestine of the cat (83), very little information is available concerning production and/or availability of glycerol for reuterin production from these sources in the gut's complex and dynamic ecosystems. Production of reuterin within the gut will be difficult to quantitate for many reasons, primarily because the β-hydroxy moiety of reuterin renders its aldehyde function highly reactive, capable of spontaneous reaction with available amino- and sulfhydryl- functional groups, among others. These reaction targets are believed to be abundantly available in the lumen contents and surrounding mucosal tissues, thus rendering futile attempts to quantify *in vivo* reuterin production. Of course, if these reaction targets are the amino- and sulfhydryl- moieties associated with an enterobacterial species, one could link decreased viability of those species with reuterin production and its antimicrobial activity.

This logic was used in a series of *in situ* experiments designed to show that reuterin can be formed in the GI tract. Uniformly labeled ^{14}C-glycerol and 10^5 cfu of a naladixic acid (Nal^r) and novabiocin (Nov^r) resistant strain of *Salmonella typhimurium* were injected into the ceca of two groups of newly sacrificed mice. One group had been previously mono-colonized with a mouse strain of Reuteri, the other with a mouse strain of *L. acidophilus* that does not produce reuterin. After 2 to 3 hr incubation under these '*in situ*' conditions the cecal contents were removed

and analyzed for (a) 10% trichloroacetic acid insoluble ^{14}C-residue and (b) change in the numbers of *S. typhimurium* present. Higher ^{14}C-labeled residue and lower *S. typhimurium* counts were seen in the Reuteri animals in comparison to the *L. acidophilus* control animals, results expected if reuterin production had occurred in the Reuteri-treated animals (unpublished studies).

Reuteri's ability to antagonize other members of the gastrointestinal microbiota is not limited to its ability to secrete reuterin. Its heterofermentation of sugars yields lactic and acetic acids, both well known for their anti-microbial activities (81). Reuteri has also been shown to produce H_2O_2 and bacteriocins. *L. reuteri* LA 6, isolated from infant feces, produces Reutericin 6, a >200 kDa bacteriocin shown to have both bacteriocidal and bacteriolytic activity against *L. acidophilus* and *L. delbrueckii* spp. (84).

B. Surface Properties and Colonization Factors. Reuteri strain 1063, isolated from pig jejunal tissue, is a strongly autoaggregative strain shown to have a relatively hydrophobic surface (85) and ability to bind fibronectin immobilized on glass beads (86). A gene (*agg*H) encoding a 56 kDa protein which mediates autoaggregation in this strain has been cloned and sequenced (86a), revealing that the corresponding protein has extensive homology to a large family of ATP-dependent DEAD-box RNA helicases. This protein is believed to be a key factor in this strain's autoaggregating ability. Aggregative and coaggregative abilities have also been described in other *Lactobacillus* species (85), and it has been suggested that this may be an important factor in determining probiotic efficacy. For example, this factor may be important in colonizing the gut and/or to coaggregating with and subsequently remove intestinal pathogens as one of their probiotic functions (87). A connection between aggregation and genetic exchange in lactobacilli has also been proposed (88, 89).

Reuteri strain 1063 encodes a 358 kDa protein which mediates adhesion to mucus isolated from both pig and chicken intestine (90). Sequence analysis of this gene, designated *mub*, revealed that the corresponding protein is extremely large, repetitive, and possesses features typical for cell surface proteins of Gram-positive bacteria. The strongest sequence similarities of the Mub protein were found to an antigen from a hepatitis virus and a human ocular epithelial protein. Although the function of these proteins is unknown, both may be located in environments where mucus is abundant, perhaps reflecting a common mechanism for interaction with mucus. Mub has both the typical cell wall anchoring sequence and a membrane spanning region in the C-terminus region. An N-terminal secretion signal sequence was identified, confirming that Mub is an extracellular protein. A strong positive correlation was found between the presence of this gene in different Reuteri strains and their adhesion to mucus components. Other studies on Reuteri's surface properties are in progress. In addition, the existence of a collagen-binding protein in Reuteri has been reported (91, 92). It is presently unknown how these apparent adhesion factors affect either Reuteri's colonizing ability or its probiotic efficacy.

A pig *Lactobacillus* strain previously classified as *L. fermentum* strain 104R has recently been re-classified as *L. reuteri* strain 104R (92a). It had been shown to colonize the mucus layer in the ileal region of pigs (92b), where it was also capable of reducing the adhesion of the enterotoxigenic *E. coli* K88 to pig ileal mucus (92c). Evidence has been obtained showing that this strain releases (presumably after death) a 1700 KDa extracellular substance that inhibits adhesion of the *E. coli* K88 cells to porcine intestine (92d). This strain is reported to have a high affinity for porcine small intestine mucus and gastric mucin, and this is believed to be a host-specific and multifactorial function, involving saccharides, lipoteichoic acids, and proteins. An adhesion operon has been identified consisting of four genes encoding: a cystathione-κ-lyase, a membrane protein, an ATP-binding protein, and a mucus adhesion promoting protein (MapA), respectively (92a).

There are no definitive ecological studies in either animal or human GI tracts to pinpoint Reuteri's preferred colonization site(s). When formulations containing Reuteri are orally administered to mice, chickens, turkeys, or pigs, Reuteri can be found in all regions of the gut. In a recent thesis by Bjorkman (93) a probiotic strain of Reuteri was found adhered to the colonic epithelium (ascending and transverse colon) in 1 of 7 colonic biopsies obtained from human subjects.

Reuteri (strain DSM 12246) was found to be unique among 47 strains of lactobacilli, including commercially available strains, recently screened for desirable probiotic characteristics (93a). Screening was based on *in vitro* functions deemed important predictors of a culture's potential to survive *in vivo* passage and colonize the human gastrointestinal tract. These *in vitro* predictors included (a) resistance to pH 2.5 and the 0.3% Oxgall (bile), (b) strong adhesion to an intestinal cell line (Caco-2 cells), and (c) antimicrobial activity against enteric pathogenic bacteria but not against the normal microbiota of the gastrointestinal tract. Only 5 of the 47 strains met all these *in vitro* requirements, and only 3 of these, Reuteri included, exhibited good *in vivo* survival in humans. Reuteri was particularly unique among these 47 strains in exhibiting the strongest antimicrobial activity toward pathogenic bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, and *Yersinia enterocolitica*. And, it was the only *Lactobacillus* strain that did not exhibit antimicrobial activity against any of the bacterial species considered normal residents of the gastrointestinal tract—a characteristic deemed beneficial for maintenance of a normal gut microbiota.

C. Miscellaneous Physiological and Molecular Properties of Reuteri. In comparison to other lactic acid bacteria (LAB), particularly species within the *Lactococcus* and *Streptococcus* genera, relatively little is known about the molecular biological properties of Reuteri. As shown for all other species of LAB, plasmids can be found in some strains of Reuteri, and some of these plasmids have been shown to encode antibiotic resistance markers (94–105, 105a). Of particular importance in this regard, however, is the fact that Reuteri, like many other lactobacilli, is resistant to vancomycin, the 'last resort' antibiotic available for human use when resistance to other antibiotics is encountered. Clearly, a probiotic possessing transmissible vancomycin resistance would be unacceptable for either human or animal use as a probiotic. It was therefore necessary to ascertain the nature of vancomycin resistance in Reuteri. With this in mind, five Reuteri strains were examined for plasmid content and presence of the *van*A gene cluster (*van*A, *van*H, *van*R, *van*S, *van*X, *van*Y, and *van*Z) (101). Three of the strains were devoid of plasmids (type strain DSM 20016, pig strain 1063, and turkey strain T1), and two (mouse strain 11284 and the human strain SD2112) had 5 and 6 plasmid, respectively, ranging in size from 1.5 to 33 Mda. None of the strains possessed any of the *van*A genes as determined by PCR amplification and Southern hybridization.

Fragments of chromosomal DNA from *L. paracasei* subsp. *paracasei* CG11 capable of functioning as promoters were isolated (using the broad host range, promoter-probe vector pGKV210) and expressed in Reuteri (106). Similarly, a constructed vector (pPSC22) containing the alpha-amylase gene of *Bacillus stearothermophilus* was cloned and expressed in Reuteri (107).

Some heterofermentative lactobacilli, including Reuteri, are able to utilize citrate for production of succinate during cofermentation with glucose. Although enzymatic evidence is missing, the endproduct pattern in this cofermentation suggests operation of a citrate lyase and succinic acid pathway (71). Kaneuchi, et al. (108) found that 23 of 39 strains of Reuteri isolated from fermented cane molasses produced succinic acid from citrate. A nickel containing acid urease from Reuteri has been partially purified and characterized (109). The enzyme consisted of three polypeptides with molecular weights of 68,000, 16,000 and 8,800 and its isoelectric point was 4.7. It was most active at pH 2 and around 65C, but was stable between pH 3 and 8 and below 50 C.

Straub, et al. (110) investigated the formation of biogenic amines by resting cells of various fermentative bacteria suspended in phosphate buffer at pH 5.5. They showed that some strains of various *Lactobacillus* species were able to produce a variety of biogenic amines. Two strains of Reuteri were shown to decarboxylate L-histidine to form histamine under these conditions. High levels of maltose phosphorylase activity were reported in a Reuteri strain associated with sourdough fermentation (111). Another Reuteri sourdough starter culture was reported to produce ethanol as the primary endproduct during glucose fermentations at pH levels ranging from 4.3 to 6.5 (112). Gobbetti, et al. (113) surveyed the esterolytic and lipolytic activities of mesophilic and thermophilic lactobacilli isolated from different cheeses. These activities were found to be species specific and mainly intracellular, with Reuteri and *L. fermentum* strains exhibiting activity in a cell wall associated fractions. Yamato, et al. (114) studied the type strain of Reuteri and speculated on the role of an intracellular protein (termed spirosin) as a sensor component of a bacterial two-component regulatory system. No extracellular enzymatic activities (e.g., proteases, lipases, nucleases, amalyases, etc.) have been reported for Reuteri. However, human strains of Reuteri have been shown to produce hydrogen peroxide (Casas, unpublished data).

Van Geel-Schutten, et al. (114a) recently reported the ability of wild-type (LB 121) and mutant strains of Reuteri growing on sucrose to synthesize large amounts of a unique glucan (D-glucose) and a fructan (D-fructose) with molecular masses of 3,500 and 150 kDa, respectively. Spontaneous exopolysaccharide-negative mutants, lacking one or both of these synthetic abilities, were isolated following growth of the wild type strain under different conditions in a chemostat. An invertase, proven to be a beta-fructofuranosidase, was purified and characterized from a strain (CRL 1100) of Reuteri (234), while another strain (CLR 1098) was shown to transport fructose through an inducible fructose-specific phosphotransferase and glucose mainly through a proton motive force-driven permease (235).

VI. Requirements for a Probiotic. Before reviewing studies designed to determine whether or not Reuteri has probiotic efficacy, two other issues need to be addressed. First, what criteria must a probiotic meet to be considered safe and efficacious for human and/or animal use? Secondly, how are probiotics to be judged as efficacious given the many intrinsic and extrinsic factors that influence attempts to measure their effectiveness as probiotics? Concerning criteria for determining probiotics efficacy, Barrow (7), Havenaar, et al. (6), Tannock (5) and others recommend that all future research on probiotics be guided by the same principles of critical appraisal which underlie all other scientific disciplines. They proposed that henceforth probiotic studies provide full details concerning:

1- Culture identification. All cultures should be properly speciated using state of the art classification methods including molecular taxonomy whenever possible. Culture source(s) must be identified, their nutritional and physiological character defined, and genetic stability of relevant activities ascertained. It is recommended, but not required, that probiotic cultures be host-specific.

2- Colonization information. Attempts should be made to understand the ecology of the GI tract in sufficient detail

to properly assess the ability of each probiotic culture to survive GI tract passage and to colonize specific regions therein.

3- Experimental design for efficacy testing. Each experiment should be designed with strict attention to proper controls, use of sound scientific methods, and recognition that all results be evaluated statistically and interpreted realistically.

4- Field testing. Successful laboratory results may or may not indicate a probiotics ability to deliver positive results in the 'real world'. A probiotic's true efficacy can be determined only after it has been subjected to appropriate field tests with animals or clinical trials with humans.

5- Publication of results. Only conclusions derived from scientifically sound studies should be given serious consideration, and then only after the studies have been rigorously critiqued and reported in peer reviewed publications.

6- Viability and amenability to commercial-scale production. In addition to meeting the criteria listed above, a probiotic strain must be amenable to large scale, cost-effective production. Furthermore, it must be maintained in a viable state up to time of application.

Reuteri has been shown to meet all these requirements. Strains isolated from different host species have been well characterized and their colonizing abilities evaluated. Probiotic formulations have been developed and tested under controlled laboratory experiments, and 'real-world' field trials have been conducted. And it has been determined that lyophilized or frozen pure cultures maintain sufficient viability for commercial-level production and storage. Experimental model systems have been developed in which the 'variability factor' (discussed below) can be brought under reasonable control, thereby providing a credible means to evaluate Reuteri's probiotic's efficacy and initiate mode of action studies.

VII. Dealing with the Variability Factor. A method was developed early on in our studies to specifically enumerate Reuteri cells based on their unique ability to produce reuterin from glycerol (53). This method enabled us to carry out colonization studies on a quantitative basis. When applied to commercial flocks of chickens and turkeys it was discovered that these birds were only sparsely colonized with Reuteri, and that probiotic administrations of host-specific Reuteri resulted in improved colonization (61–63). There were additional incentives to initiate these studies on poultry. First, there are few, if any, demonstrably effective probiotics available for poultry (7). Secondly, these animals are susceptible to stress-associated growth depression that can be moderated by application of growth promoting antibiotics—a practice believed contributory to the serious antibiotic resistance problems that have emerged around the world (115). A probiotic capable of alleviating this growth depression could become an alternative to the current use of growth promoting antibiotics in the food animal industries.

However, if there is one issue on which researchers and practitioners in the probiotic field concur, it is that results obtained from efficacy tests conducted in the past have tended to yield inconsistent or highly variable results from test to test (116). Probiotic research has long been trapped in a 'Catch 22' situation—an inability to properly assess a strain's probiotic efficacy owing to absence of a dependable assessment system.

A number of reports have addressed this matter. Barrow noted (7), for example, in those cases where some efficacy (e.g., increased weight gain, decreased death rate) was demonstrated, the paired controls tended to exhibit substandard performances. It was not clear whether such results indicated a purely biological variation between the paired groups, or if the probiotics being tested were effective only when the test subjects were poorly managed or subjected to others detrimental/stressful conditions. Barrow and others have argued that implementation of sound scientific practices are needed to resolve this matter. Fuller (117) claimed that although the probiotic mode of action is unknown, "it seems likely that in the case of growth promotion of farm animals it is operating by suppressing the growth or metabolism of a growth-depressing organism." This argument was bolstered by the well-known growth promoting effects of antibiotics and other antimicrobial agents. Their ability to reverse growth depression in chickens purportedly caused by the presence of large numbers of *Enterococcus hirae* in the duodenum (118) is cited as a prime example in this regard. Conversely, if the suppressing organism(s) either is not present or its effects are neutralized in some manner, growth of the animal would not be depressed and the probiotic treatment would appear ineffective. As pointed out by Fuller, factors independent of mode of action may also account for a probiotic's inconsistent performance. The probiotic species or strain used, for example, may be ill suited for a particular host. Lack of proper quality control could result in use of a probiotic product having poor viability. In addition, ineffective dosing regimens, the host's age, diet, and environment can also influence the probiotic response. These are points well taken but ancillary to the probiotic 'Catch 22' dilemma.

As early as 1878 Pasteur, in one of his classic experiments, demonstrated the deleterious effects of the combined stressors, cold stress and pathogen challenge, on growth and mortality in chickens (119). The depressive effects of stressors on animal growth have thus been recognized for over a hundred years, but only recently have we begun to understand the nature of stressor-associated diseases. Improvements in production of food animals and their products have occurred since then, particularly during the past few decades. This is most evident in poultry production where the development of high-performance breeds of chickens and turkeys, improved dietary formulations, and hygienic management practices have

resulted in a productive, efficient, and cost-effective industry. On the other hand, some of these practices have had counter-productive consequences. For example, cost-effectiveness in the industry requires placement and grow-out of birds at such high population densities that the animals are subjected to an assortment of environmental stressors that reduce productivity. Crowding, post-hatch servicing, pre-treatment holding, transporting, sub-optimal environmental temperatures, poultry house dust, litter dampness, ammonia generation, and many other stressors produce an 'alarm reaction' that can retard an animals growth or initiate an actual weight loss (119–135). Newly hatched birds are particularly sensitive to these stressors, and growth retardation and tissue damage incurred during this period can persist into adulthood (121).

Stressor-associated growth depression in commercial turkey production was recently demonstrated by Barnes (136, 137), who coined the term 'poult growth depression' (PGD). He calculated that suboptimal growth associated with this syndrome might be the most costly "disease" affecting the turkey industry. This was clearly established in a multi-year study in which commercial breeds of turkeys (hatched from fully randomized eggs) were divided into two groups. One group was brooded under normal, large-scale, commercial farm conditions, and the other group brooded in clean facilities (College of Veterinary Medicine, North Carolina State University, Raleigh, NC) that are used for this purpose just once each year. The

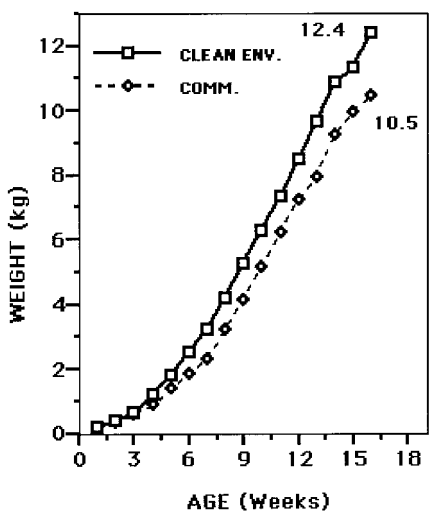

Fig. 6. Growth depression in turkeys associated with commercial brooding. In studies conducted during four successive years, it was determined that "Exposure of young turkeys to organisms present in the environment of commercial farms in continuous production reduces their growth, which in turn decreases their overall productivity at processing" (136, 137). The results shown in this figure are the respective average body weights (four year average) of turkeys grown in the clean environment at the School of Veterinary Sciences research farms (open squares) or under normal commercial conditions (open diamonds).

results obtained from this study (Figure 6) determined that the commercially brooded birds, however well managed, suffer from PGD. This depression commences soon after hatch, and poor growth observed during the first month posthatch is not compensated by market age. It's relationship to other diseases causing poor growth is unknown, but it is known that deaths occurring during the first week posthatch are directly related to stressor intensities. Uncommon or mild on new farms or in first flocks on depopulated farms (or in a clean laboratory environment), PGD is most common and often severe on farms in continuous production. This syndrome has been described in chickens as well (138–141). Recently, Klasing, et al. (142) and Klasing and Ping (1143) reported that it may be immunologically mediated, most likely caused by continual exposure of young birds to intense microbial stress, a view consistent with that proposed by Barnes (137). Hereafter in this review, we refer to growth depression and mortalities associated with this 'disease' in both chickens and turkeys as 'avian growth depression,' or AGD.

A major factor in environmental stress is the continual exposure of animals, young animals in particular, to a wide variety of microorganisms. The Barnes study described above (137), for example, showed that even young turkeys grown in a clean environment suffered growth depression when exposed to used litter or intestinal contents from growth-depressed, commercially brooded, but otherwise healthy, poults. The more intense this exposure, the more devastating the stress, the consequences of which can be further exacerbated by co-presence of unfavorable physical factors (i.e., cold, dampness, etc.). Klasing and colleagues (142–145) showed that a chick's immune response to a wide variety of antigens, including infectious challenges (which may or may not result in clinical disease depending on the virulence potential of the challenging microorganism), causes significantly lower rates of growth and decreased feed efficiency. For example, injections of sheep red blood cells (SRBC), a strain of rapidly cleared *Escherichia coli*, or *E. coli*/*S. typhimurium* lipopolysaccharide (LPS) cause a decrease in the rate of skeletal muscle protein synthesis, and an increase in the rate of protein degradation (144, 145). Experiments on young chickens have implicated both corticosteroids and interleukin-1 (IL-1) as mediators of these metabolic changes. It was shown that injections of crude IL-1 preparations depress growth as much as the antigens themselves. Evidence of their combined involvement is consistent with other findings that have identified an immunomodulatory role of the immunopituitary axis with feedback regulatory interactions between IL-1 and corticosterone (146, 147).

VIII. Effect of Reuteri Administrations on Animal Health. In the studies reviewed below, the 'variability factor' was brought under reasonable control by intentionally exposing animals to environmental and/or microbiological stressors applied under controlled conditions. The

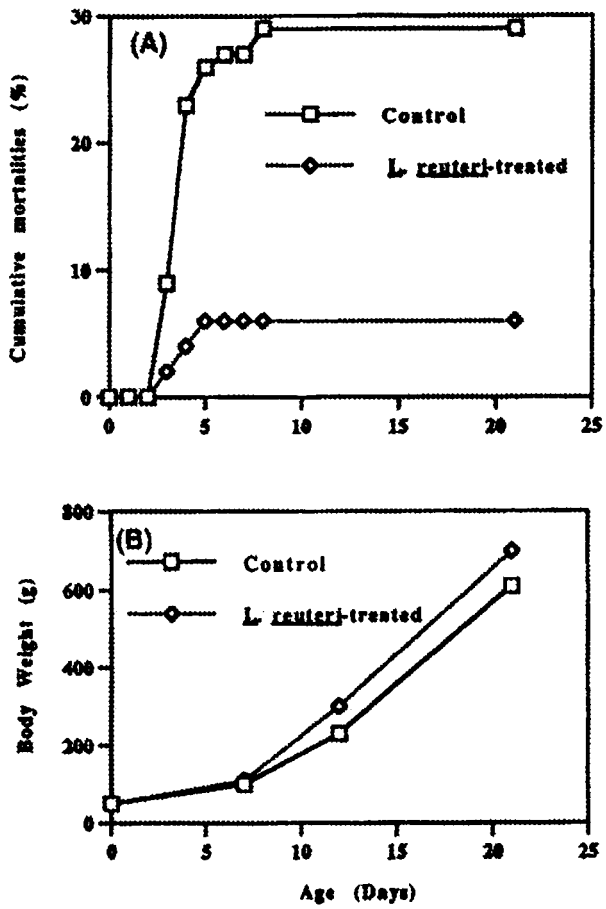

Fig. 7. Protective effect of *Lactobacillus reuteri* administration on growth and death rates in cold stressed, *Salmonella*-challenged chickens. Shown are typical results obtained from a series of experiments demonstrating that Reuteri probiosis effectively moderated stressor-induced growth depression and deaths in chickens. Graph A: Cumulative deaths in stressed birds vs. *Lactobacillus reuteri*-treated, stressed birds; Graph B: Body weight growth rates of stressed birds vs. *Lactobacillus reuteri*-treated, stressed birds. (Reproduced from reference 15 with permission of the publisher).

deaths, growth depression, and/or other negative consequences of the stress-challenge became relatively predictable under these conditions, and our ability to evaluate a potential probiotic's effectiveness in moderating these effects was thereby considerably improved.

A. Avian Growth Depression (AGD) in Chickens and Turkeys. Dunham, et al. (63) showed that AGD induced in young chickens by exposing them to combined stressors (i.e., mild cold stress and *Salmonella*), could be significantly moderated by probiotic treatments (described earlier) with a chicken-specific strain of Reuteri. For example, (Figure 7) when control chicks were exposed to a mild cold stress during the first 48 hr posthatch and approximately 10^6 cfu *S. typhimurium* gavaged into their crop on day 1 posthatch, deaths generally ensued and the survivors had a significantly reduced average body weight. However, when these birds were sprayed with Reuteri at hatch and the probiotic also added to their feed, fewer deaths occurred and the birds exhibited increased body weight growth in comparison to control birds. On the other hand, if these stressors were not applied and the birds were brooded in an ideal environment in terms of temperature, nutrition, and in a hygienic environment, AGD did not occur and Reuteri treatments had no discernable effect. Essentially the same stressor-induced, Reuteri alleviated AGD was observed in studies using turkey poults as the experimental animals (61, 69, 70), although lesser stressor levels needed to be applied to turkeys to obtain the same effects as seen in chickens. Apparently, commercial breeds of turkeys are considerably more susceptible to the deleterious effects of stress than are commercial breeds of chickens, but in either case Reuteri treatments moderated these effects. Furthermore, in both species of birds, Reuteri moderated AGD even when the cold treatment and the *Salmonella* challenges were applied individually (63). This suggested that beneficial effects of Reuteri are not based solely on bacterial vs. bacterial interactions in the gut, generally referred

Table 2

Effect of in ovo Lactobacillus reuteri treatments on performance of chicks challenged at hatch with Salmonella typhimurium

Treatments	Day 6 posthatch		Day 40 posthatch	
	% Mortality	Body weight (g)	% Mortality	Body weight (g)
Challenged	36[a]	72[b]	41[a]	1,728[b]
Challenged + *L. reuteri*	6[b]	107[a]	9[b]	1,934[a]

[a,b]numbers with unlike superscripts indicate a significant difference ($p < 0.05$) from the control. Chicks were challenged with 10^3 cfu *S. typhimurium* per chick by gavage at hatch. Half served as challenged controls and the other half received *L. reuteri* in ovo (at embryo day 18) and were given an *L. reuteri*-supplemented feed (ad libitum) thereafter. (Table reprinted from reference 15 with permission of published.)

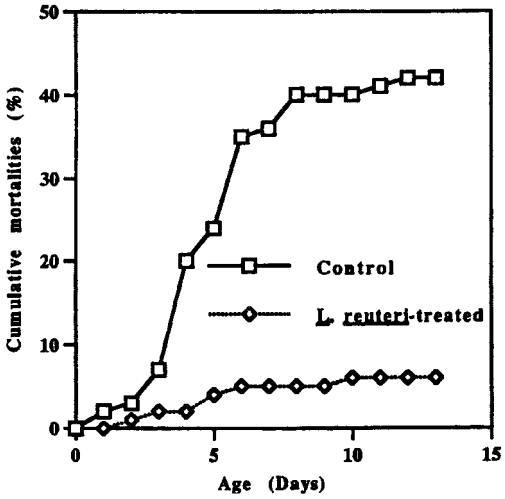

Fig. 8. Protective effect of *Lactobacillus reuteri* administration on mortality of young turkeys exposed at hatch to *Salmonella typhimurium*. As described in the text and shown here, Reuteri probiosis proved consistently effective in limiting a pandemic contagion resulting from *Salmonella* aerosols created as infected poults (or chicks) are hatched. Only a few infected birds can result in an infectious aerosol sufficient to decimate their sibling population.

to as the competitive exclusion (CE) effect described by Nurmi and Rantala (30).

B. In Ovo Administration to Chickens. Positive effects were also observed when Reuteri was administered in ovo (10^6 per embryo at embryonic day 18 and 22, respectively) and subsequently as GAIAfeed® to chicks challenged at hatch with 10^3 cfu (by gavage) *S. typhimurium* (strain

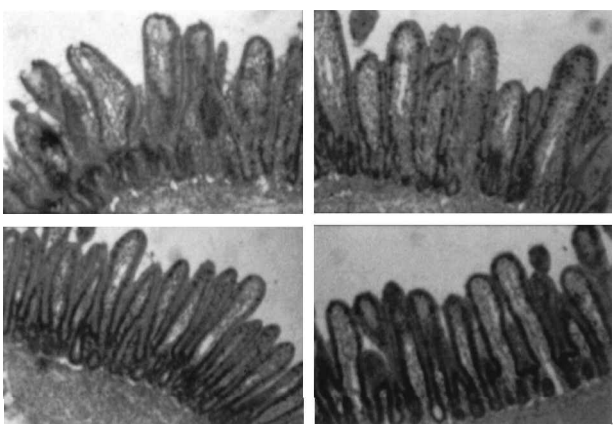

Fig. 9. Protective effects of *Lactobacillus reuteri* and gentamicin administrations (in ovo) on ileal villi in young chickens challenged at hatch with *Salmonella typhimurium*. Representative photomicrographs of ileum sections obtained 6 days post-infection from: infected chicks (upper left panel); infected, gentamicin-treated chicks (upper right panel); infected, *L. reuteri*-treated chicks (lower left panel); infected, *L. reuteri*- plus gentamicin-treated chicks (lower right panel). (Reproduced from reference 15 with permission of the publisher).

Table 3

Resistance to in-hatcher enteropathogenic Escherichia coli infection in broilers receiving in ovo Lactobacillus reuteri and gentamicin

Treatments	Body weight (g)	% Mortality
Absolute control	842[b]	1.42[b]
E. coli-challenged:		
Control	803[b]	9.52[a]
+gentamicin	819[b]	4.26[a]
+*L. reuteri*	847[a]	3.56[b]
+gentamicin and *L. reuteri*	882[a]	0.00[b]

[a,b]numbers with unlike superscripts indicate a significant ($p < 0.05$) difference from the control. *L. reuteri* (10^6 cfu per embryo) and/or gentamicin (0.2 mg per embryo) were co-administered in ovo at age 18 into the air sac and amnionic fluid, respectively. The enteropathogenic E. coli challenge was administered as an in-hatcher aerosol generated by four seeded embryos that were inoculated with 10^4 cfu *E. coli* at the time of pipping (i.e., hatching). Absolute controls (no treatments, no challenge) and *E. coli*-challenged controls were included. The body weight and mortality measurements were made at 22 days posthatch. GAIAfeed® was added to the diet of the *L. reuteri*-treated chicks. (Table reproduced from reference 15 with permission from publisher).

ST-10) (62). The beneficial effects of the probiotic on both mortality and body weight gain were clearly evident as early as 6 days posthatch and by 40 days posthatch 41% of the *Salmonella*-challenged chicks died, but only 9% died if administered Reuteri (Table 2). In other experiments along these lines using poults (15), the *Salmonella*-challenge was administered in ovo. Two groups of 100 poult eggs each were treated as follows: three eggs in each group were inoculated in ovo with 50 cfu of *S. typhimurium* ST-10. One group served as controls, the other was administered Reuteri using GAIAspray® and GAIAfeed®. Typical results obtained from these experiments are shown in Figure 8. In this particular experiment, the *S. typhimurium* aerosol that developed during hatching of the challenged birds caused an in-hatcher pandemic resulting in over 40% mortality. Reuteri probiosis reduced the cumulative mortality to less than 10%.

In the USA, gentamicin is used in the poultry industry as an effective antibiotic for salmonellosis. A number of experiments have shown that Reuteri's probiotic efficacy exceeds gentamicin's efficacy in protecting poultry from enteropathogen challenges (15). In one such experiment, Reuteri and/or gentamicin were administered in ovo to chicks followed by *S. typhimurium* challenge (10^3 cfu by gavage) immediately after hatching. At 1, 3, and 6 days post hatch the inflammatory changes in ileal villi were visualized by fixing and staining these tissues with periodic acid-Schiff reagent. Representative photomicrographs of these tissue sections are presented in Figure 9. It was observed that while the inflammatory damage caused by the infection was somewhat moderated by gentamicin alone, it appears to have been completely abated by

Reuteri or a combination of Reuteri plus gentamicin. Another interesting observation was made during the course of this particular experiment. Ileal tissues were obtained from birds that had received Reuteri and/or gentamicin regimens in ovo as described above, but were sacrificed just before challenge with *S. typhimurium*. It was observed that the gentamicin treatment alone caused a blunting of the ileal villi, and that this blunting was eliminated by the co-presence of Reuteri (data not shown). This is one among many observations indicating that Reuteri's protective activities are not directed solely to microbiological threats. Other studies to be discussed below have shown that Reuteri can protect gut tissues against damage caused by other non-biological moieties, such as acetic acid (158) or methotrexate (166).

In ovo colonization by Reuteri was found to be as effective against *E. coli* (15, 62) as it was against *Salmonella* challenges. As seen from the study summarized in Table 3, Reuteri preformed as well as gentamicin, indeed better if body weight gain is taken into account, in protecting broiler chicks from the mortality and morbidity effects of an in-hatcher epidemic caused by release of an enteropathogenic strain of *E. coli*. In this experiment Reuteri (10^6 cfu/embryo) was administered in ovo with or without gentamicin (0.2 mg/embryo). At 72–84 hr prior to hatching, *E. coli* cells (10^4 cfu/embryo) were injected into the air cell of 4 eggs that were then placed into the hatcher consoles assigned to the challenge treatments. Each treatment group was placed in different rooms to avoid cross-contamination with unchallenged animals. The results obtained showed that while both gentamicin and Reuteri reduced the *E. coli*-induced chick mortality significantly, only Reuteri was effective in preventing the AGD caused by the microbiological challenge.

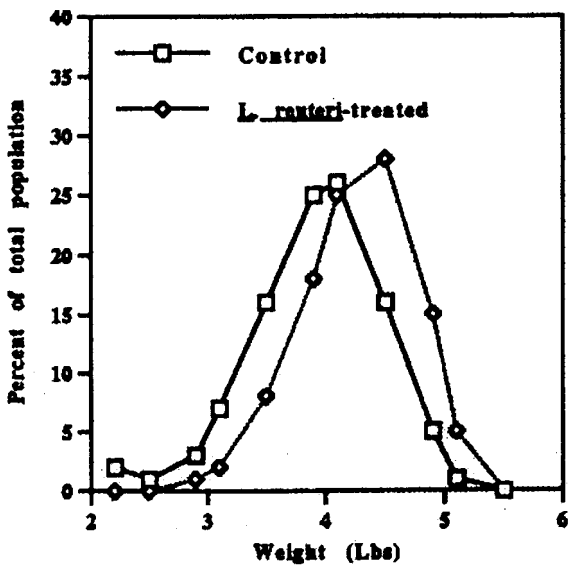

Fig. 10. Effect of *Lactobacillus reuteri* administrations on body weight distribution profile of 42 day-old commercial turkeys. These results were obtained when commercially grown Reuteri-treated and untreated turkeys (572 turkeys in each group) were weighed as they were being transferred from their brooding to grow-out barns. (Reproduced from reference 15 with permission of the publisher).

C. Commercial Poultry Field Trials. The experiments described above provided effective and relatively consistent test model systems to determine whether or not Reuteri had a protective effect on stressor-induced disease in poultry. They could not however answer the more important question, namely, is Reuteri able to manifest these health-enhancing effects in a 'real world' environment? It is one thing to show that Reuteri alleviated AGD when stressors were applied under laboratory-controlled conditions. It is quite another to determine if beneficial probiosis would occur in a commercial field trial environment. In this regard, probiotic studies are much like Pasteur's intentions to not only understand the microbial world but to apply its vast powers whenever possible to solution of practical problems. With this in mind, Casas, et al. (69) conducted 16 controlled field trials comprising approximately 280,000 turkeys. The beneficial effects of Reuteri treatments were observed shortly after placement of the poults, with typical stimulation of body weight growth. A comparative body weight distribution profile is shown in Figure 10. In this particular study, 572 randomly selected turkeys from each group were weighed as they were being transferred from their brooding houses to the grow-out houses at 42 days posthatch. The positive effect of Reuteri on growth of these animals was clearly evident. The control birds weighed an average of 3.8 lbs whereas the treated birds averaged 4.2 lbs for a significant 9.5% improvement in body weight. This shift in the body weight distribution pattern attributable to Reuteri probiosis has been observed consistently in both laboratory and field trials. Of the 16 commercial trials conducted, 12 yielded improved flock performance at market age (i.e., at approximately 4 months posthatch) as indicated by significant improvements in livability, feed conversion, body weight, and Grade A quality carcasses. Reuteri probiosis successfully demonstrated under laboratory-controlled conditions was thus confirmed under 'real world' conditions. This was shown also to be the case in human clinical trials to be discussed later.

D. Effect on Dietary Protein Deficiency in Chickens. Is growth retardation in young birds caused by a deficiency in dietary protein also a form of AGD that can be moderated by Reuteri? A preliminary experiment along these lines was conducted on chicks brooded from hatch to day 21 posthatch (63). To our surprise, the results showed that growth retardation resulting from a deficiency in dietary protein was significantly moderated by prophylactic treatment with either Reuteri or a combination of monensin (an ionophore used as a coccidiostat) and the antibiotic bacitracin. Neither treatment had an effect when

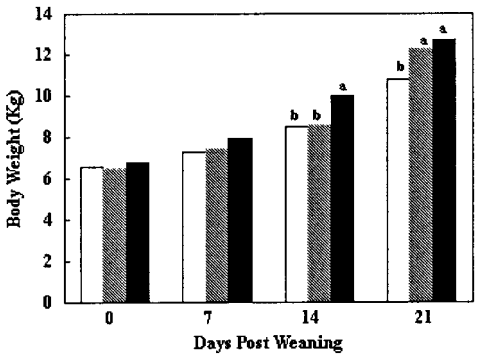

Fig. 11. Comparative effects of *Lactobacillus reuteri* vs antibiotic growth promoters on growth of post-weaning pigs.

the birds were grown for 20 days posthatch under ideal environmental conditions with access to an optimal diet containing 19.9% crude protein. On the other hand, growth was significantly depressed when the dietary protein level was reduced to 16.1%. Reuteri administration was able to significantly moderated this nutrition-associated growth depression. This novel probiotic effect warrants further attention for a number of reasons. It suggests, for example, that a host's dietary nitrogen requirements can be influenced by the composition its gut microbiota, suggesting important theoretical and practical implications concerning the role of the gut microbiota in a host's health and nutrition. It may also address some of the core issues concerning probiotic efficacy and mode of action. Furthermore, it also suggests, as others have done (21–24), that inasmuch as antibiotic and probiotic prophylactic treatments have similar growth promoting effects, they may share a common mode(s) of action. Use of Reuteri as a biological alternative to growth promoter antibiotics could have important implications for human health in light of the fact that development and transfer of antibiotic resistance from animal microbiota to human pathogens has been linked to this prophylactic use of antibiotics (115).

E. Reuteri: A Biological Alternative to Growth Promoting Antibiotics. In addition to experiments on poultry described above (Section VIII B) comparing Reuteri to growth-promoting antibiotics (15, 62), similar studies have also been carried-out on swine (59). A well-controlled study was conducted to assess the efficacy of Reuteri (pig strain 1063) applied orally to piglets during pre-weaning and as a top dressing added to the feed of lactating sows and post-weaned pigs. Three treatment groups were included in this study which was carried-out under commercial conditions using commercial feeds. One group ($n = 44$) served as controls with no copper or antibiotics added to their feed. A second group ($n = 41$) was administered commercial antibiotic regimens (175 mg copper and 100 mg Enterdox/Kg feed in pre-weaner feed and later in post-weaner feed: 175 mg copper and 40 mg Tylamix/Kg feed). And the third group ($n = 53$) was administered the control diets but supplemented with approximately 10^8 cfu Reuteri per day per pig. Sows we fed their respective diets for a period of 10 days prior to farrowing and during lactation. Piglets received oral applications of Reuteri until weaning and then received top dressing of Reuteri daily on their feed until 21 days post weaning. Birth weights and growth rates of piglets were recorded until weaning, and growth rates and feed conversion rates were recorded on weaner pigs for 21 days post weaning.

Although no beneficial effects from either the antibiotic or probiotic treatment were noted during the pre-weaning period, during the 21-day postweaning period, the Reuteri-treated group had significantly improved in performance as measured by growth rate (Figure 11). This group also exhibited improved cleanliness and health scores. The overall improvements in this group were identical to the group receiving diets containing the antibiotic, indicating that Reuteri may indeed become a biological alternative to use of growth promoter antibiotics in certain animal production industries.

What effect does Reuteri have on other hosts? Given its purported universal distribution in the gut of animals

Table 4

Effect of feeding Lactobacillus reuteri on fecal shedding of Cryptosporidium parvum oocytes and colonization of the distal ileal epithelium of mice immunosuppressed by prior inoculation with retrovirus LP-BM5 and challenged with Cryptosporidium parvum

Group*	Fecal shedding+ (number oocytes× 10^3 per g ± SEM)			Ileal colonization (number oocytes× 10^3 per cm of intestines ± SEM
	Day 0	Day 7	Day 14	
A	0.00	0.00 ± 0.00^a	0.00 ± 0.00^c	0.00 ± 0.00^a
B	0.00	1.58 ± 0.24^b	9.19 ± 4.29^b	4.00 ± 1.13^b
C	0.00	0.00 ± 0.00^a	0.00 ± 0.00^c	0.00 ± 0.00^a
D	0.00	1.34 ± 0.33^b	0.00 ± 0.13^a	0.00 ± 0.00^a

*10 mice per group (5 mice per cage). Groups C and D were supplemented with *L. reuteri*; groups B and D were challenged with *C. parvum*. + Days after *C. parvum* challenge. a,b,c values within same column with unlike superscript symbols differ significantly ($p < 0.05$). (Table reprinted from reference 148 with permission from publisher.)

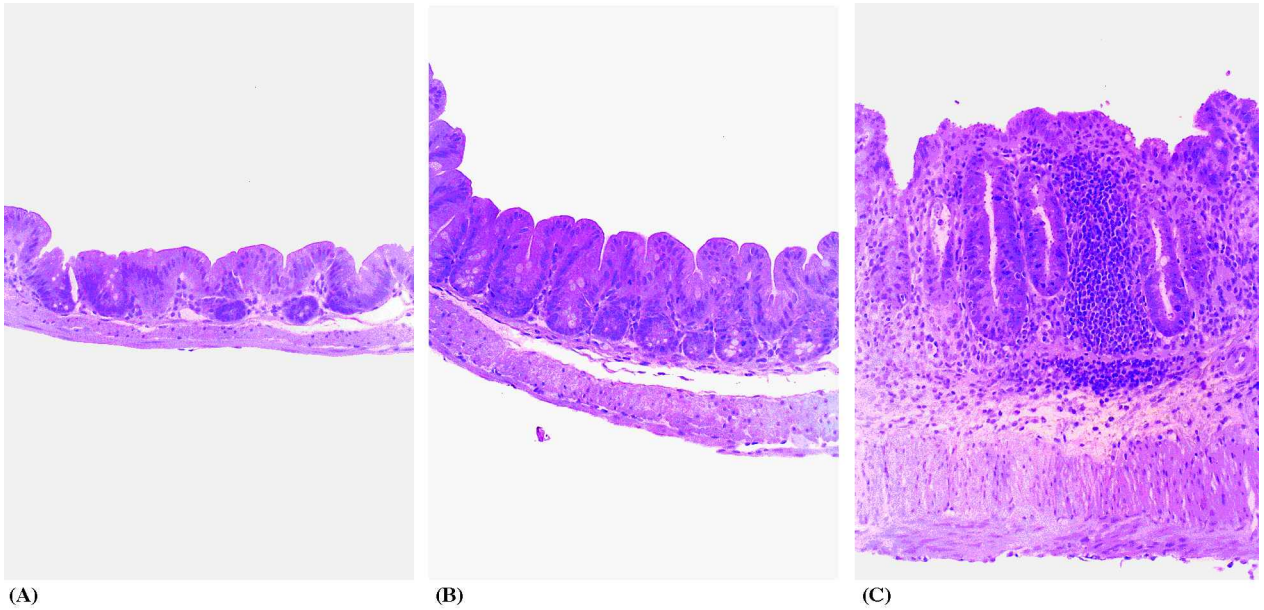

Fig. 12. Lactobacillus reuteri treatment diminishes hyperplastic and inflammatory cecal lesions of TCR-a-deficient mice resulting from infection with *Cryprosporidium parvum.* Cecal sections of mice receiving only Reuteri (graph A), Reuteri and *C. parvum* (graph B), only *C. parvum.* Note effect of *C. parvum* on mononuclear lamina propria infiltrates and thickness of mucosa (between arrowheads).

ranging phylogenetically from avians to humans (57), does its probiotic efficacy obtain in all its hosts? In other words, is the Reuteri-host relationship a truly symbiotic phenomenon, applicable to all animal species hosting Reuteri? The following studies emerging from laboratories around the world indicate a positive answer to these questions.

F. Effect of Reuteri on Cryptosporidium parvum Infections in Immunodepressed Mice. Efficacy of Reuteri as a probiotic for the control of *C. parvum* infection was evaluated by Alak, et al. (148) using C57BL/6 female mice that had been immunosuppressed by intraperitoneal inoculation with the LP-BM5 leukemia virus. Four months after virus inoculation, these mice developed lymphadenopathy, splenomegaly, and susceptibility to *C. parvum* infection. However, after daily feeding with Reuteri (10^8 cfu per day) for 10 days prior to challenge with 6.5×10^6 *C. parvum* oocytes (and continued Reuteri administrations), the mice cleared the parasites from the gut epithelium. The untreated, *C. parvum*-challenged mice shed high levels of oocytes in the feces (Table 4). These studies showed that Reuteri's probiotic effectiveness was not limited to bacterial infections; it extends to protection from a protozoal disease as well. Furthermore, it suggested that Reuteri might help protect immunodeficient subjects, who are particularly susceptible, from this protozoal disease. In a later study both Reuteri and *L. acidophilus* strains were shown to be efficacious in reducing fecal shedding of oocytes (236).

C. parvum may cause diarrheal disease in a variety of mammals, including humans and economically important livestock. The disease is especially severe in immunocompromised hosts, such as the immunosuppressed mice discussed above and humans with acquired immunodeficiency syndrome (AIDS). Mechanisms underlying immunity to *C. parvum* are not well understood but several *in vivo* studies suggest that both $CD4^+$ T lymphocytes and IFN-gamma are critical in resistance and recovery from infection (149–151). As discussed by Famularo, et al. (116) and De Simone, et al. (152), the gastrointestinal microbiota has a protective role as well, but by a mechanism not involving IFN-gamma. Along these lines (and described below in the present report) we have shown (15) that Reuteri-treated chickens have a significantly higher number of $CD4^+$ cells

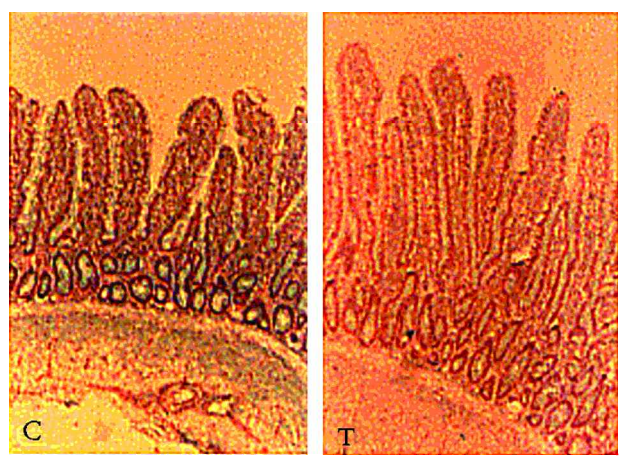

Fig. 17. Effect of *Lactobacillus reuteri* administrations on development of ileal villi in 3 day-old chicks. Shown are representative sections of ileal tissues (prepared and analyzed as described in reference 15) obtained from control chicks (left panel) and Reuteri-treated chicks (right panel). (Reproduced from reference 15 with permission of the publisher).

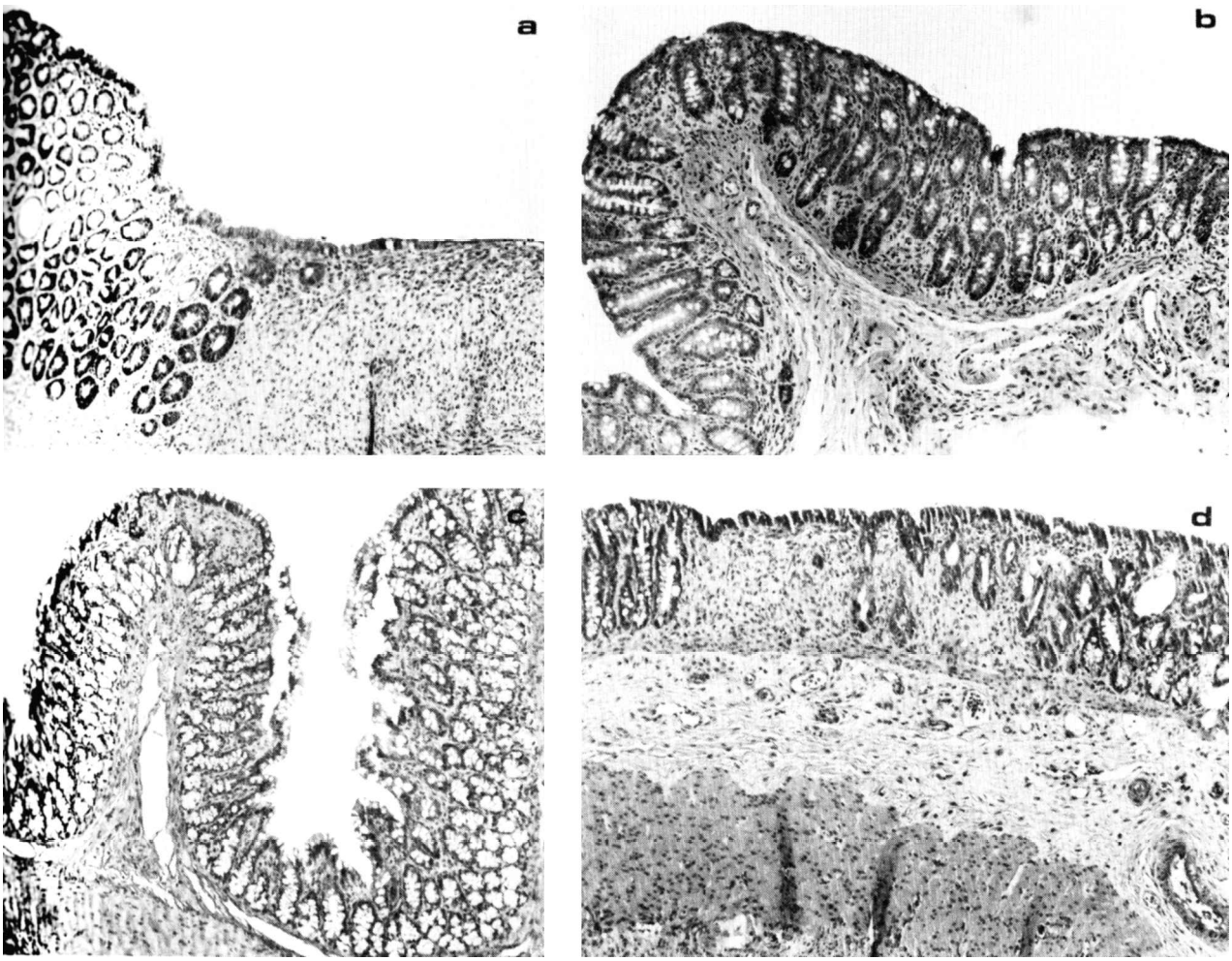

Fig. 13. Lactobacillus reuteri prevents development of acetic acid-induced colitis in a rat model. Representative photomicrographs of eosin-hematoxylin-stained rat colonic mucosa prepared 4 days after acetic acid challenge. Panel A = Acetic acid-challenged control mucosa; Panel B = Unchallenged control mucosa; Panel C = *L. reuteri*-treated (immediately), challenged mucosa; Panel D = *L. reuteri* treated (1 day post challenge), challenged mucosa. (Reprinted from reference 158 with permission of the publisher).

in their ileal lamina propria than do their non-treated controls. Although no conclusions are possible at the present time, these combined observations suggest that Reuteri-conferred protection against enteric disease may involve activation of host $CD4^+$ functions.

G. Effect on Cryptosporidium parvum-induced Diarrhea in Piglets. For comparative animal studies on human GI diseases, the piglet is an animal of choice because its GI tract is reported to be similar in many respects to the human GI tract. Piglets deprived of mother's colostrum, and fed only a cow's milk-supplemented diet, are susceptible to *C. parvum*-induced diarrhea. A model system was developed using such colostrum-deprived piglets as another model to evaluate efficacy of Reuteri as a probiotic for control of cryptosporidiosis (Gomez and Casas, unpublished data). Control (unsupplemented) and Reuteri-supplemented piglets were challenged orally 5 days after birth with *C. parvum* oocysts. Onset and severity of diarrhea was scored during the following two weeks. In comparison to the unsupplemented controls, significantly fewer Reuteri-supplemented piglets exhibited diarrhea throughout the experimental period. These findings were consistent with the immunosuppressed mouse experiments described above, and provided additional evidence that Reuteri prophylaxis can moderate onset and duration of *C. parvum*-associated disease.

H. Effect on Cryptosporidium parvum-associated Inflammatory Bowel Disease in TCR-η-deficient Mice. A similar protection by Reuteri from *C. parvum* infection has been observed in an ongoing study using adult gnotobiotic TCR-η-deficient mice (153). When these mice are challenged with *C. parvum*, a persistent infection is established as well as inflammatory bowel disease-like lesions of the cecum. The cecal lesions are characterized by inflammatory cell infiltrates within the lamina propria and extensive epithelial cell hyperplasia. It was shown that when these mice are pre-colonized with Reuteri and then challenged with *C. parvum*, fewer *C. parvum* are detected (7 weeks

post challenge) in the ileal and cecal sections than detected in mice not receiving the Reuteri. Hyperplastic and inflammatory cecal lesions due to *C. parvum* colonization were also diminished by Reuteri (Figure 12). These findings suggest that Reuteri treatment is important in the maintenance of intestinal mucosal integrity and in decreasing the parasite burden upon infection of immunodeficient hosts such as these TCR-η-deficient mice infected with *C. parvum*.

I. Effect on Development of Spontaneous Colitis in Interleukin 10 (IL-10) Gene-deficient Mice. IL-10 gene-deficient mice (generated in a 129 Sv Ev genetic background) housed under conventional conditions spontaneously develop a chronic colitis similar to human Crohn's disease. This disease does not occur in the normal mice or in the IL-10 deficient mice if they are raised under germfree conditions, suggesting that luminal bacteria play an essential role in the initiation of colitis in this model. Madsen, et al. (153a) showed that these mice have an increased mucosal adherence or invasion of aerobic bacteria in the colon that precedes development of colitis. And, that this increased bacterial adhesion is coupled with a dramatic reduction in the number of colonic luminal lactobacilli. The colonic inflammation that develops in these mice is characterized by patchy, transmural, acute, and chronic inflammation, accompanied by mucosal ulceration and epithelial hyperplasia. Mucosal inflammation is not present at birth, nor does it appear before weaning at 3 weeks of age. At approximately 4 weeks of age, however, the colonic mucosa become inflamed, and there is subsequently a gradual increase in the severity of inflammation accompanied by development of frank ulceration.

Based on observations that IL-10 gene-deficient mice (a) do not develop this inflammation when raised under germfree conditions, (b) at 2 weeks after birth have significantly increased levels of aerobic bacteria either adherent to or translocated within the colonic mucosa (in comparison with control mice despite being raised in the same environment), and (c) exhibit a deficiency in luminal levels of lactobacilli coincident with the increased level of adherent/translocated aerobic bacteria, it was hypothesized that if the colonic lumen were repopulated with control levels of appropriate lactobacilli, the defective mucosal adherent/translocated bacterial pattern would become normalized and development of colitis thereby prevented. Madsen, et al. (153a) chose Reuteri to repopulate the colon because (a) control mice which did not develop colitis were predominantly colonized by this species, in comparison to the deficient mice which, for reasons unknown, were predominantly colonized by *L. johnsonii*, and (b) Reuteri had previously been shown to antagonize intestinal inflammatory disease in other rodent model systems (158,166, also see below). Reuteri was administered to the Il-10 gene-deficient mice beginning at 1 week of age with a single enema, followed by a daily rectal swabbing with a broth containing Reuteri. As a result, the defective mucosal adherent/translocated bacterial pattern was normalized and the development of colitis prevented. A similar attenuation of the colitis was observed when the prebiotic lactulose was added (0.06%, wt/vol) to the drinking water. Addition of this prebiotic, like addition of the probiotic, also increased the colonic *Lactobacillus* population, including in this instance *L. johnsonii* and two unidentified *Lactobacillus* species.

J. Effect on Candida albicans Infection in Mice. Wagner, et al. (154) assessed four probiotic bacteria, *L. acidophilus*, *L. rhamnosus* GG, *Bifidobacterium animalis* and Reuteri, for protection of athymic bg/bg-nu/nu and euthymic bg/bg-nu/+ mice from mucosal and systemic candidiasis caused by oral and anal inoculation with *Candida albicans* (1×10^7 cfu per ml). Each of the four probiotic species and the fungus *C. albicans* colonized the GI tracts of both strains of mice. The presence of the probiotic bacteria in the GI tract prolonged the survival of adult and neonatal bg/bg-nu/nu mice compared to that of isogenic mice colonized with *C. albicans* alone. The incidence of systemic candidiasis in the probiotic-associated mice was also significantly reduced. The immunologic and nonimmunologic mechanisms purported to underlie these biotherapeutic effects will be discussed later (see Section XI: Speculations on Reuteri's Mode(s) of Action).

K. Effect on Acetic Acid-induced Colitis in Rats. Bengmark and colleagues at Lund University medical hospital in Sweden conducted a series of experiments showing that Reuteri had positive effects on maintenance of mucosal integrity in the GI tract. The rat was used as the experimental animal, and instead of using microbial agents to challenge the GI tract, the animals were challenged with chemical or surgically-induced stressors. Fabia, et al. (155) reported that exposure of excluded rat colonic tissue to 4% acetic acid for 15 s induced a uniform and reproducible colitis resembling human ulcerative colitis. Although differences exist between this model and human inflammatory bowel disease (IBD), the pattern of arachidonic acid metabolism and inflammatory response mediators observed in this model is almost identical to that observed in human IBD (156). Fabia, et al. (157) subsequently noted similar changes in the colonic mucosa-associated microbiota both human patients with active colitis and in rats with acetic acid-induced colitis. When compared to patients with inactive ulcerative colitis, those with active ulcerative colitis exhibited significant decreases in total anaerobic bacteria, anaerobic Gram-negative bacteria, and lactobacilli. These same microbiological changes occurred in rats with acetic acid-induced colitis, indicating that a reduction in the number of anaerobic bacteria and lactobacilli is a common feature in active colitis regardless of origin.

On this basis, Fabia, et al. (158) used their acetic acid-induced colitis model to determine if administration

of exogenous lactobacilli had any beneficial effect on development of the syndrome. Intracolonic administration of a rat-specific strain of Reuteri applied immediately after the acetic acid administration (at a dose of 5 to 7× 10^7 cfu Reuteri per ml) prevented development of colitis (Figure 13). The challenge-associated morphologic damage, increase in luminal myleoperoxidase activity (an index of neutrophile infiltration), and mucosal permeability (determined as plasma exudation into the lumen) were almost normalized by the Reuteri treatment. If the Reuteri were administered 24 hr after the acetic acid or in lower doses, a less protective effect was reported.

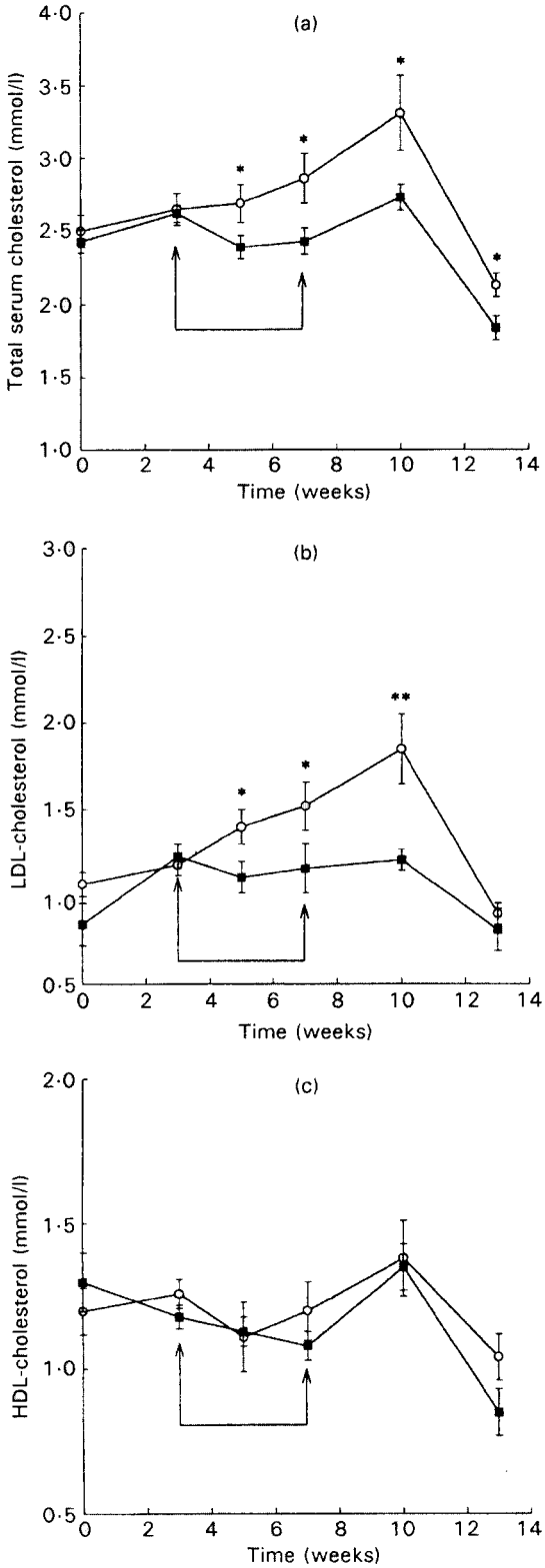

Fig. 14.

L. Effect on Bacterial Translocation from the Gut Following Subtotal Liver Resection in Rats. The intestinal mucosal barrier is the first line of defense preventing translocation of enteric bacteria, endotoxins, and other unwanted substances from the gut to extraintestinal sites. Translocation occurs when humans or animals are subjected to stressors such as burn injury, surgery, radiation exposure, anti-inflammatory steroid applications, liver failure following hepatitis, toxic insults, or liver surgery. Uncontrolled translocation can lead to generalized sepsis, organ infections, and/or assorted immunopathological complications, debilitations, and death in some cases. A well-balanced intestinal microbiota has a positive effect on maintenance of gut mucosal integrity and can thereby prevent or minimize translocation of undesirable substances from the gut (159–161). Conversely, a disturbed, unbalanced enteric microbiota can result in increased translocation of these substances to extraintestinal sites with associated ill effects on the host's health and well being (162).

Wang, et al. (163) demonstrated microbial ecology disturbances in the gut followed by severe bacterial translocation when rats were subjected to acute liver failure (ALF) induced by subtotal (90%) liver resection. This model was used to study the effect of rat-specific (strain R2LC) Reuteri-fermented oatmeal on maintenance of gut integrity in these animals as monitored by bacterial overgrowth, translocation, and enterocyte protein contents. The number of anaerobic bacteria, Gram-negative anaerobes, and lactobacilli decreased significantly in the distal small intestine and colon in the hepatectomized animals treated with saline or unfermented oatmeal, as compared to sham operation or hepatectomized animals treated with the Reuteri-fermented oatmeal. The incidence of bacterial translocation to the systemic circulation was nil and 17% in rats subjected to sham operation with saline or 90%

Fig. 14. Effect of *Lactobacillus reuteri* administrations on total serum cholesterol, LDL-cholesterol, and HDL-cholesterol levels in pigs. It can be seen from these experiments with pigs that administrations of Reuteri during the indicated probiotic feeding period significantly reduced the total serum cholesterol and LDL-cholesterol, but not the HDL-cholesterol. Closed squares: Reuteri-treated pigs; open circles: control pigs. Panel A: total serum cholesterol; Panel B: LDL-cholesterol; Panel C: HDL-cholesterol. (Data reprinted from reference 170 with permission of the publisher.)

hepatectomy with the Reuteri-fermented oatmeal, respectively, and 80–90% and 34–50% in rats subjected to hepatectomy with saline or unfermented oatmeal, respectively. They concluded that administration of the Reuteri-fermented (but not the unfermented) oatmeal contributed to maintenance of gut integrity and prevented ALF in this model.

In another study using a different ALF model, Adawi, et al. (164) investigated the effect of rectal administration of arginine and probiotic bacteria (five *Lactobacillus* strains including rat- and human-specific strains of Reuteri) on bacterial translocation and the extent of liver failure in rats. Arginine was included in these experiments because it is known to have an effect on the immune system, particularly after trauma. It also has a secretagogue effect on several endocrine glands whose hormones have a trophic effect on the intestinal mucosa. Furthermore, arginine is a precursor of polyamines which are considered important mediators of cell growth and differentiation. ALF was induced 8 days after the probiotic administrations by intraperitoneal injection of D-galactosamine (1.1 g per kg body weight). Bacterial translocation was evaluated by culturing the portal and arterial blood, mesenteric lymph nodes, and the liver. Bacterial load in the cecum and colon was determined and liver histological and enzymatic changes studied. These studies showed that administrations of the lactobacilli with or without arginine significantly modulated the extent of liver failure and reduced bacterial translocation. Beneficial effects of arginine alone indicated a possible role of nitric oxide and/or polyamines in the moderating ALF in these animals. Reuteri significantly reduced the incidence and extent of bacterial translocation in this model system, providing additional evidence that this species contributes to maintenance of gut mucosal integrity and prevention of sepsis even when mucosal integrity is threatened by chemical stressors. In another experiment using this D-galactosamine-induced ALF model, pretreatment with Reuteri injected intraperitoneally three days, one week, and two weeks before induction of ALF had no beneficial effect (165), indicating that Reuteri's effectiveness is manifested from within the GI tract, not elsewhere.

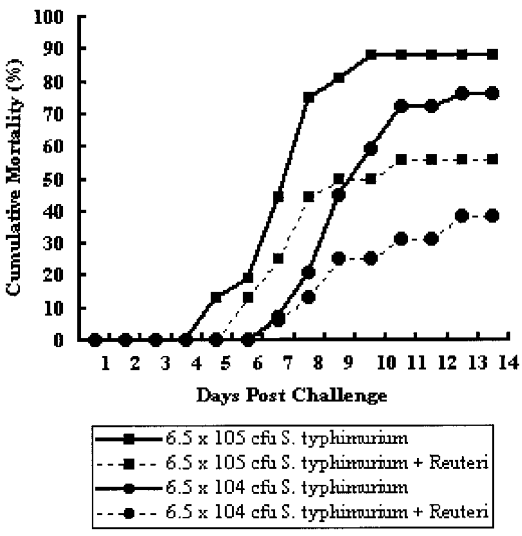

Fig. 15. Effect of *Lactobacillus reuteri* administrations on preventing deaths in *Salmonella typhimurium*-infected BALB/c mice. Reuteri administered in drinking water (3×10^7 cfu per ml) protects mice from deaths caused by oral (gavage) inoculation with 6.5×10^4 and 6.5×10^5 cfu *S. typhimurium*

M. Effect on Methotrexate-induced Enterocolitis in Rats. Methotrexate (MTX) is a chemotherapeutic prescribed as an antineoplastic agent for human cancer patients as an antirheumatic agent for juvenile rheumatoid arthritis. However, it causes cytotoxic injury to gastrointestinal mucosa and disruption of the intestinal microecology resulting in a severe enterocolitis. Intraperitoneal administration of MTX to rats on an elemental diet causes a severe nonperforative enterocolitis associated with significant body weight loss, mucosal mass loss, and generalized disruption of the intestinal barrier. Mao, et al. (166) used this rat model system to evaluate the effects of oral administrations of host-specific Reuteri (strain R2LC) and *L. plantarum* DSM9843 (vectored in oat-base formulations) on this experimental MTX-induced enterocolitis. Severity and progress of the enterocolitic inflammation was monitored on the basis of weight loss, increased intestinal permeability, bacterial translocation from the gut to extraintestinal sites, and intestinal myloperoxidase levels. Both *Lactobacillus* species helped restore the intestinal microecology, decreased the body weight loss, and decreased intestinal permeability concomitant with decreased bacterial translocation. It was concluded that administration of these lactobacilli was helpful in reducing the severity of MTX-induced enterocolitis in rats.

In another report using this same MTX-induced rat model for enterocolitis, Mao, et al. (166a) evaluated the effects of this drug on the gut immune response. All rats received continuous intragastric infusion of an elemental diet with or without supplementation of fibers (pectin or oatbase) and Reuteri (strain R2LC) or *L. plantarum* DSM 9843 from the beginning of the study. The control rats received normal chow throughout the study. On day three, the rats received intraperitoneal injections of either MTX (20 mg/kg), or normal saline, and sampling was done on day six, including measurements of (a) ileal and colonic secretory IgA levels, both soluble and insoluble fractions, and (b) gut lamina propria $CD4^+$ and $CD8^+$ lymphocyte counts. It was determined that administration of MTX significantly diminished both the intestinal secretory IgA levels and the gut lymphocyte numbers. Addition of Reuteri or the *L. plantarum* (but not the pectin or oatbase) significantly increased the ileal and colonic secretory IgA levels, both soluble and insoluble fractions, and elevated $CD4^+$ and $CD8^+$ numbers compared with the enterocol

tis rats. It was concluded that enhancements of gut immune functions by these lactobacilli might be important processes through which their probiotic efficacy was manifested. The authors of the present review reached a similar conclusion (15) based on findings that Reuteri probiosis in young chicks increased the lamina propria $CD4^+/CD8^+$ ratio in the ileum region of the animals (see Section XI C).

N. Effect on Serum Cholesterol and HDL Lipids in Animal Models. Lactobacilli are believed to be significant contributors to bile salt hydrolase (BSH) activity found in the ileum and cecum of the mouse and other animals (167). Gilliland (168) proposed that if lactobacilli possessing high levels of BSH activity accessed the GI tract, BSH activity in the intestine would increase accordingly. Increased proportions of the deconjugated bile salts, which are less water soluble in the gut, would result in their excretion via the feces. Consequently, like the proposed mechanism for serum cholesterol lowering by cholestyramine (and other bile salt sequestrants), decreased amounts of bile salts would return to the liver. It is believed that this would result in a loss of feedback inhibition of bile salt synthesis and an increased conversion of cholesterol to bile salts (169).

This proposed mechanism for microbial control over serum cholesterol levels is consistent with a recent study by De Smet, et al. (170) using pigs and a pig strain of Reuteri possessing an active BSH. During a 13-week experiment, 20 pigs were fed a high fat, high cholesterol, low fiber diet for the first 10 weeks, and a regular pig diet for the last 3 weeks of the experiment. One group of pigs received twice daily, ca 10^{11} cfu Reuteri for 4 weeks (from week 3 to week 7). Subsequently, this treated group was again fed the same diet as the control group. The Reuteri administrations caused a significant lowering of the total and LDL-cholesterol concentrations in the treated pigs compared with the control pigs, while no change in HDL-cholesterol was observed (Figure 14). Fecal output of neutral sterols and bile salts increased in the treated pigs. During the final 3 weeks of normalization to the regular diet, cholesterol concentrations in both groups and the differences in total and LDL-cholesterol concentrations between the groups largely disappeared. The positive relationship observed between increased fecal neutral sterols and bile salts on the one hand and serum cholesterol lowering on the other tends to confirm the purported interaction between gut BSH activity and serum cholesterol levels. These authors believe that long-term human trials are warranted. And, that fermented foods containing BSH active lactobacilli may be an effective means for cholesterol control by people conscious of their unhealthy diet and as a valid alternative to pharmaceutical intervention in hypercholesterolaemic individuals.

Similar results suggesting a similar mode of action by Reuteri were obtained by Taranto, et al. (171) using mice fed a diet enriched with fat to produce hypercholesterolemia. They found that administration of Reuteri at a concentration of only 10^4 cfu per day for 7 days decreased blood total cholesterol by 38%, resulting in serum cholesterol levels similar to those found in the control group fed a normal, non-fat diet. Reuteri also caused a 40% reduction in triglycerides and a 20% increase in the ratio of high-density lipoprotein to low density lipoprotein without translocation of the indigenous microbiota to the spleen and liver. Based on these findings and the 1 to 2 rule (172), it was concluded that a significant positive effect could be obtained for patients suffering from elevated cholesterol by ingesting Reuteri and thereby improving their gut BSH activity. The 1 to 2 rule states that a 1% reduction in the serum cholesterol level causes a 2% lowering of the risk for coronary heart disease.

Tannock and McConnell (173) reconstituted lactobacilli-free mice with BSH active *Lactobacillus* strains, but found their cholesterol-lowering effect to be insignificant. However, they did not report how many viable lactobacilli were used, and it is known that the 'minimum effective dose' and 'dose-response' relationship' of cholestryamine treatment, for example, differed in different test subjects (138, 139). Du Toit, et al. (174) on the other hand, found a 'probiotic mixture' containing 2 strains of *L. johnsonii* and 1 Reuteri strain (isolated from pig feces as only 3 out of 297 isolates possessing high BSH activity) effective in lowering serum cholesterol in minipigs after 3 weeks of probiotic feeding. Along these same lines, but involving use of a non-*Lactobacillus* species, Ling, et al. (175) showed that *Eubacterium coprostanoligenes* administered to germfree mice daily for 1 week had a transient effect on lowering blood cholesterol levels. This species converts cholesterol to coprostanol which, like deconjugated bile salts, is poorly absorbed by the GI tract.

O. Aflatoxin Binding In Vitro and In Situ. Preliminary studies by Edens, et al. (176) have shown that viable Reuteri cells are able to bind aflatoxin B_1 (AFB_1). To determine if *in situ* conditions could negate this binding, sealed intestinal loops (in anaesthetized chickens) were injected with a Reuteri suspension containing bound AFB_1. Ten min after this injection the loop was cut, the contents discharged and centrifuged, and the supernatant fraction analyzed for AFB_1. It was shown that AFB_1 remained bound to the Reuteri surface.

P. Effect on Salmonella typhimurium Infection and Translocation in the BALB/C Mouse. Studies employing mouse models were initiated in our laboratories primarily for two reasons. First, to determine if the protective effect of Reuteri on enterobacterial challenges seen in chickens and turkeys could be demonstrated in mammals as well. And secondly, to establish a 'state of the art' animal model system that could be used in our attempts to delineate Reuteri's mode of action. Both germfree (purchased from the University of Wisconsin, Madison WI, USA) and specific pathogen free (SPF) BALB/c mice (Taconic labo-

Table 5

Effect of Lactobacillus reuteri biotherapy on rotavirus-induced diarrhea in children (1995 and 1996 clinical trials)

A. Duration of Watery Diarrhea (Days):		
	Placebo (n = 46) Mean (± SD)	*L. reuteri*-treated[a] (n = 40) Mean (± SD)
Before treatment	2.9 (± 1.5)	3.2 (± 1.4)
After treatment	2.7 (± 1.9)	1.6 (± 1.3) (p = 0.002)
B. Percent of Patients with Persisting Watery Diarrhea (%):		
	Placebo	*L. reuteri*-treated
Day 0	100	100.0
Day 1	100	82.5 (p = 0.003)
Day 2	80.4	37.5 (p = 0.000)
Day 3	47.8	20.0 (p = 0.006)
Day 4	26.1	7.5 (p = 0.020)

[a]The *L. reuteri* dose was 10^{10} to 10^{11} cfu per day for duration of trial.

ratories, Germantown MD, USA) have proven useful on both accounts. Upon arrival, the SPF BALB/c mice are assigned to appropriate isolators and/or cages and both intestinal tissues and feces are examined for presence of Reuteri. It has been determined that while every mouse has a well-established enterolactobacillus population, they are devoid of Reuteri and therefore can serve as controls in these studies.

The ability of Reuteri to protect BALB/c mice from salmonellosis has now been experimentally determined (177, 178). As seen in Figure 15, Reuteri added to drinking water at a concentration of 3×10^7 cfu per ml for two weeks before challenge (and continued post-challenge) confers significant protection from deaths caused by oral administration (by gavage) of 6.5×10^4, or 6.5×10^5 cfu of *S. typhimurium* strain 14028. It was observed that Reuteri probiosis decreases both the rate and extent of death resulting from the infection with this pathogen. Experiments are presently underway to determine which of the many presumably innate defense mechanisms available to these animals are enhanced by the Reuteri administrations. To date, we have only determined that the probiotic treatment appears to enhance maintenance of gut mucosal integrity concomitant with significantly decreased translocation of *S. typhimurium* from the gut to the mesenteric lymph node, liver, spleen, and other extraintestinal sites.

IX. Reuteri is a Safe and Effective Colonizer of Humans and Animals. Based on the above studies, it was concluded that host-specific strains of Reuteri may colonize their respective hosts and enhance protection against detrimental effects of certain microbiological, chemical, and physical stressors. Following are the results of clinical trials conducted using a human strain of Reuteri isolated from breast milk of a young healthy mother who was nursing her child.

A. Children as Subjects. The first human clinical safety trial involving Reuteri was conducted in 1995 at the Tampere (Finland) University Hospital (Shornikova, unpublished data). The objectives of the trial were to determine (a) if oral administrations of a human strain (SD2112) of Reuteri would colonize the human GI tract, (b) the dosage needed to obtain colonization, and (c) if any adverse effects occurred as a result of these administrations. Children aged 6 to 36 months were enrolled with parental consent in the study after being hospitalized in the infectious disease ward of the hospital. The children were hospitalized with presumed viral or mild bacterial infections; some received antibiotics, others did not. These subjects were included in a four-group randomized double-blind design receiving the following treatments: (a) Reuteri administered at a dose of 1 to 3×10^8 cfu per day for 5 days, (b) administered at a dose of 10^{10} cfu per day for 5 days, (c) administered as a dose of 10^{10} cfu once, and (d) placebo administered for 5 days. Total lactobacilli and Reuteri were enumerated in stool samples to monitor GI tract colonization. Baseline stools were collected before administration of Reuteri or placebo. Enumerations were conducted on samples collected 1 and 3 days after initial administration and 10 days after terminating the treatments. Based on these trials it was concluded that the Reuteri administrations had no adverse effects, and that good gastrointestinal colonization by this species was observed (based on fecal counts) for both the low and high dose administrations.

A second tolerance/safety and dose response/fecal colonization clinical trial was conducted in Mexico with 72 children, ages 12 to 36 months (179). The children were randomized into four groups receiving either placebo or a low (10^6 cfu per day), medium (10^8 cfu per day), or high (10^{10} cfu per day) dose of a probiotic beverage containing a blend of Reuteri, *L. acidophilus*, and *Bifidobacterium animalis*. Intake of beverage and tolerance were monitored daily. Evaluations included: incidence of vomiting, abdominal discomfort, gas and stool characteristics, total lactobacilli and Reuteri fecal counts. The study consisted of an entry evaluation, a 3-week feeding evaluation, and a post-feeding evaluation. It was concluded that the probiotic blend was well tolerated, that colonization occurred based on the presence of high numbers of Reuteri in feces of the treated children, and that clinical evaluations revealed no statistical differences among the four groups.

B. Healthy Adults as Subjects. A clinical trial was conducted on 30 healthy male subjects (age 18 to 75 years) using a two-group, double-blinded, parallel design to evaluate the safety, tolerance, and colonization potential of Reuteri as a probiotic for adult humans (180). The subjects consumed for 21 days two gelatin capsules containing either a freeze-dried preparation with a cryoprotectant, or a placebo. The administered Reuteri dose was 10^{11} cfu per day for a 21 day period. A physical examination and

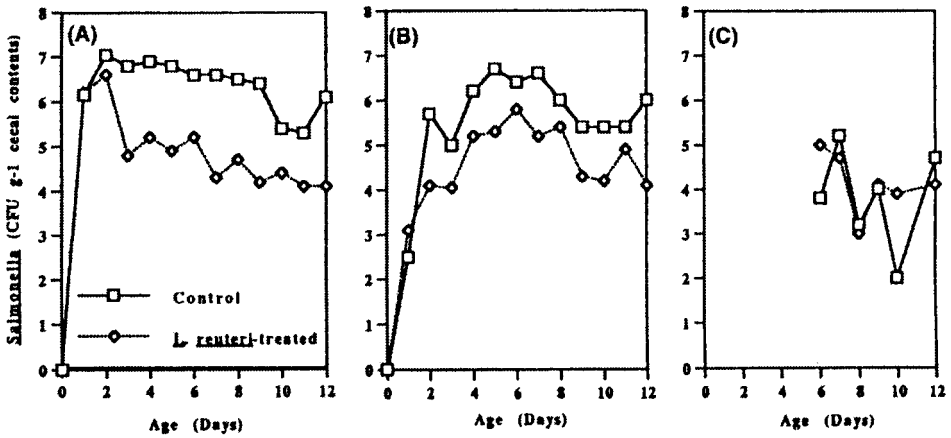

Fig. 16. Effect of *Lactobacillus reuteri* administrations on *Salmonella typhimurium* colonization in the cecum of turkeys. Both controls and Reuteri-treated turkeys were orally inoculated (by gavage) with *Salmonella typhimurium* (2×10^6 cfu per bird) at hatch (panel A), on day 1 posthatch (panel B), and on day 5 posthatch (panel C). The cecal samples were analyzed daily for cecal *Salmonella* in surviving animals.

urinalysis parameters were determined on days 0, 21, and 28; stool samples were obtained on days 0, 14, 21, 28, and 77 for enumeration of total lactobacilli and Reuteri. It was concluded that Reuteri may be fed at 10^{11} cfu per day without any clinically significant safety or tolerance problems. This dosage resulted in colonization within 7 days of consumption and was maintained for at least 7 days post-consumption, indicative of good colonization; however, colonization judged by fecal examination was lost within 2 months after cessation of consumption.

C. HIV Positive Adults as Subjects. A safety and tolerance double-blind, placebo controlled clinical trial was conducted to evaluate the effects of probiotic administrations of Reuteri on an adult population infected with the human immunodeficiency virus (HIV) (181). Thirty-nine adult (male and female) subjects (ages 23 to 50 years) were randomly assigned to one of two experimental treatments within their respective block (AZT use or no antiretroviral therapy). The study was conducted for 35 days with the subjects consuming placebo or Reuteri (10^{10} cfu per day) for the first 21 days followed by a 14 day washout period. To evaluate safety, blood samples were taken at baseline, day 21, and day 35 for serum chemistry, hematology and immunology profile analyses. Urine samples were collected for routine urine analysis and physical examinations were conducted. Blood, urine, and sputum were collected for bacterial analyses, and subjects completed daily questionnaires evaluating bowel functions, gastrointestinal tolerance, and tolerance in general. Weekly fecal samples were collected for enumeration of total lactobacilli and Reuteri. It was concluded that no clinically significant changes could be seen in any of the safety parameters measured. Consumption of Reuteri by these subjects increased fecal levels of this microorganism from baseline, although it was noted that fecal levels of both Reuteri and total lactobacilli were 2 and 3 logs lower, respectively, than observed in healthy male adults. Overall, this study indicated that Reuteri may be administered at 10^{10} cfu per day without clinically significant safety or tolerance problems. This study also documented a low level of fecal lactobacilli in the HIV positive population. The reason(s) for this are unknown, but it may be a clue to further our understanding this disease.

X. Effect of Reuteri Probiosis on Human Health. Clinical evidence to this effect was first obtained showing that Reuteri is effective as a therapeutic agent capable of moderating acute rotavirus diarrhea in children. Subsequently, clinical evidence was obtained showing a prophylactic effect as well. It was shown that consumption of a beverage containing a blend of probiotic cultures, including Reuteri, *L. acidophilus*, and *Bifidobacterium animalis*, significantly reduced the risk of young children developing diarrhea.

A-Therapeutic Efficacy for Rotavirus-induced Diarrhea in Children. Diarrheal diseases are one of the most common health problems encountered during childhood worldwide. During periods of acute diarrhea, the normal gastrointestinal microbiota is radically changed, including decreases in *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species (182–184). Several studies have indicated that *Lactobacillus* probiosis can accelerate normalization of the host's microbial balance and thereby moderate acute episodes of diarrhea (185–187). Among these, *L. rhamnosus* GG has been shown to promote clinical recovery from rotavirus gastroenteritis in children and enhance intestinal immune responses (188–190).

Shornikova, et al. (191) used a Reuteri strain isolated from human breast milk to study 40 patients between the ages of 6 and 36 months hospitalized with acute diarrhea; 75% of the diarrheas were diagnosed as rotavirus-induced. The patients were randomized to one of two treatment groups to receive either 10^{10} to 10^{11} cfu Reuteri or a matching placebo daily for the length of the hospitalization or up to 5 days. It was concluded from this clinical trial that Reuteri is effective as a therapeutic agent in acute

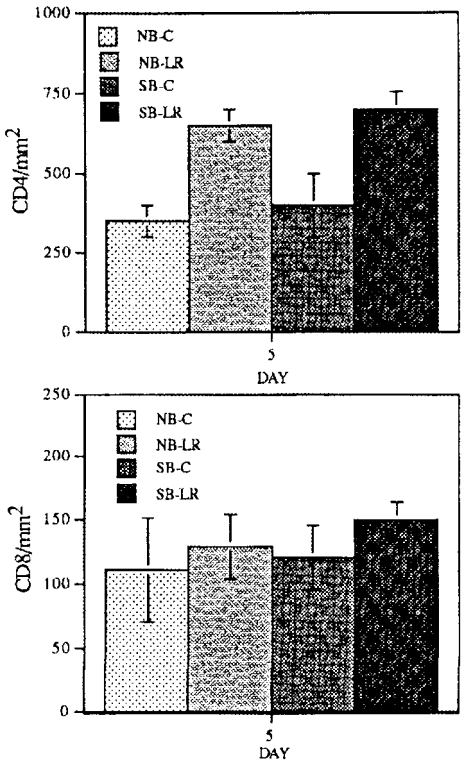

Fig. 18. Effect of *Lactobacillus reuteri* administrations on $CD4^+$ and $CD8^+$ T cells in the lamina propria of the ileum in 5 day-old chicks. Upper panel: number of $CD4^+$ T cells per mm^2 of tissue; Lower panel: number of $CD8^+$ T cells per mm2 of tissue. NB-C: Control chicks brooded under normal conditions; NB-LR: Reuteri-treated chicks brooded under normal conditions; SB-C: Control chicks brooded under stressed conditions (mild cold stress and *Salmonella* infection as described in reference 15); SB-LR: Reuteri-treated chicks brooded under stressed conditions. (Reproduced from reference 15 with permission of the publisher).

rotavirus diarrhea in children. This was based on observations that (a) the mean duration of watery diarrhea after treatment was 1.7 days in the Reuteri group and 2.9 days in the placebo group, and (b) on the second day of treatment only 26% of patients receiving Reuteri had watery diarrhea, compared with 81% of those receiving placebo. Reuteri accounted for $>75\%$ of the total lactobacilli found in the children fed this probiotic.

Reuteri's efficacy in rotavirus-induced diarrhea in children was confirmed in another clinical trial by Shornikova, et al. (192) investigating the dose dependency of the effect of Reuteri. In this study children between the ages of 6 and 36 months admitted for rotavirus-associated diarrhea were randomized into three groups to receive either 10^7 or 10^{10} cfu Reuteri or a matching placebo once a day for up to 5 days. It was determined that the main effect of Reuteri was that it significantly reduced the duration of watery diarrhea after initiation of treatment. The duration was 2.5 days in the placebo group vs. 1.9 days in the group receiving 10^7 cfu and 1.5 days in the group receiving 10^{10} cfu. The correlation between the dosage of Reuteri, recovery in the feces, and the beneficial clinical effects were significant. A summary of the beneficial effects of Reuteri on these children during studies conducted in 1995 and 1996 is shown in Table 5.

B-Prophylactic Efficacy for Community-acquired Diarrhea in Children. As noted above, documented clinical studies have shown certain probiotics to be safe and effective biotherapeutic treatments for childhood diarrhea. However, there are no reported controlled clinical trials evaluating their effectiveness in preventing childhood diarrhea. Ruiz-Palacios and colleagues (193, 194) recently conducted two blinded, controlled studies to evaluate the effect of Reuteri in preventing community-acquired diarrhea in 12 to 36 month-old children living in Mexico. In the first study, 243 children were fed for 14 weeks placebo or a probiotics mixture containing Reuteri. More Reuteri-fed children were free of diarrhea during the 14 weeks than were those placebo-fed (90/119 vs. 77/120 respectively; $p = 0.04$). In the second study, three groups comprising a total of 319 children were studied for 16 weeks. One group (placebo) received two 120 ml servings of whole milk daily as a supplement to their diet. The second group received this milk regimen supplemented with a probiotic blend developed by BioGaia Biologics (Stockholm, Sweden) containing: Reuteri (1.5×10^7 cfu per g), *L. acidophilus* (3.6×10^8 cfu per g), and *Bifidobacterium infantis* (5.1×10^8 cfu per g). The third group received a probiotic blend developed by Chr. Hansen's Laboratory (Milwaukee, WI, USA) containing L. acidophilus (3.2×10^8 cfu per g) and *B. animalis* (1.1×10^9 cfu per g). Compared to the placebo group (62 episodes of diarrhea), the Reuteri-fed group had a significantly lower incidence of diarrhea (47 episodes; $p = 0.04$), and non-Reuteri fed group had a near significance reduction in episodes (47 episodes; $p = 0.135$).

XI. Concerning Reuteri's Mode of Action. As evidence has accumulated pointing to the broad-spectrum nature of Reuteri's probiotic efficacy, questions emerged concerning its underlying mode of action. Are Reuteri's probiotic effects limited to interactions with other microorganisms in the gut ecosystems? It has long been assumed that a probiotic's primary role is to maintain balance among the gastrointestinal microbiota (1, 4, 21–24). However, it is possible that Reuteri and/or its products may also have some direct effect on activities of the host's enterocytes and/or immunocytes. In fact, there is evidence that some lactobacilli play a role in the host's mucosal immune defenses (195, 196). Some of the studies cited above show Reuteri to be effective in preventing translocation, however provoked, of microorganisms from the gut to extraintestinal sites. Does Reuteri play a role in maintaining gut mucosal integrity? If so, how is this accomplished? On the other hand, Reuteri's beneficial effects to some extent may depend on its ability to regulate other microbe-associated activities within the luminal environment. Clearly,

questions concerning Reuteri's mode of action far outweigh answers at this time. Some answers will emerge only after a thorough examination of the 'cross talking' that is believed to occur between Reuteri cells, the gastrointestinal microbiota in general, and target host tissue cells. Following are a number of observations indicating that multiple modes of action may underlie Reuteri's probiotic efficacy.

A. Competitive exclusion (CE). CE is a process whereby certain members of the gastrointestinal microbiota are able to prevent or antagonize pathogens from adhering to gut mucosa and initiating disease. It derives from the work of Nurmi and Rantala (30) who showed that colonization of *Salmonella* in the chicken gut is thwarted by oral administration of cecal extracts obtained from non-infected, healthy chickens. As mentioned, our poultry studies showed that under certain conditions Reuteri administrations were capable of exerting a CE effect (15). For example (Figure 16), whereas *S. typhimurium* was observed to undergo rapid growth in the poult gut up to about 5 days posthatch, this growth could be decreased 1 to 2 log units by Reuteri prophylaxis. However, in other experiments a CE effect by Reuteri was delayed until after the bird's life was no longer threatened by *Salmonella* (63), indicating that while Reuteri can function as a CE agent, this is unlikely its sole mode of action.

B. Effect on Ileal Villi Development in Chickens and Turkeys. Comparative morphometric analyses were carried out on duodenal, jejunal, and ileal tissue obtained from 3 day-old control and Reuteri-treated chicks grown under optimal conditions. The results of these analyses showed that Reuteri stimulated development of longer villi and significantly deeper crypts, specifically in the ileal region of the gut (15). Photomicrographs of representative sections of ileal tissues obtained from control and Reuteri-treated poults are shown in Figure 17. This enhanced ileal mucosal development caused by Reuteri occurred in turkeys as well, and the effect was retained until the birds reached market age (197). Thornbecke, et al. (198) showed that the ileum and cecum of chickens exhibited the greatest difference of any tissues when conventional (CV) birds were compared to germfree (GF) birds. Cook and Bird (199), studying villus area and epithelial cellular migration in CV and GF chicks, reported that by day 7 posthatch the villus area and the crypt depth were significantly larger and deeper, respectively, in CV chicks than in the GF chicks. It is interesting to note that a positive correlation has also been established (at least in rats) between villus height and gain in body weight (200).

The mechanisms underlying gut mucosal cell development are not well understood, but it is believed that mucosal T cells play a role. Studies on nude vs. euthymic mice have shown that mucosal T lymphocytes influence epithelial cell renewal and differentiation in pathological states, and appear also to regulate enterocyte growth under normal conditions as well, indicating a close relationship between the intestinal epithelium development and its associated lymphoid elements (201). Furthermore, the gut-associated lymphoid tissue (GALT) is no longer believed to be a secondary, but rather a primary lymphoid organ. This view is based on a rapidly expanding body of evidence showing that bone marrow precursors can home to the gut epithelium, rearrange their T cell receptor genes and further differentiate in the mucosal microenvironment (202). Given the gut microbiota's antigenic potential and its proximity to this primary lymphoid tissue, it would not be surprising if a variety of regulatory and 'cross talk' interactions co-evolved between a host and certain members of its microbiota. Probiotic efficacy may be based on such interactions. Reuteri mono-associated BALB/c mice are being used to determine the effect of Reuteri on development of the murine gut. Preliminary results (described below) point to a similar role for Reuteri on ileal tissue development in both murine and avian systems.

C. Effect on Chicken Lamina Propria $CD4^+/CD8^+$ T Cell Ratio. This effect of Reuteri on ileal tissue development and the purported role of gut lymphocytes in this regard, led to an examination of lymphocyte subsets in gut tissues of control and Reuteri-treated chicks (15). Only limited analyses of the avian mucosal immune system were

Fig. 19. Effect of host-specific strains of *Lactobacillus reuteri* on development of ileal tissues in monocolonized gnotobiotic BALB/c mice. Left panel: germfree controls; middle panel: monocolonized with mouse-specific strain of Reuteri; right panel: monocolonized with human-specific strain of Reuteri.

possible when these experiments were undertaken owing to a paucity of required immunological reagents. Nevertheless, some interesting results were obtained as shown in Figure 18. It was observed that, in direct comparison to 5 day old untreated chicks, Reuteri-treated chicks had significantly more $CD4^+$ (helper T cells) T cells but not $CD8^+$ (cytotoxic T cells) T cells in the lamina propria of the ileal region of the GI tract (15). The Reuteri-treatment increased the $CD4^+/CD8^+$ ratio in the ileum from approximately 2 to 3.5, but it had no effect on either of these T cell populations in the duodenal or jejunal regions of the gut. Nor did treatment have an effect on the B cell population in either the duodenum or ileum (data not shown). The observation that the Reuteri-associated stimulation of $CD4^+$ lymphocytes in the chick's ileum occurred whether the birds were grown under stressed or non-stressed conditions appear to be another clue as to the mechanism(s) underlying Reuteri's role in protecting these hosts from AGD.

Are these effects of Reuteri on the avian ileum causally related? Does the presence of Reuteri in the avian gut stimulate $CD4^+$ T cell proliferation/activity in the lamina propria, which in turn stimulates crypt mitotic activity and proliferation of ileal epithelial cells? Although one can only speculate at this time, Ferreira, et al. (203) have shown that activated T lymphocytes in the human small intestinal lamina propria are involved in enhancing proliferation of intestinal epithelial cells. It remains to be seen if Reuteri plays a role in T cell activation.

Additional information and speculations concerning the role of probiotic microorganisms (and the normal gastrointestinal microbiota in general) on a host's mucosal tissues and overall immune functions are available in reviews by Freter and Nader de Macias (204) and Famularo, et al. (116). Included among the many propositions cited by these authors is the opinion that while "pathogenic bacteria entering the lamina propria from the gut lumen may proliferate and translocate to other organs,—a normal indigenous microflora counteracts this." And that "the entry of bacteria from the physiological indigenous microflora into the mucosa and their subsequent translocation to other organs is currently regarded as a crucial step for the development of the normal mucosal and systemic immunity." Does Reuteri enter into the mucosa and thereby contribute to the development of the normal mucosal and systemic immunity? Could such a 'contribution' account, at least in part, for Reuteri's probiotic efficacy?

D. Effect of Reuteri on Avian Humoral Responses to Salmonella Infection. Famularo, et al. (116) and Perdigon and Alvarez (195) reviewed information showing that lactobacilli and other lactic acid bacteria resident in the GI tract may act as adjuvants to certain humoral immune responses. Using the *Salmonella* antibody agglutination method of Williams and Whitmore (205), our studies on *Salmonella*-challenged poults showed that gut colonization by Reuteri does in fact enhance the avian humoral response to this pathogen (15). Sera obtained from the Reuteri-treated and untreated poults challenged with *S. typhimurium* at hatch, on day 1 and day 5 post hatch were analyzed for antibodies to the *S. typhimurium* challenge strain. It was found that Reuteri-treated poults infected with *S. typhimurium* (10^6 cfu per bird by gavage into the crop) at time of hatch or on day 1 post hatch had significantly higher antibody titers by days 15 and 16 than did the untreated poults. However, no significant antibody response was induced in either the Reuteri-treated or untreated poults that had already by day 5 posthatch become resistant to the *Salmonella*-challenge. Reuteri's apparent 'adjuvant effect' on antibody production appeared to be limited to the period when these birds were actively responding to the *Salmonella*-challenge.

E. Reuteri as Adjuvant and Regulator of Cytokine Expression in BALB/c Mice. Evidence that Reuteri possesses intrinsic adjuvanticity and can influence gut mucosal cytokine expression was recently reported by Maassen et al (206). Female BALB/c mice (6-10 weeks of age) were orally administered eight different common lactobacilli, including (a) three obligatory heterofermentative strains (mouse *L. reuteri* ML1, pig *L. fermentum* 104R [recently re-classified as *L. reuteri* 104R], and mouse *L. brevis* ML12), (b) four facultative heterofermentative strains (mouse *L. murines* CNRZ, cheese *L. casei* ATCC393, sauerkraut *L. plantarum*, and human *L. plantarum* NC1B8826), and (c) one obligate homofermentative strain (mouse *L. gasseri* ML21). Each *Lactobacillus* strain was evaluated with respect to mucosal induction of pro- and anti-inflammatory cytokines, IgA-producing plasma cells in the gut, as well as systemic IgG antibody responses against parenterally administered haptenated chicken gamma globulin (TNP-CGG).

Immunohistochemical analysis of cytokine-producing cells in the gut villi showed no significant induction of the cytokines Il-1η, IFNκ, IL-4 or IL-10 after oral administration of the obligate homofermentative and facultative heterofermentative lactobacilli. In contrast, oral administrations of the obligate heterofermenters, *L. reuteri* ML1 and *L. brevis* ML12, induced expression of the proinflammatory/Th1 cytokines TNF-η, IL-2 and /or IL-1β. These same two heterofermenters and *L. reuteri* 104R also significantly enhanced the IgG response against parentally administered TNP-CGG. The non-obligatory heterofermenters did not show this adjuvanticity. The observation that *L. reuteri* 104R exhibited less adjuvant activity than *L. reuteri* ML1 could be attributed to host-specificity factors, noting that 104R is a pig strain and ML1 is a mouse strain. Additional indications of the importance of host-specificity in selecting lactobacilli for probiotic use are cited throughout this review.

These results provide evidence that: (a) oral lactobacilli administration can influence local cytokine production af-

ter parenteral immunization with a pathogen virus (UV-inactivated Chikungunya virus), (b) certain strains, notably obligate heterofermenters such as Reuteri, are able to nonspecifically enhance the humoral response (adjuvanticity) to TNP-CGG, and (c) adjuvanticity can be correlated with induced gut cytokine profiles. These findings imply that certain *Lactobacillus* strains induce distinct mucosal cytokine profiles and possess differential intrinsic adjuvanticity. A relation between mucosal cytokine production and systemic antibody responses has been demonstrated (206a, 206b). In addition, due to different kinetics of expression of cytokines, analysis at other timepoints may show induction of other cytokines, such as IFNκ.

F. Effect of Host-specific Strains of Reuteri on Development of Ileal Villi and Immune Response in BALB/c Mice. As reported above, Reuteri has been shown to (a) stimulate development of ileal villi and crypts and to increase the $CD4^+/CD8^+$ T cell ratio in the lamina propria of the avian ileum. Also, host-specific Reuteri has been shown to function as an adjuvant for systemic antibody responses and to induce distinct cytokine production in the murine gut. Recent studies in our laboratory comparing germfree BALB/c mice monocolonized with either mouse or human strains of Reuteri revealed similar effects on development of ileal tissues in these mammals as determined by scanning electron microscopy (Figure 19), morphometric measurements, and cytokine profiling. After 45 days of stable monoassociation, ileal villi (but not stomach or cecal tissues) were approximately 20% longer ($p < 0.05$) and more fully developed in mice monoassociated with the mouse Reuteri when compared to either the germfree controls or to mice monoassociated with human Reuteri. Sixty days after monocolonization, when all three groups of mice were subsequently conventionalized by oral inoculation with cecal contents obtained from mice that had been administered altered Schaedler's flora, it was found that the human Reuteri strain was rapidly excluded; whereas, the mouse Reuteri strains persisted in their respective hosts. After 45 days of monocolonization and 30 days of 'conventionalization', spleen cells from the three treatment groups were cultured in the presence of the inducing agents (concanavalin A, lipopolysaccharide from *Salmonella typhimurium*, phorbol-12-myristate-13-acetate, ionomycin, and heat-inactivated Reuteri) for cytokine determinations (IL-2, IL-4, IL-10, IL-12, and INFκ). No difference was found in the cytokine profile between the germfree mice and the mice monocolonized with the mouse or human Reuteri. Spleen cells from mice from the three treatment groups responded in the same way to the inducer signals. However, after 'conventionalization' with altered Schaedler's flora, the cytokine profile changed. Spleen cells from the mice colonized with mouse Reuteri produced higher concentrations of INFκ, IL-4, and IL-10 in the presence of mitogens (including the heat-treated Reuteri) than did spleen cells from either the control or human Reuteri-colonized mice. The cytokine most stimulated under these conditions was INFκ. These findings indicate that host-specificity factors play a role in Reuteri's ability to colonize its respective hosts and to elicit mucosal responses that may underlie its effectiveness as a probiotic. A preliminary report of these findings has been presented (58). A full report will be presented upon completion of evaluating the influence of Reuteri on (a) intestinal CD4 + /CD8 + T cell ratios, and (b) cytokine (IL-2, Il-4, Il-10, Il-12, IL-18 and INFκ) production by cells isolated from Peyer's patches, the MLN, and peripheral blood.

XII. Methods for Administering Reuteri to Humans: Reuteri-containing Functional Foods. A number of practical considerations must be taken into account if a bacterial strain is to qualify as a probiotic. At a minimum it must maintain a high degree of viability after being produced on a commercial scale in a cost-effective manner. The Reuteri strain used in all human clinical trials is now also commercially available as a functional food ingredient. After growth in commercial fermenters and harvesting, Reuteri cells are formulated as either lyophilized or frozen preparations with guaranteed viability if consumed within indicated time frames. For clinical trials Reuteri was administered either in capsule form, containing known cfu per capsule, or at known concentrations suspended in various beverages or dairy products.

Reuteri was first introduced into the human functional foods market in Sweden in 1991 as BRA milk and BRA fil (a fermented milk) dairy products. The BRA term derived from the fact that these products contained a probiotic mixture of *Bifidobacterium animalis*, Reuteri, and *L. acidophilus*. Subsequently, Symbalance (Toni AG, Switzerland) yoghurt and drinking yoghurts containing Reuteri and other probiotic cultures together with inulin as a prebiotic were introduced into Switzerland and Japan markets. Several dairy products, juices, and other Reuteri-containing functional foods are now also available in Finland and the USA with markets growing in other countries as well (207, 208). These products are formulated to deliver to the consumer approximately 5×10^5 to 10^6 cfu per ml or per g of product. For products requiring heat treatments (e.g., UHT milk or juice) sufficient to inactivate Reuteri, special aluminum-sealed lyophilized preparations of Reuteri are placed in a LifeTop® packet mounted on the product's package or bottle. Upon puncture of the LifeTop® packet, viable Reuteri is delivered to the product at specified levels.

A. Prebiotics and Probiotics. It is known that probiotic activity in the GI tract is not determined solely by the inherent properties of the probiotic. Many host factors, including the diet, play major roles in determining probiotic efficacy. Oyofo, et al. (209), for example, showed that addition of mannose or lactose (sugars not absorbed and therefore not metabolized by avian species) to the diets of

Table 6

Lactobacillus reuteri as a functional food component

Fruits and Vegetables as Sources of Phyto-Protectants: Fruits and vegetables contain fiber, antioxidants and numerous health-promoting phytochemicals, e.g., sulfides, phytates, flavonoids, glucatates, carotenoids, cumarins, monoterpines, lignans, phenolic acids, indoles, isothiocyanates, phthalides, polyacetylenes, etc. (reference 223).
Dairy Products as Sources of Lacto-Protectants: Various fresh and fermented dairy products contain lactochemicals such as conjugated linoleic acid (CLA), sphingomylen, ether lipids, butyric acid, and extracted plant phytochemicals, etc (reference 230).
Meats as Sources of Corpro-Protectants: Meats, particularly those obtained from ruminant animals are a rich source of the corproprotectant, conjugated linoleic acid (CLA) (reference 228).
***Lactobacillus reuteri*-containing Fruits, Vegetables, Dairy Products, and Meats as Sources of Microbio-Protectants:** These foods supplemented with viable, host-specific cultures of Reuteri are sources for the protectants present in the respective foods plus the health-enhancing products (e.g., lactic and acetic acids) and broad-spectrum functions that have been shown to be associated with probiotic administrations of Reuteri.

broiler chickens had no effect on their growth rate but significantly reduced intestinal colonization by *S. typhimurium*. On the other hand, glucose, maltose, sucrose and other sugars readily metabolized by the host were unable to enhance this CE effect. Bailey, et al. (210), also using *S. typhimurium*-challenged chickens, observed that compared to controls, significantly fewer birds were colonized with this enterobacterial species when fed diets containing 0.75% fructooligosaccharides (FOS). They observed an 80% reduction in the level of *Salmonella* present in the ceca of FOS fed birds testing positive for this enteropathogen. Probiotics were not used in either of these studies and the basis for the CE enhancing effect of the added sugars remains unknown. It is presumed, however, to be mediated at least in part by enhancing the antagonistic activities (e.g., acid production) of certain members of the indigenous gastrointestinal microbiota, and in part by competition for receptor sites on both bacterial and tissue surfaces (211). Enhanced efficacy in poultry production was also demonstrated by Casas, et al. (70) using a probiotic-prebiotic formulation containing Reuteri and lactose, respectively. As mentioned earlier, a number of probiotic formulations designed for human consumption also incorporate prebiotics (e.g., inulin in the Toni AG yoghurts) into their commercial products. Gibson and Roberfroid (212) developed the concept of 'synbiotics', i.e., the combined use of probiotics and prebiotics as a means to better manipulate the composition of the gut microbiota.

B. Perspectives on Reuteri as a Functional Food Component. Whatever its specific mode(s) of action, Reuteri's health-enhancing activities appear to be ultimately focused on reinforcing the host's mucosal defense barriers against invading pathogens or substances capable of injuring intestinal tissues, inciting inflammatory damage, and thereby contributing to loss of intestinal mucosal integrity. A healthy GI tract with a healthy, well-balanced microbiota allows only nutrients to pass into the bloodstream; whereas, a 'leaky gut' allows incompletely digested molecules (e.g., proteins, fats, etc.), bacteria, fungi, viruses, and other undesirable microorganisms and substances to translocate across the intestinal barrier to extraintestinal sites. A variety of physiological insults (e.g., antibiotic use, radiation treatments, gut infections, improper diets, etc.) have been shown to induce this 'leaky gut' syndrome. Among the serious health problems that emerge when the gut microbiota is not properly balanced and when gut integrity compromised is the translocation of microorganisms (primarily but not exclusively Gram-negative, endotoxin-bearing bacteria) to extraintestinal sites. This septic state can lead to serious immunopathologies and even death in some cases (214–217). In the present report we reviewed evidence pointing to Reuteri's functional role in helping to maintain and/or repair the gut mucosal barrier when its integrity is threatened by a variety of biological and chemical stressors.

Given that Reuteri and other truly efficacious probiotics function to enhance their hosts defense capabilities, their current classification as functional food components appears reasonable (213, 218). Historically, this classification can be seen as a convergence of two ancient concepts, namely, Metchnikoff's probiotic concept proclaimed approximately 100 years ago and Hippocrates vision recorded over 2,400 years ago:

> "Let food be your medicine and medicine your food." Whosoever gives these things no consideration and is ignorant of them, how can he understand the diseases of man." (Hippocrates, ca. 400 BC).

Hippocrates' vision has become today's paradigm for efforts to promote human health and wellbeing through increased consumption of healthy (functional) foods together with decreased consumption of unhealthy foods and dependency on pharmaceutical interventions (219, 220). And it is now enjoined by Metchnikoff's recommendation to 'absorb large quantities of useful microbes—'. It is important to also note that probiotic microbes are not the only microbial contributors to the 'functionality' of our foods. Lactic acid bacteria have been used throughout history to preserve virtually all commodity groups including dairy products, vegetables, fruits, meats, and cereals (221). During this century, it was shown that they enhance the nutritional value of food as well, for example, by increasing levels of vitamins and amino acids in some foods or by decreasing their mutagen content (222, 222a). It is also known that microbes play essential roles in gut ecosystems as providers of SCFAs and other microbe-

derived nutrients for development and maintenance of a healthy gut (4). And just during the past few decades, it was discovered that some species are sources of functional food ingredients per se.

Most notable among functional food components are the nonnutritive plant metabolites, termed phytochemicals (phyto-protectants) which are naturally present in relatively small amounts in most fruits and vegetables. Approximately 14 classes of dietary phytochemicals have recently been identified in common foods. Together with certain plant fibers (e.g., oat fiber), they have been targeted as functional food ingredients based on overwhelming epidemiological evidence linking reduced cancer risk, in particular, with increased consumption of fruits and vegetables containing these phytochemicals (223). However, plants are not the only sources of functional food ingredients. Certain substances found in meats and dairy products (we suggest the terms, corpro-protectants lactoprotectants, respectively) have also been targeted as functional food ingredients (230). The long chain n-3 polyunsaturated fatty acids found in marine plants and fish oils, for example, have been shown effective in preventing and treating cardiovascular disease, hyperlipidemia, thrombosis and embolic phenomena, and in modulating immune responses (224). Conjugated linoleic acid (CLA) is another chemoprotective fatty acid found in both animal tissues and milk. This potent substance is a microbial product. In ruminant animals, it is produced by a rumen bacterium, *Butryivibrio fibriosolvens*, as a metabolic intermediate in formation of other rumen-derived fatty acids by rumen bacteria (225). It has been reported that a strain of Reuteri isolated from rat intestine synthesizes linoleate isomerase, an enzyme that catalyzes conversion of linoleate to CLA (226, 227). Thus, Reuteri may prove to be an important source of *in situ* produced CLA in monogastric animals, including humans. CLA has been targeted as functional food ingredient, based on extensive animals model studies showing that it has anti-inflammatory properties and confers broad-spectrum chemoprotection against mammary tumors, colon cancer, and artherosclerosis (228, 229). Clearly, functional food components are derived directly and indirectly from microbial sources as well as from fruits, vegetables, grains and dairy products (Table 6).

There is yet another interesting perspective to be considered in our attempts to understand the health-enhancing effects of probiotics and functional foods. It derives from an integrated view of microbial pathogenesis and virulence recently proposed by Casadevall and Pirofski (231). They take both host and pathogen factors into account in analyzing the onset and progress of a microbe-induced disease. Included among the host factors are contributions of the indigenous microbiota, probiotics, and both the nutritional and functional aspects of the host diet. In their view, a microbe-induced disease occurs when the host sustains sufficient damage to perturb homeostasis, with damage being an inclusive term encompassing cell, tissue, and organ damage, mediated either by the pathogen, or the host, or both. They propose that host-pathogen interactions can be best analyzed using host damage as the common denominator for characterizing the importance of the host response to the outcome of the host-microbe interaction. Accordingly, the course and outcome of any particular disease is subject to modification by many factors, including the host's genetics, nutritional status, and status of its gastrointestinal microbiota, as well as the nature of the pathogen, its inoculum intensity, and its route of infection. The health-enhancing effects of probiotics and probiotic-containing functional foods appear more readily discernable from this perspective.

This brings us full circle to the primary focus of this present report. Namely, that a search for functional food ingredients to enhance human and animal health should not be limited to plant and animal sources. Microorganisms, their products, and their activities need to be included in this search (232, 233), particularly those important probiotic activities carried-out by species that reside in GI tracts in a symbiotic relationship with their human or animal hosts. As mentioned earlier and based on the recent explosion of citations (keyword $<$probiotics$>$) in the MEDLINE database of the USA National Library of Medicine (Figure 1), this inclusion is well underway. Metchnikoff's probiotic concept appears to be 'alive and well' and is being rapidly transformed into microbial products that will significantly enhance human and animal health. The authors have reviewed information indicating that *Lactobacillus reuteri* is the prototype species for future research and development of probiotic-containing functional foods.

XIII. Summary and Conclusions. Experimental and clinical studies cited in this report indicate that:

1. Reuteri is a symbiotic bacterial species well adapted to colonize human and animal GI tracts, with hosts' spanning the phylogenetic spectrum from avian to mammalian species, including humans.
2. Reuteri is unique among probiotic microorganisms in its ability to produce and secrete a metabolic intermediate (reuterin) capable of antagonizing pathogenic microorganisms.
3. Probiotic administrations of Reuteri have been shown to 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 protozoal diseases, (b) certain chemically-induced and stressor-induced diseases, and (c) hypercholesterolemia caused by a high fat diet.
4. Metchnikoff's probiotic concept has been validated, based on evidence of Reuteri's unique broad-spectrum,

probiotic efficacy derived from well-controlled laboratory experiments, field trials with animals, and clinical trials with human subjects.

5. Reuteri can exert biotherapeutic effects as well as prophylactic protection.
6. Reuteri can be grown on a large commercial scale, and methods have been developed to preserve its viability for extended periods of time, allowing for the production of Reuteri-containing functional foods for human and animal applications.

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