The role of limosilactobacillus reuteri strains on human health

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The Role of *Limosilactobacillus reuteri Strains* on Human Health

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Acronyms and Abbreviations

AhR	Aryl hydrocarbon receptor
ASD	Autism spectrum disorder
C-section	Cesarean section
CNS	Central nervous system
EPS	Extracellular polymeric substances
GI	Gastrointestinal
GMSCs	Gingiva mesenchymal stem cells
HMOs	Human milk oligosaccharides
ILCs	Innate lymphoid cells
MHFD	Maternal high fat diet
MSCs	Mesenchymal stem cells
MUBs	Mucus-binding proteins
NK cell	Natural killer cell
PPI	Proton-pump inhibitor
RANKL	Receptor activator of nuclear factor kappa beta ligand
RCT	Randomized control trial
TNFα	Tumor necrosis factor- α
Tregs	Regulatory T lymphocytes
3-HPA	3-Hydroxypropionaldehyde

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Abstract

In recent years the human gastrointestinal microbiota has proven to play a critical role in host health and disease. The majority of human microbiota resides in the large intestine and is composed of a diverse array of microorganisms, including bacteria, fungi, and archaea. Among these, *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri*), a gram-positive, heterofermentative lactic acid bacterium has gained attention as a probiotic due to its potential health benefits. Several strains of *L. reuteri* have been identified, each with its unique set of functional genes contributing to its probiotic effects. The beneficial properties include gastrointestinal microbiota modulation, pathogen colonization resistance, cobalamin synthesis, as well as positive endocrine, musculoskeletal, integumentary effects, anti-inflammatory, and neurodevelopmental effects.

Many chronic diseases have been associated with states of microbiota imbalance called dysbiosis. In contrast to the conventional pharmaceutical management of chronic diseases, probiotics such as *L. reuteri* could serve as important treatment options to rehabilitate perturbed microbial ecosystems and its functionally active metabolites, thus improving the safety and efficacy of current clinical practices. With more data gathered, *L. reuteri* could become a valuable tool for treating chronic conditions and restoring human health.

Key words: *Limosilactobacillus reuteri*, probiotic, gastrointestinal microbiota, dysbiosis, treatment

Sažetak

Posljednjih godina istraživanja su pokazala da mikrobiota probavnog trakta igra ključnu ulogu u zdravlju i bolesti ljudi. Većina ljudske mikrobiote nalazi se u debelom crijevu i sastoji se od raznolikog niza mikroorganizama, uključujući bakterije, gljivice, i arheje. Među njima se ističe gram-pozitivna, heterofermentativna bakterija mliječne kiseline *Limosilactobacilllus reuteri* (ranije poznata kao *Lactobacillus reuteri*) koja je privukla pozornost kao probiotik zbog svojih potencijalnih zdravstvenih koristi. Identificirano je nekoliko sojeva *L. reuteri*, koji svaki sa svojim jedinstvenim skupom funkcionalnih gena pridonose specifičnim probiotičkim učincima. Tu spadaju modulacija gastrointestinalne mikrobiote, otpornost na kolonizaciju patogena, sinteza kobalamina te pozitivni učinci na endokrini, mišićno-koštani, pokrovni i imuni sustav te na neurorazvoj.

Mnoge kronične bolesti povezuju se sa stanjima neravnoteže mikrobiote koja se nazivaju disbiozom. Za razliku od konvencionalnog farmaceutskog liječenja kroničnih bolesti, probiotici poput *L. reuteri* mogli bi poslužiti kao važne terapijske opcije u oporavku narušenih mikrobnih ekosustava i njegovih funkcionalno aktivnih metabolita te tako unaprijediti sigurnost i učinkovitost trenutnih kliničkih praksi. S više prikupljenih podataka, *L. reuteri* bi mogao postati vrijedan alat za liječenje kroničnih stanja kao i za unapređenje ljudskog zdravlja.

Ključne riječi: *Limosilactobacillus reuteri*, probiotik, crijevna mikrobiota, disbioza, liječenje

1. Introduction

The World Health Organization defines probiotics as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (1). *Lactobacillus reuteri*, recently renamed *Limosilactobacillus reuteri (L. reuteri)*, is a well-studied microorganism known to induce numerous probiotic effects in its host. In addition, *L. reuteri* has fulfilled the necessary safety and efficacy criteria to be termed probiotic. These criteria include the capacity to survive in the gastrointestinal (GI) tract, a high resistance to gastric acids, the lack of any transferable antibiotic resistance genes, and the capacity to exert clear benefits to the host (2). Multiple studies have demonstrated *L. reuteri* supplementation to be safe, even at doses of as high as $2{,}9 \times 10^9$ colony-forming units (CFU)/day, in adults, children, infants, and even in the HIV-infected demographic (3–7).

L. reuteri has been shown to be an indigenous member of the human microbiota (8) and is found in multiple body sites, including the gastrointestinal tract, urinary tract, skin, and breast milk (9). However this beneficial species is becoming exceedingly rare in humans (10), probably due to the microbiome disrupting factors such as a low fiber diet, ultra-processed foods, antibiotics, oral contraceptives, etc. Although microbiota are not genetically transmitted, they still follow a hereditary pattern since offspring microbiota are maternally derived via vaginal delivery amongst other mechanisms. In this manner the loss of microbial diversity could be compounded over subsequent generations (11).

The human body is actually a super-organism composed of an approximately 1:1 ratio of microbial cells and human cells (12) with over 99% of the unique genes in human genome are bacterial and less than 1% are human (13). The emerging fields of metagenomics, meta-transcriptomics, and metabolomics are beginning to elucidate the intimacies between probiotic organisms and host physiology. A central understanding in the area of microbiota research is that prebiotics are metabolized by probiotics to yield postbiotics. Postbiotics are defined as “soluble factors, metabolic products or by-products, secreted by live bacteria, or released after bacterial lysis providing physiological benefits to the host” (14). For example, 90% of serotonin and 50% of dopamine is produced in the gastrointestinal tract by probiotic organisms (15).

In the past, microbiota research has yielded inconsistent results. This is largely attributed to the failure to identify microorganisms on the strain level. Therefore, current probiotic research ensures to differentiate microbes on a strain level. The aim of this review will be to amalgamate the beneficial effects of various *L. reuteri* strains on human physiology, by examining both animal and human studies.

2. *Limosilactobacillus reuteri* Probiotic Characteristics

2.1 Gastrointestinal Tract Adherence and Colonization

In addition to the commonly known functions of the gastrointestinal tract, such as digestion of food and absorption of nutrients, the gastrointestinal tract also has the function of killing pathogenic microbes entering with food. These mechanisms include low pH of the stomach and the action of bile salts in the proximal small bowel. Multiple strains of *L. reuteri* were found to be resistant to acidity and bile salts. Researches attributed this to *L. reuteri's* ability to form biofilms (16), as well as, a gene cluster for cytoplasmic urease enzyme (17). *H. pylori* uses cytoplasmic urease to convert urea into carbon dioxide and ammonia. Ammonia neutralizes gastric acid upon entering the outer bacterial membrane, thus averting acidification at the inner membrane (18). The presence of cytoplasmic urease in the genome of *L. reuteri* is believed to serve the same function.

The majority of commercial probiotics on the market today exert a transient effect. Meaning they are unable to adhere and colonize the gastrointestinal tract and hence their presence in host microbiota is transitory (19). Specific strains of *L. reuteri* exhibit colonization effect in specific vertebrates, including humans (20). It is believed that microbe host adherence is achieved through bacterial surface proteins binding elements present in host mucus layer. The *L. reuteri* genome contains genes coding for clusters of orthologous protein adherence mediators, or so-called adhesins, also called mucus-binding proteins (MUBs) and MUB-like proteins (21). These bacterial proteins bind elements in mucus secreted by enterocytes of the gastrointestinal tract, facilitating the first stage of colonization, adherence.

Due to the anatomy and physiology of the gastrointestinal tract, different parts of the GI tract have different environments in terms of acidity, bile acids, digestive enzymes, oxygen availability, epithelia types, and competitive effect of native host microbiota. Due to these topographical

differences, individual microbiota have evolved to colonize their regional niche. When examining the microbiota topographically, *L. reuteri* is found in the proximal digestive tract of the host (22). In fact, host GI epithelia are able to differentiate between strains of the same microbial species, further supporting the notion that vertebrates and their microbial symbionts are highly coevolved and exclusive. Once a microbe has successfully adhered to host mucus, biofilm formation ensues, ensuring the stability of the colony.

2.2 Biofilm Activity

A biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (23).

The biofilm formation activity of *L. reuteri* has been studied in a variety of contexts, including in the gut, on dental surfaces, and on medical devices. The ability of *L. reuteri* to form biofilms on dental surfaces was studied and the researchers found that *L. reuteri* was able to adhere to dental surfaces and form a biofilm, which was characterized by the presence of EPS and the ability to inhibit the growth of pathogenic bacteria (24).

On a mechanistic level, *L. reuteri* produces several proteins and enzymes that are involved in biofilm formation, including aggregation substance, cell surface proteins, and extracellular surface protein. These proteins and enzymes work together to initiate the production of EPS and to form the mature biofilm structure (25).

Recently, *L. reuteri* was delivered as a biofilm on microsphere and such delivery was found to promote the adherence of *L. reuteri* to intestinal epithelium and enhance its probiotic property (26,27). This system of using a probiotic organism's endogenously produced biofilm as a capsule in order to better survive hostile host conditions, mainly acidity and bile acids, has been termed fourth generation probiotics (16). When compared to non-bond (planktonic) state probiotics, biofilm delivered probiotics have demonstrated significantly more pronounced short and long term probiotic effects in animal models (28).

Besides *L. reuteri's* ability to form its own biofilm, it also has the ability to infiltrate biofilms of other bacterial species. In one study regarding vaginal flora, uropathogenic *Escherichia coli* enveloped in a mature biofilm was exposed to *L. reuteri* RC-14. The study reported that *L. reuteri* was able to first integrate its own biofilm into the pre-existing *E. coli* biofilm, then penetrate to inner *E. coli* colony, followed by significantly reducing *E. coli* bacterial counts inside the biofilm (29). This antimicrobial activity of *L. reuteri* has been well documented and is attributed to its metabolite production profile. A particularly potent antimicrobial compound secreted from *L. reuteri* is called reuterin.

2.3 Production of Bacteriocins

Bacteriocins are proteinaceous or peptic toxins produced by bacteria in order to inhibit the growth of select microorganisms. Reuterin (3-hydroxypropionaldehyde, 3-HPA, is an organic compound containing both hydroxy and aldehyde functional groups exhibiting a wide range of antimicrobial activity against foodborne pathogens and spoilage microorganisms (Figure 1) (30).

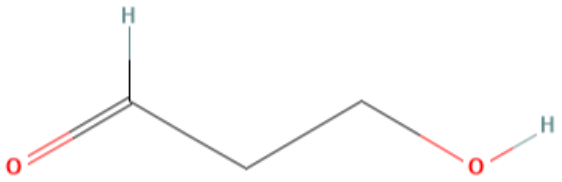

Figure 1. Chemical Structure of Reuterin *(31)*

The name reuterin is derived from *L. reuteri*, which produces reuterin biosynthetically from glycerol as a broad-spectrum antibiotic (bacteriocin) (32). Reuterin is an intermediate in the metabolism of glycerol to 1,3-propanediol catalysed by a coenzyme B12-dependent diol dehydrase (32).

In terms of anti-microbial spectrum, reuterin was found to kill a range of enteropathogenic organisms including yeasts, fungi, protozoa and viruses, without exerting effect on commensal gastrointestinal flora species (33). One study found that gram negative bacteria were especially susceptible to reuterin (33). Another study found that reuterin extracts were effective in decontaminating meat contaminated with of *E. coli* O157:H7 MRK 1452 and *L. monocytogenes* ST121 (34). The antimicrobial activity of reuterin is at least in part attributed to the spontaneous

conversion of 3-HPA to acrolein, a cytotoxic electrophile (35). The addition of lactic acid was found to synergistically increase this bacteriolytic effect (34). In addition to producing reuterin, *L. reuteri* is known to produce lactic acid, acetic acid, ethanol, reutericin, and reutericyclin, which also have proven antimicrobial and bacteriocin effects (36,37).

2.4 Activity Against *Helicobacter pylori*

Helicobacter pylori is a gram-negative, microaerophilic, spiral (helical) bacterium commonly found in the stomach. It was estimated that over 50% of the world's population will have *H. pylori* in their upper gastrointestinal tracts at some point throughout their lives, being more common in developing countries (38). Due to this exceedingly high prevalence of *H. pylori* epidemiologically speaking, researchers have investigated if *H. pylori* can also function as a commensal member of the stomach/ duodenum flora. One study found that non-pathogenic strains of *H. pylori* improve stomach acid secretion and satiety signalling (39), another found *H. pylori* to regulate the colonization of other stomach flora (40). However, for those colonized with pathogenic strains of *H. pylori*, the term infection not colonization is more appropriate.

Pathogenic *H. pylori* infection can result in chronic gastritis, peptic ulcers, and malignancy. There are multiple pathophysiological mechanisms involved in this process, these include adaption to acidity, mucus degradation, host immune response, and cytotoxin-associated gene A driven malignancy (41).

L. reuteri has demonstrated promising anti-helicobacter activity. Three RCTs of adult patients infected with *H. pylori*, encompassing 73 patients, determined that *L. reuteri* DSM 17648 alone significantly reduced *H. pylori* load in patients and helped alleviated mild symptoms of dyspepsia with a favorable safety profile (42–44). Additionally, when combined with a PPI *L. reuteri* DSMZ 17648 has been shown to have similar efficacy to standard triple therapy (pantoprazole, clarithromycin and amoxicillin or metronidazole) in the eradication of *H. pylori*, 65.22% and 73.91% respectively (45). Another RCT showed that when *L. reuteri* was given during seven day triple therapy vs. just triple therapy, eradication rate increased from 66% to 88% (46). One meta-analysis published in 2020 (47) encompassing 40 studies and 5792 patients concluded that *L. reuteri* probiotic supplementation in addition to triple or quadruple therapy improved the eradication rate by approximately 10% relative to the control groups. Additionally, they found a

44% reduction rate in the incidence of adverse reactions. Similarly, a 2019 meta-analysis (48) examining 11 RCTs and 724 patients found that adjuvant *L. reuteri* supplementation increased eradication rate by an average of 16% compared to controls only receiving triple or quadruple therapy. They also found adjuvant *L. reuteri* supplementation reduced the incidence of taste disorders by 64%. Table 1 summarizes the effectiveness of *L. reuteri* supplementation in the treatment of *H. pylori* infection as sole therapy and as adjuvant therapy up to the year 2021.

Table 1. Clinical Efficacy of *L. reuteri* Strains as Sole Therapy and as Adjuvant Therapy Against *H. pylori* Infection

Strain	**Treatment**	**Subjects**	**Result**	**Reference**
ATCC 55730	10 days *L. reuteri*	Children (Symptomatic)	Improvement of GI symptoms	(50)
DSM 17938	20 days triple therapy + *L. reuteri*	Adults (Symptomatic)	93.3 % eradication rate	(51)
DSM 17648	14 days *L. reuteri*	Adults (Symptomatic)	Significant decrease of pathogen load in stomach	(42)
SD2112	4 weeks *L. reuteri*	Adults (Symptomatic)	Significant decrease of urea breath test and pathogen density	(52)
DSM 17938	8 weeks *L. reuteri* + pantoprazole twice daily	Adults (Symptomatic)	13.6% eradication rate	(53)
DSM 17938, ATCC PTA 6475	28 days *L. reuteri* then 7 days *L. reuteri* + triple therapy	Adults (Symptomatic)	13% eradication rate with *L. reuteri* alone. 75% eradication rate with *L. reuteri* + triple therapy	(54)

DSMZ17648	14 days *L. reuteri*	Adults (asymptomatic colonization)	Reduced *H. pylori* load in asymptomatic carriers	(43)
DSM 17648	4 weeks *L. reuteri* +triple therapy	Adults (Symptomatic)	82.69% eradication rate	(55)
DSM 17648	4 weeks *L. reuteri* + triple therapy	Adults (Symptomatic)	86.2% eradication rate, Improved GI symptoms	(56)
DSM 17648	28 days *L. reuteri*	Adults (Symptomatic)	22.5% reduction in *H. pylori* load measured by urea breath test	(44)
DSMZ17648	28 days *L. reuteri* *+/- eradication therapy* (omeprazole + amoxicillin + metronidazole + bismuth for 10 days)	Adults (Symptomatic)	50% eradication rate with *L. reuteri,* 60% eradication rate with *L. reuteri* + eradication therapy	(57)
DSMZ17648	8 weeks *L. reuteri* + Pantoprazole	Adults (Symptomatic)	65.22% eradication rate	(45)
DSMZ17648,	14 days *L. reuteri* + liquorice and calcium carbonate	Adults (Symptomatic)	54.3% eradication rate	(58)

There are multiple proposed mechanisms by which probiotics decreases *H. pylori* bacterial load (Figure 2). Firstly, probiotics have been shown to improve GI barrier function, specifically by upregulating tight junctions, promoting mucus secretion, and increasing growth factors (59). The GI epithelium and associated mucus are integral parts of the innate defence against pathogenic bacteria entering through the GI tract. Second, probiotics reduce the host inflammatory response caused *by H. pylori* infection (60). The sustained expression of pro-inflammatory factors, such as IL-8, TNF-α, by *H. pylori* can lead to a chronic inflammatory response which is a key pathophysiological step in the manifestation of *H. pylori* gastritis. Probiotics have been shown to

mitigate this inflammatory response by inhibiting pro-inflammatory pathways such as NF-κB. Third, as previously mentioned, *L. reuteri* is known to produce a wide range of antimicrobial substances, such as lactic acid, acetic acid, ethanol, reutericin, and reutericyclin, which have been shown to effective in inhibiting a wide range of pathogenic organisms (33). This likely suppresses *H. pylori* through direct toxicity, as well as, indirectly with changes in local pH. Furthermore, *L. reuteri* has been shown to interfere with *H. pylori* gastric colonization (61). This is achieved by competitive binding to adhesion receptors of *H. pylori*, specifically two glycolipids found on gastric epithelium called gangliotetraosylceramide and sulfatide. Without pathogen adhesion, it is far less likely to manifest a clinical picture of disease. Similarly, *L. reuteri* DSM17648 has shown to the unique ability to effectively chelate *H. pylori* (44). This is achieved by specifically binding to *H. pylori* surface proteins to form *reuteri/pylori* co-aggregates. The aggregated *H. pylori* are not capable of adhering to gastric mucosa and have significantly reduced motility. As a result, *H. pylori* co-aggregates are subject to peristaltic contractions and are enterically excreted. The *L. reuteri* DSM17648 strain is currently available on the market for reducing the load of *H. pylori* in the stomach.

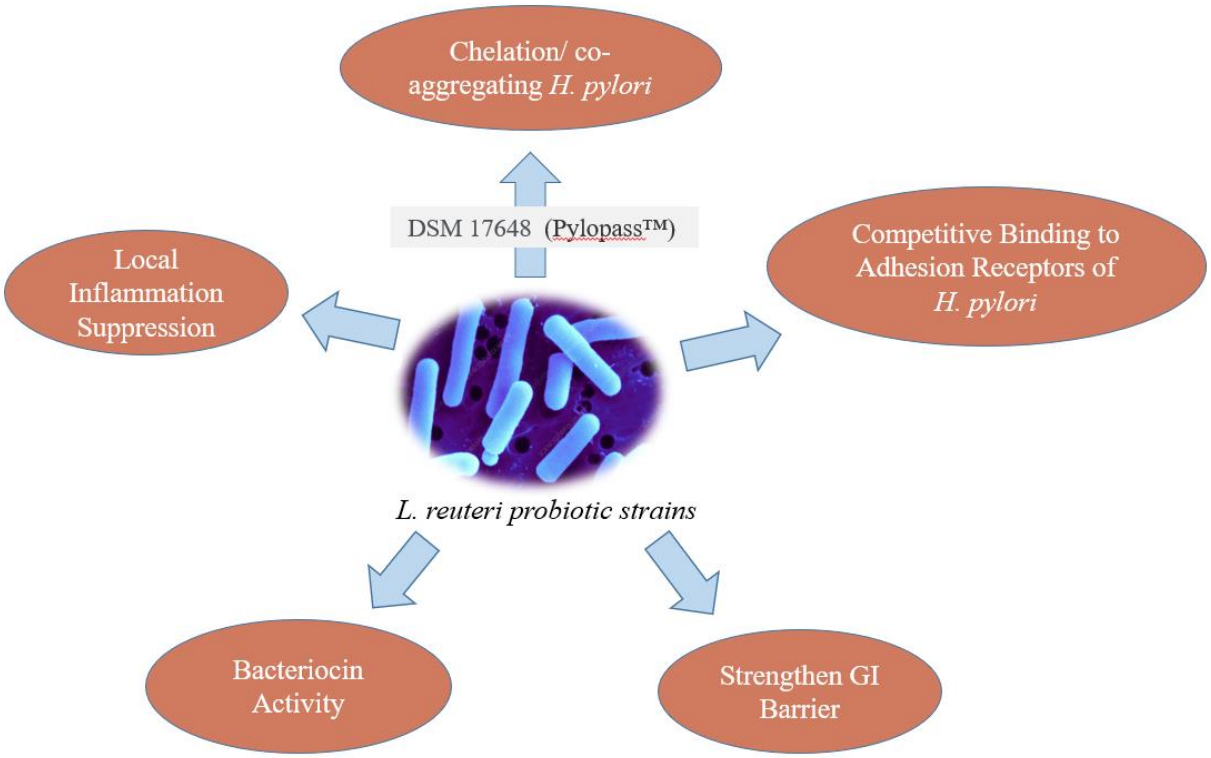

Figure 2. Schematic Diagram of *L. reuteri* Probiotic Mechanisms of Action Against *H. pylori*

Additionally, *L. reuteri* treated individuals have shown a marked decrease in antibiotic-associated adverse effects. One meta-analysis concluded that *L. reuteri* supplementation during triple therapy decreased antibiotic-associated side effects by 20–30% compared to controls only receiving triple therapy (62). Antibiotic-associated adverse effects encompassed diarrhoea, abdominal pain, nausea, taste disturbances, vomiting, and constipation.

2.5 Effect on Accelerated Wound Healing

The four classical stages of wound healing are haemostasis, inflammation, proliferation, and remodelling. Direct topical application of probiotics and their products in skin wounds has been shown to promote wound healing (63–65). This local effect primarily relies on the antibiotic properties of probiotics and their interaction with local inflammatory cells and proliferating epidermis. Considering this, researchers examined if orally consumed probiotics residing in the gut could also exert wound healing properties in distant tissue such as skin.

In one mouse study (66), researchers evaluated the healing properties of orally consumed live *L. reuteri* ATCC-PTA-6475. A standardized 2.0-millimeter full thickness dorsal skin excision was made, and then the wound was microscopically examined at three, six and twelve days after excision. Mice orally receiving *L. reuteri* exhibited complete epidermal closure in half the time required for matched control animals. When examining wound area, female mice consuming *L. reuteri* experienced a more rapid increase in healing compared to male mice. In the *L. reuteri* group, further examination of the wound bed showed accelerated maturation of granulation tissue, accelerated collagen deposition and significantly increased numbers of regulatory T lymphocytes (Tregs). Tregs are a specialized subpopulation of T cells that down-regulate host inflammatory responses and have been shown to prevent excessive inflammation surrounding tissue wounds (67). To further determine the significance of this increase in Tregs, researchers transferred purified Treg cells originating from *L. reuteri*-supplemented donor mice into mice genetically lacking T and B lymphocytes. The transferred Treg cells were sufficient to recapitulate the rapid cutaneous wound healing in the hosts.

Additionally, significantly increased plasma levels of oxytocin, known to be required in the normal mammalian wound healing processes (68–70), were demonstrated in *L. reuteri* dietary

supplemented mice. Intriguingly, *L. reuteri* supplemented mice who were subject to vagotomy did not show elevated plasma oxytocin nor accelerated wound healing. Therefore, the oxytocin-driven acceleration of wound healing is vagus nerve dependent. Researchers concluded that *L. reuteri's* presence in the GI tract influences the immune system and oxytocin levels to exert the systemic effect of accelerated skin wound healing.

Another similar study in mice (71) evaluated the wound healing effects of topical *L. reuteri* extracts. All mice were subjected to a full thickness wound 1mm x 2mm in the area located on the mesial gingiva of the first maxillary molar. Then mice were randomly distributed into to two groups: local injection of 0.9% sodium chloride (control group) or local injection of *L. reuteri* extracts. The goal was to determine whether bacterial extracts could regulate the functions of gingiva MSCs (GMSCs) and thus promote wound healing. The authors discovered that local injection of *L. reuteri* extracts increased the proliferation of gingival MSCs, as well as, their capacities of migration, expression of stem cell markers and osteogenic differentiation. Accelerated wound healing was evident histologically and macroscopically. Immunohistochemistry staining indicated that *L. reuteri* extracts promoted GMSCs migration via PI3K/AKT/β-catenin/TGFβ1 pathway, thus accelerating wound healing.

2.6 Influence on Integumentary Health

It is well established that skin and mucosal surfaces of mammals are colonized by millions of bacteria (72). Mechanistically, microbes and their metabolites interact with immunological (73), metabolic (74), and neuroendocrine pathways (75) that modify stress-related responses in the skin through a gut-brain-skin axis (76). Mouse and human studies suggest that supplementation with probiotic bacteria has many beneficial effects on the integumentary system (63,77–80).

A study in mice investigated the integumentary effects of *L. reuteri* ATCC 6475 yogurt supplementation (81). After 24 weeks, mice showed a significant increase in dermal thickness when compared to controls. This was histologically confirmed to be attributed to additional collagen and subcutaneous fat deposition. Furthermore, probiotic-fed mice expressed an average of 12 hair follicles to every control group rat's hair follicle (a 1,200% increase), thus increasing fur density. When examining the hair cycle stages of probiotic-fed mice, a dramatic anagenic shift was found. Controls demonstrated 36% anagen phase while probiotic group showed 70% anagen

phase hair follicle cycle distribution. Additionally, when measuring the pH of the skin, oral cavity, rectum, and vagina, all mucocutaneous surfaces were slightly more acidic particularly in female mice.

These microscopic changes resulted in visibly thicker and shinier fur. Researchers measured hair luster by reflectometry and found a 100% increase in light reflectivity. The experiment was repeated comparing *L. reuteri* yogurt vs *L. reuteri* drinking water vs controls in order to rule out any confounding factors present in the yogurt (82). The results demonstrated increases in dermal thickness, folliculogenesis, sebocytogenesis, anagenic follicular shift, and fur luster in both the yogurt and drinking water groups when compared to controls. Therefore, these integumentary effects observed were attributed to *L. reuteri* dietary supplementation. The health conveying phenotype demonstrated in *L. reuteri* supplemented mice has been coined "glow of health."

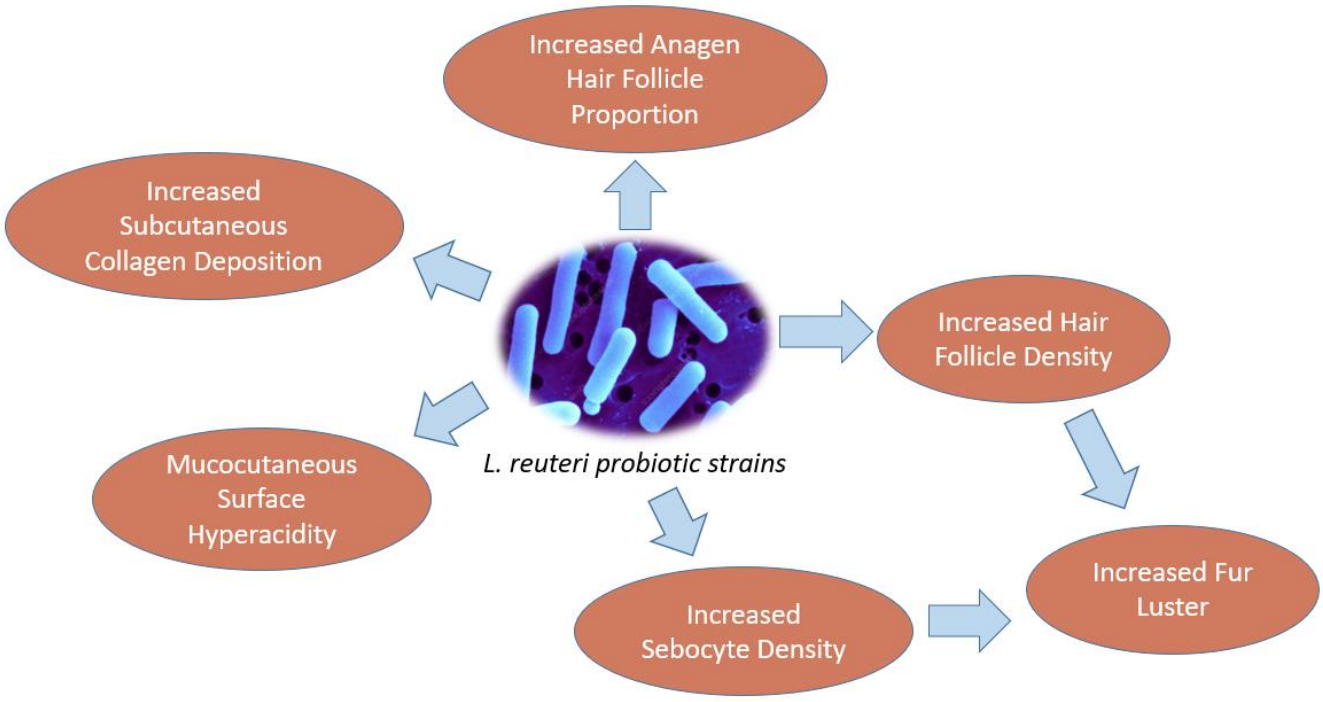

Figure 3. *L. reuteri* Effect on Integumentary Health

2.7 Testicular Health

One mouse study found that oral ingestion of *L. reuteri* ATCC 6475 over one year increased testicular weight nearly 40% versus control groups (83). Histological examination revealed that this increase in total testicular weight was due to increased seminiferous tubule cross sectional area and total germ cell volume. Likewise, Leydig cell density and individual Leydig cell volume was significantly increased. Given that the Leydig cell is the main cellular source of testosterone, researchers measured the serum testosterone after 5 months of *L. reuteri* supplementation. A 500% increase in serum testosterone was found. No analogous studies have been conducted in humans. Researchers observed the same testicular-increasing effects of *L. reuteri* when they administered an IL-17 antibody. This suggests that *L. reuteri's* effects are mediated through increasing IL-10, thus decreasing IL-17 levels.

2.8 Effects on Immune Function

Multiple studies have shown that *L. reuteri* induces anti-inflammatory Treg cells (84–86). It is believed that *L. reuteri's* ability to promote Tregs is a key contributor to the myriad of beneficial effects of *L. reuteri* demonstrated in both diseased and non-diseased conditions. The Treg-inducing property of *L. reuteri* is strain-dependent. However, the immunomodulatory effects of *L. reuteri* are not solely mediated by Treg induction, as *L. reuteri* suppressed Th1/Th2 proinflammatory responses in Treg-deficient mice (87). Also, specific *L. reuteri* strains can reduce the production of many pro-inflammatory cytokines. For instance, *L. reuteri* GMNL-263 can reduce serum MCP-1, TNFα, and IL-6 levels in mice fed with high fat diet (88). In fact, the immunomodulatory effects of *L. reuteri* appear to rely on its metabolites. One mouse study found that the *L. reuteri* BM36301 culture supernatant alone reduced TNFα production from human myeloid THP-1 cells (89).

Interestingly, when *L. reuteri* catabolizes tryptophan, the metabolite, indole-3-aldehyde, acts as an agonistic ligand for the aryl hydrocarbon receptor (AhR). The AhR is expressed on cells of the innate immune system, specifically macrophages, dendritic cells, NK cells, B lymphocytes and certain subtypes of T cells as Th17 and Treg cells (90). One mouse study found that activating the AhR upregulates IL-22 production from innate lymphoid cells (ILCs) (91). This upregulation in IL-22 was shown to induce immune tolerance to known probiotic species, while also increasing

colonization resistance to pathobionts such as *Candida albicans*. Therefore, it is postulated that this form of probiotic metabolite – host immune system interaction has evolved as a mechanism to ensure selective commensalism of the host.

Secretory IgA is an antibody component of the innate immune system, specifically, produced by mucus membranes of gastrointestinal, respiratory, and urogenital tracts (92). One mouse study found that dietary supplementation of *L. reuteri* increased free secretory IgA levels (93). However, this secretory IgA upregulation was not evident in vitamin A-deficient rats, suggesting a vitamin A-dependent mechanism. It is currently unclear how exactly *L. reuteri* induces B cells to upregulate IgA production.

One randomized control study followed 262 employees at a company in Sweden for 80 days (94). The goal was to measure the amount of total sick-leave caused by respiratory or gastrointestinal infections, between the probiotic supplementing and control groups. The authors found the probiotic group *L. reuteri* ATCC 55730 decreased the number of reported sick days from 26.4% (controls) to 10.6% (probiotic) over the course of the study. This clinically demonstrated *L. reuteri'*s immune system augmenting effect.

2.9 Oral Health Benefits

After the gut, the oral cavity has the second most diverse microbiota community consisting of over 700 species of bacteria (95). Oral dysbiosis is a risk factor in the development of caries, periodontal disease, halitosis, and cardiovascular disease (96–98). A major etiological factor in the development of caries is the dysbiotic proliferation of a bacteria called *Streptococcus mutans* (99). This bacteria thrives by fermenting dietary sugars, leading to a reduction in pH levels that leads to tooth demineralization (100).

One RCT examining the oral health effects of *L. reuteri* ATCC 55730 in children found the prevalence of caries was significantly lower in the probiotic group (0.67 ± 1.61) than controls (1.53 ± 2.64) (101). Additionally, the probiotic group had a lower incidence and severity of gingivitis compared to controls. Similarly, an *in vitro* study accessed the effect of *L. reuteri* ATCC 55730 on *S. mutans* (102). An inverse relationship was found between the relative abundance of *L. reuteri*

and *S. mutans*, suggesting that the presence of *L. reuteri* has an inhibitory effect on *S. mutans* colonization.

Another RCT examined the effect of *L. reuteri* ATCC PTA 5289 & DSM 17938 dual strain supplementation on residual pocket depth in periodontitis patients (103). After twenty-four weeks, the overall probing pocket depth in the probiotic lozenges group (2.64 ± 0.33 mm) was significantly lower compared to the control lozenges (2.92 ± 0.42 mm). This difference was even more pronounced in moderate (4-6 mm) and deep (≥7 mm) pockets. A similar study with chronic periodontitis patients found that after twelve weeks of oral *L. reuteri* ATCC PTA 5289 & DSM 17938 dual strain supplementation, pocket depth was significantly reduced. Researchers also reported a reduction in the population density of *Porphyromonas gingivalis*, a bacteria implicated with periodontal disease (104).

2.10 Influence on Bone Health

Estrogen deficiency is a major risk factor for osteoporosis that is associated with bone inflammation and resorption (105). Mouse studies have shown dietary supplementation of *L. reuteri* ATCC PTA 6475 significantly suppresses bone loss associated with estrogen deficiency in ovariectomized mice (106). Osteoclast bone resorption markers and activators (Trap5 and RANKL), as well as, osteoclastogenesis are significantly decreased in *L. reuteri*-supplemented mice.

An analogous RCT conducted in postmenopausal women (107) assessed tibial bone mineral density in *L. reuteri* group vs placebo, in both pre-existing osteoporosis patients and healthy patients after twelve months of treatment. In the osteoporosis comparison, the *L. reuteri* group had a 1,02% mean average reduction in bone loss (−0,83% vs −1,85%). Similarly in the non-osteoporosis comparison, the *L. reuteri* group showed a 0,93% mean average reduction (−0,93% vs −1,86%). Hence, both *L. reuteri* and placebo group experienced a reduction in bone mineral density, however this loss of density was significantly less in the *L. reuteri* group in both osteoporosis patients and non-osteoporosis patients. Researchers concluded that *L. reuteri* 6475 should be further explored as a novel approach to prevent age-associated bone loss and osteoporosis.

2.11 Vitamin Synthesis

The human genome is not capable of synthesizing vitamins required for it's cellular physiology (108). Therefore, thirteen vitamins have been termed essential because they must be exogenously sourced. It is common knowledge that many of the essential vitamins can be attained through a variety of dietary sources, however some vitamins are also actively produced by gut microorganisms as metabolites. This can be achieved symbiotically by having select vitamin producing bacteria colonize the host GI tract, or entirely exogenously by consuming fermented foods. Thus, fermented foods have been called bifunctional foods since they already contain the liberation of microbial metabolites (bioactive effect) (109).

Currently, *L. reuteri* strains CRL1098, JCM1112, DSM 20016, and ZJ03 have demonstrated the ability to produce vitamin B12 (cobalamin) (110–112). Genome analysis of *L. reuteri* has identified a cluster of cobalamin biosynthesis genes, specifically coding for the CobA, CbiJ, and CbiK enzymes (110). It is now understood that *L. reuteri* requires cobalamin in the biosynthesis of reuterin. Specifically, the conversion of 3-hydroxypropionaldehyde to reuterin is catalyzed by an enzyme called 3-hydroxypropionaldehyde reductase, which requires cobalamin as a cofactor to function properly. Therefore, all strains of *L. reuteri* which produce reuterin also produce cobalamin. In one mouse study, the administration of *L. reuteri* CRL1098 together with a diet lacking vitamin B12 was shown to ameliorate pathologies in B12-deficient pregnant female mice and their offspring (113). In addition to B12, *L. reuteri* strains 6475 and JCM1112 have been found to synthesize vitamin B9 (folate) (111,114).

2.12 Microbiota Modulation

Numerous metabolic, autoimmune, endocrine, neuropsychiatric, and oncological conditions have been associated with perturbances in the microbiota (115). This heterogeneous group of microbiota imbalances have collectedly been labelled dysbiosis. Hallmarks of dysbiosis include alteration in microbial composition (bacterial, archaea, fungi), metabolome, anatomical microbe localization, reduced diversity, opportunistic shift, and pathogen colonization (116–119). Studies have demonstrated disease specific gut microbiota signatures on the metagenomic, meta-transcriptomic and metabolomic levels of various chronic diseases such as inflammation bowel disease, colorectal cancer, obesity, type 2 diabetes, atherosclerosis, and allergy (120–123).

Keystone taxa are highly connected microbial taxa that individually or in a guild exert a considerable influence on microbiota structure and functioning irrespective of their abundance (124). *L. reuteri* has shown significant evidence to be considered a keystone taxa, as it is able to influence the diversity, composition, and metabolic function of gut microbiota in mice, piglets, and humans.

One study assessed oral administration of *L. reuteri* DSM17938 to mice, which were induced to have gut microbial dysbiosis due to FOXP3 gene mutation. FOXP3 gene mutation impairs functioning of Treg cells, manifestations of this include multiorgan inflammation, which in humans is called "immune dysregulation, polyendocrinopathy, enteropathy with X-linked inheritance" (or IPEX syndrome) (125). The results demonstrated that *L. reuteri* was able to prolong the lifespan of these mice and reduce multi-organ inflammation while remodelling the gut microbiota (126). The disease-ameliorating effect of *L. reuteri* was attributed to the remodelled gut microbiota, notably increased phylum Firmicutes and the genera *Lactobacillus* and *Oscillospira*. The study also demonstrated that *L. reuteri* supplementation resulted in inosine production (postbiotic effect). Through adenosine A2A receptor engagement, inosine can reduce Th1/Th2 cell responses and their associated pro-inflammatory cytokines. These results suggest that the *L. reuteri* – gut microbiota – inosine – adenosine A2A receptor axis could serve as a potential therapeutic method for Treg-deficient disorders. Likewise, in mice studies *L. reuteri* C10-2-1 has been shown to increase microbiota alpha diversity (93,106). Alpha diversity is a term used to describe the diversity, in terms of taxa number, within a single sample. High alpha diversity, i.e. high number of different taxa, with regard to the gut microbiome, is emerging as an important indicator of health (127–130).

A study in piglets examining the microbiota modulating effects of *L. reuteri* ZLR003 supplementation found increased alpha diversity when compared to the antibiotic-treated and control groups. Specifically noteworthy, the *L. reuteri* group showed significant microbiota shift in the jejunum with regard to asserting Proteobacteria as the dominant phylum (131). In another piglet study, the authors found that three weeks of *L. reuteri* TMW1.656 fermented fed supplementation increased alpha diversity, especially in the Firmicutes phyla, while reducing the abundance of *Enterobacteriaceae*, when compared to controls (132). Researchers concluded that

the microbiota modulating effect of this strain was due to its ability to produce the bacteriocin reutericyclin.

2.13 Effects of *L. reuteri* on Infant Health

There are many reports demonstrating beneficial effects of *L. reuteri* on different aspects of infant health, from infant colic, acute infectious gastroenteritis to general microbiota modulation leading to prevention of atopic phenotypes.

L. reuteri is an effective treatment against infant colic. One meta-analysis encompassing four double-blind RCTs involving 345 infants with colic (174 probiotic and 171 placebo) found that *L. reuteri* DSM17938 supplementation reduced crying and/or fussing time by 25% after 21 days when compared to controls (133). Similarly, the probiotic group was almost twice as likely as the control group to completely resolve colic at all time points. This significant treatment effect was only found in breast fed infants and was absent in formula fed infants. Therefore, it is speculated that a prebiotic nutrient in human breast milk, likely human milk oligosaccharide (HMOs), provides the microbial substrate to enable microbial symbiotic effects (134).

L. reuteri has been shown to significantly reduce symptom duration of acute infectious diarrhoea in children. One RCT of children (6-36 months of age) hospitalized for watery diarrhoea studied the effect of *L. reuteri* DSM 17938 on symptom duration. On day two and three of treatment, watery diarrhoea persisted in 82% and 74% of the placebo and 55% and 45% of the *L. reuteri* recipients respectively (135). Hence, the probiotic group saw expedited infectious recovery. An analogous study also found that application of *L. reuteri* DSM 17938 reduced the duration of acute diarrhoea in hospitalized children. The prevalence of diarrhoea-free children, *L. reuteri* vs controls, after 24 hours was 50% versus 5% and after 72 hours 69% versus 11% (136). One systematic review concluded *L. reuteri* DSM 17938 oral administration reduces the duration of diarrhoea and increases the likelihood of cure. In preventive settings, *L. reuteri* has the potential to reduce the risk of community-acquired diarrhoea in otherwise healthy children (137).

Additionally, there are reports showing differences in infants' microbiota depending on the type of birth. Compared to vaginally delivered infants, Cesarean (C)-section delivered infants display a dysbiotic GI microbiota, specifically microbiota with less probiotic *Lactobacillus* and

Bifidobacterium species, and a higher relative abundance of *Enterococcus*, *Enterobacter*, and *Clostridium* species (138–140).

One study found that supplementing C-section babies with *L. reuteri* DSM 17938 from 2 weeks to 4 months of age shifted the gut microbiota composition toward the distribution pattern found in vaginally delivered infants (138). Also noteworthy, as long as the mother's GI tract is colonized with *L. reuteri*, *L. reuteri* will be present in her breast milk (10). This gastrointestinal to mammary duct translocation was shown to occur due to dendritic cells sampling bacteria directly from the gut lumen by creating openings between enterocyte tight junctions (141). Once internalized by dendritic cells (immune sequestration) bacteria may spread to other locations via the bloodstream to arrive at mucosal-associated lymphoid tissue system (141). Growing evidence suggests earlier infancy lactobacilli colonization may protect the infant from developing atopic allergy (142). Also, *L. reuteri* supplementation in children has been shown to ameliorate atopic dermatitis phenotype (143). Thus, breast milk is considered a natural synbiotic, since it contains both probiotics, as well as prebiotic fiber (HMOs) to bestow the engraftment of a eubiotic infant microbiome.

2.14 Benefits in Neuropsychiatric and Neurodevelopmental Disorders

The evidence supporting the role of gut microbiota in the pathogenesis and/or progression of many neurodevelopmental, neuropsychiatric, and neurological conditions is mounting (144). Consequently, restoring a microbiota balance with probiotics is dawning as a novel preventive and therapeutic approach for managing such disorders (145,146). Clinical trials investigating the treatment of neuropsychiatric disorders with chronic exogenous oxytocin administration have raised several issues regarding CNS absorption efficacy and safety, especially in children and adolescents (147,148). It is believed that chronic exogenous oxytocin may lead to desensitization of the endogenous oxytocinergic system (148).

Currently, *L. reuteri* is the only probiotic known to upregulate endogenous oxytocin production (66). This has led researchers to propose *L. reuteri* as a psychobiotic. Psychobiotics are probiotics that confer mental health benefits to the host when consumed in a particular quantity through the interaction with commensal gut bacteria (149). It is hypothesized that administering psychobiotics, such as *L. reuteri*, would not only elevate endogenous oxytocin levels, but rather mitigate the adverse effects driven by exogenous oxytocin administration. This is because stimulating

endogenous oxytocin production also stimulates complex homeostatic pathway involving interrelated gut, immune, endocrine, and brain functions (150). The use of *L. reuteri* supplementation has yet to be studied in human neuropsychiatric conditions.

Exposure to maternal obesity in utero is positively associated with neurodevelopmental disorders, such as autism spectrum disorder (ASD) in children (151). One mouse study demonstrated that a maternal high fat, low fiber diet induced social deficits in the offspring (152). Shotgun genomic sequencing revealed significantly different microbiota composition when compared to mice fed a regular diet. The most striking difference was the relative abundance of *L. reuteri*, which was nine-fold less abundant in the maternal high fat diet (MHFD) offspring group compared to controls. Remarkably, after four weeks of live *L. reuteri* consumption MHFD offspring significantly improved sociability and preference for social novelty (152). This suggests that *L. reuteri* may also offer benefit to human neurodevelopmental disorders.

3. Conclusion

L. reuteri has been shown to be a safe and effective probiotic for attenuating both GI diseases and diseases in remote tissues, as well as, augmenting human host physiology. These various probiotic mediated effects are strain dependant. Current research suggests that *L. reuteri* and its metabolites promote human health through direct and indirect mechanisms. Specific *L. reuteri* strains have demonstrated a myriad of probiotic effects including the ability to kill a range of enteropathogenic organisms, accelerate wound healing, increase skin thickness, increase hair follicle density and the prevalence of anagen stage hair follicles, increase serum testosterone, increase serum oxytocin, increase male germ cell volume, upregulate Tregs, increase secretory IgA, reduce the incidence of dental caries, improve periodontitis treatment, reduce age related bone loss, synthesize B12, beneficially modulate GI microbiota, reduce the duration of infectious gastroenteritis, ameliorate infant colic, and improve ASD in mice (Figure 1).

Further research strengthening clinical evidence of effectiveness for various conditions is needed to be able to use this bacterial species in the validated clinical therapeutic protocols. Additionally, to unravel the mechanisms behind the observed probiotic effects more studies evaluating transcriptome and metabolome are needed. Understanding the metabolome could be pivotal in the application of precision medicine. Dietary interventions together with edible bacterial cocktails

may be used to activate latent host genetic programs, originating from host-microbiota coevolution, as well as to treat disease and optimise health.

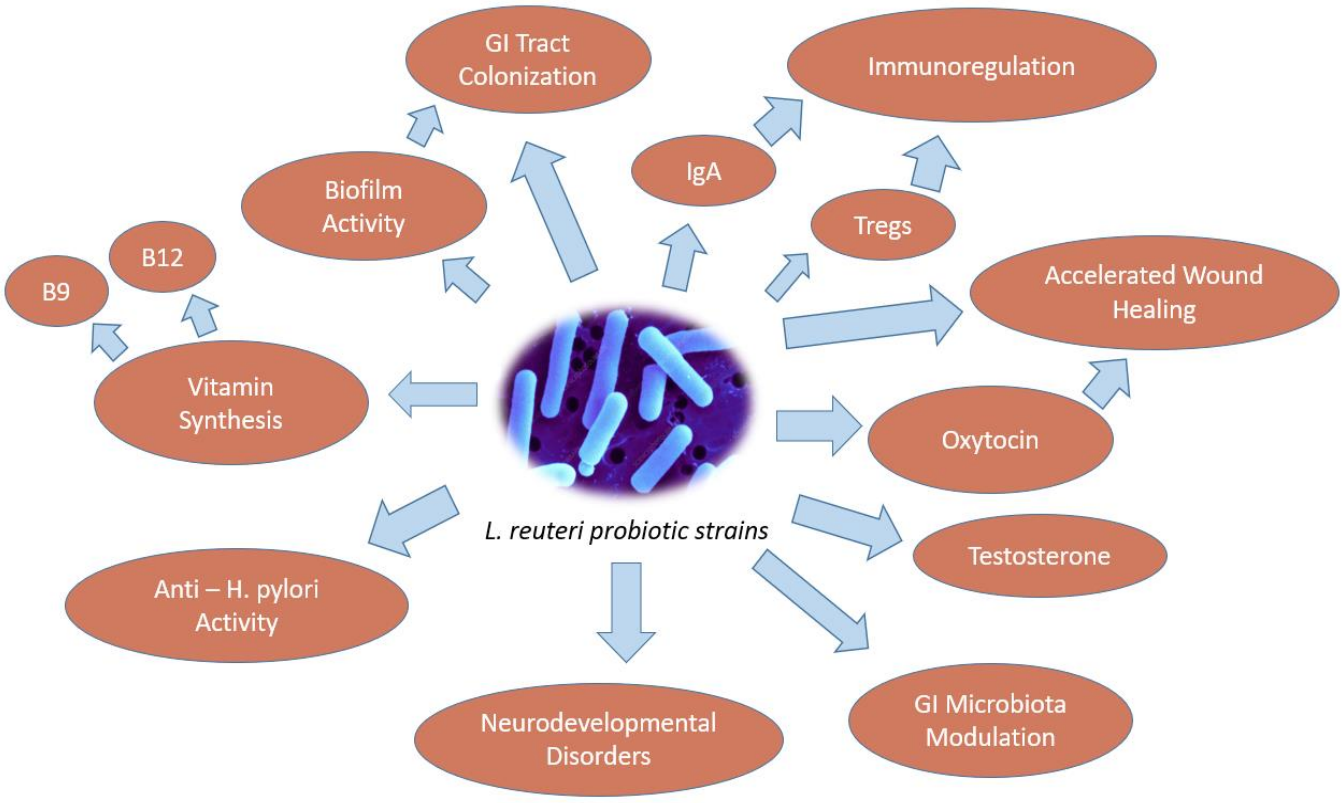

Figure 4. Probiotic Properties of *L. reuteri*

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