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THE ROLE OF THE GUT MICROBIOTA AND *LACTIPLANTIBACILLUS PLANTARUM* WJL STRAIN IN JUVENILE GROWTH DURING CHRONIC UNDERNUTRITION

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SUMMARY

The World Health Organization defines malnutrition as a deficiency, excess, or imbalance in a person's energy and/or nutrient intake. However, it is becoming increasingly clear that not only diet per se, but also the intestinal microbiota plays a fundamental role in the development of diseases associated with malnutrition. Next-generation sequencing, the renewed interest in bacterial culturing techniques, and the use of gnotobiotic models have enabled major advances in causally linking the role of the microbiota and specific bacterial strains in the aetiology/prevention of diseases associated with undernutrition, including wasting, stunting, and underweight. We have shown that the *Lactiplantibacillus plantarum* strain, selected in the gnotobiotic invertebrate Drosophila model for its growth-promoting properties, is able to increase the growth of juvenile undernourished mice in both gnotobiotic and specific pathogen-free animals, and that this effect is dependent on the expression of the NOD2 receptor by intestinal epithelial cells. These results suggest that administration of selected bacterial strains may represent a novel strategy for alleviating the persistent stunting that is one of the long-term consequences of undernutrition, which still affects millions of children under five years of age in low- and middle-income countries.

ACUTE MALNUTRITION

When a developing young organism faces specific nutritional challenges such as undernutrition (low-calorie or low-protein diet), it becomes stunted, that is, it is too small for its age. Because stunting affects both physical and cognitive development, it is not just a problem in childhood; the effects often persist throughout someone's life. In addition to being too small for age, stunting is associated with a dysbiotic gut microbiota that is immature for age (*Subramanian* et al., 2014). The causal evidence for this observation comes from germ-free animal models. Smith and colleagues transplanted into germ-free mice the faecal microbiota of monozygotic Malawian twins, who had become discordant for kwashiorkor, a form of severe acute malnutrition. After feeding these mice with a suboptimal diet they observed more severe weight loss in mice colonized with kwashiorkor-associated microbiota compared to the mice colonized by microbiota of healthy twin. This phenomenon was

associated with different metabolic profile and more labile responses to the renutrition regime of the kwashiorkor-associated microbiota (*Smith* et al., 2013). In a subsequent study, the same group showed that microbiota from undernourished children is also able to transmit impaired growth, altered bone morphology, and metabolic abnormalities in the muscle, liver, and brain to recipient gnotobiotic mice (*Blanton* et al., 2016). Moreover, they found that dysbiotic microbiota of these undernourished children is permissive for the invasion of bacterial taxa from the healthy microbiota and these invading bacterial taxa could improve growth impairments. Supplementing the microbiota of undernourished donors with cultured representatives of these invasive species again ameliorated growth and metabolic abnormalities of recipient mice (*Blanton* et al., 2016). Along these lines, Tidjani Alou and colleagues combined culturomics and metagenomics methods to identify potential probiotics candidates that were present in stool samples of healthy children and absent in malnourished patients with kwashiorkor. As a result, a complex of 12 species was identified in healthy children that was missing in kwashiorkor patients. They concluded that microbiotherapy based on selected strains has the potential to improve the current treatment of severe acute malnutrition and prevent relapse and death by re-establishing a healthy gut microbiota (*Tidjani Alou* et al., 2017). In addition to administering specific bacterial strains, another way to alter the intestinal microbiome is to provide substrates that promote the growth of specific bacterial taxa. Gehrig and colleagues showed that improving therapeutic diets by supplementing them with ingredients that increase the representation of growth-promoting bacterial taxa in the microbiota of undernourished donors promoted healthy growth in both preclinical mouse and piglet models and in a subsequent human study (*Gehrig* et al., 2019). These results not only confirmed the transferability of findings from preclinical models, but also simultaneously illustrated a possible new approach for treating malnutrition in children.

CHRONIC UNDERNUTRITION

Contrary to the acute malnutrition, chronic malnutrition is a result of inadequate nutrition over a long period of time leading to the failure of linear growth and, as a result, to short and thin individuals. Its causative factors are still poorly understood (*Gordon* et al., 2012) and until recently, nothing was known about the role of the microbiota in this condition in mammals (*Schwarzer* et al., 2016). In the invertebrate gnotobiotic Drosophila model of chronic protein undernutrition, microbiota in general and specific commensal strains of *Acetobacter* and *Lactobacillus* in particular have been shown to promote host systemic growth during the juvenile phase (*Shin* et al., 2011; *Storelli* et al., 2011). Specifically, Storelli and colleagues studied different strains of one of the most abundant Drosophila commensal species, *Lactiplantibacillus plantarum* (Lp). We were able to show that a single strain LpWJL can recapitulate the growth benefit effect of the full microbiota. This effect was strictly strain-specific and relied on endocrine tissue-specific activity of the TOR kinase, whose activity is regulated by circulating amino acid levels and controls the production of systemic hormonal growth signals, such as the

insulinlike peptides, including the Drosophila analogues of mammalian insulin-like growth factor (IGF-1) (*Storelli* et al., 2011).

In mammals, the juvenile growth is governed both by the nutritional input and the activity of somatotropic axis (*Breier*, 1999). Growth hormone (GH) is released from the anterior lobe of the pituitary gland in a pulsatile pattern and acts by binding to its receptor in the membranes of the target cells in the liver or peripheral organs (*Hartman* et al., 1993). The binding leads to the initiation of a signalling cascade, culminating in the phosphorylation of transducer and activator of transcription (STAT) proteins and induction of transcription of GH-regulated genes, in particular insulin-like growth factor-1 (IGF-1), IGF-1 binding proteins (IGFBPs) and suppressors of cytokine signalling (SOCSs) (*Bartke* et al., 2013). IGF-1 is the main mediator of GH actions and it also inhibits GH release by classical negative feedback loop. Upon protein malnutrition or starvation, the GH signalling pathway is inhibited, the levels of IGF-1 decrease, and the negative feedback loop on GH production is compromised. This leads to the increased levels of GH in the circulation and individuals enter maintenance (survival) mode. The GH resistance state seems to be an adaptive response to undernutrition in order to maintain euglycemia and preserve energy (*Fazeli* and *Klibanski*, 2014).

To investigate the role of the intestinal microbiota in mammals' juvenile growth, we studied the role of the microbiota on growth kinetics of conventional mice compared to germ-free animals on standard breeding diet or under chronic undernutrition (*Schwarzer* et al., 2016). We found that under normal nutritional conditions, the microbiota of infant mice was necessary to maximize systemic weight gain and linear growth. At the same time, the microbiota improved bone growth parameters, including femur length, cortical thickness, and cortical and trabecular bone fraction. On the molecular level higher growth kinetics of CV mice was accompanied by higher level of circulating IGF-1 and IGFBP-3, its major binding protein, despite GH levels similar to GF animals. The peak of circulating IGF-1 levels at day 28 after birth, which corresponded to a spurt growth period in CV mice, was not observed in GF animals. These data suggested higher sensitivity of conventional animals to GH actions and highlighted the major role of IGF-1 in postnatal growth. Further, the importance of the intestinal microbiota and its composition for juvenile growth on both normal breeding and low-protein diets was recently shown by Darnaud and colleagues (*Darnaud* et al., 2021). Two different defined mouse minimal microbiota, Oligo-MM^{12} (*Eberl* et al., 2019) and GM15, showed an enhanced ability to buffer the deleterious effects of a low-protein diet on systemic growth in post-weaning mice compared to an SPF microbiota, with concomitant improvement in circulating levels of insulin-like growth factor-1 (IGF-1). Given the modularity, stability, and easy tractability of the minimal microbiota, this model offers new opportunities for research focused on how the microbiota affects host physiology in general and juvenile growth in particular (*Darnaud* et al., 2021). In contrast to CV or gnotobiotic consortium-colonized mice, the growth of GF animals was completely arrested when challenged by chronic undernutrition. Yet, when we monocolonized GF mice with lactobacilli strains selected in Drosophila for their different growth promoting abilities, we observed a strain-dependent increase in the magnitude of linear and ponderal growth. The growth-promoting strain LpWJL was able to recapitulate the effect of the

entire microbiota on growth. The improved mouse growth associated with the complete microbiota and a selected LpWJL strain was accompanied by restored GH sensitivity and IGF-1 production in liver and in the peripheral tissues (*Schwarzer* et al., 2016).

Our findings on the conservation of the growth-promoting properties of the LpWJL strain in both the invertebrate Drosophila and the mammalian mouse model were limited to the gnotobiotic animals. Therefore, we asked the obvious question: Is LpWJL capable of alleviating stunting in conventional juvenile mammals as well? To this end, we developed a new preclinical mouse model for nutritional stunting in conventional mice with reduced circulating IGF-1 and insulin levels (*Schwarzer* et al., 2023). Previously, consumption of similar diet low in proteins and fats during the early-stage of life, in combination with exposure to certain commensal bacterial species (*e.g*. Bacteroidales species and *Escherichia coli*) was shown to contribute to the development of environmental enteropathy (EE). EE is characterized by increased gut inflammation, intestinal permeability and villous blunting, which as a result leads to growth failure and stunting (*Brown* et al., 2015). Contrary to the study of Brown and colleagues, our model of stunting was not associated with small intestinal inflammation but with altered small intestine crypt cell proliferation. Using this model, we have shown that repeated administration of LpWJL sustains the postnatal growth of malnourished conventional animals by orchestrating metabolic and hormonal changes in the juvenile host manifested as improved circulating levels and activity of both IGF-1 and insulin. The improved IGF-1 and insulin levels and activity were in accordance with a recent clinical study in which the authors reported a significant increase in the levels of both of these hormones in severe acute malnourished Bangladeshi children, who received microbiota-directed therapeutic food treatment (*Gehrig* et al., 2019). Further, we identified administration of heat-killed LpWJL bacteria as well as its isolated cell walls as being sufficient bacterial signals stimulating animal growth. This prompted us to search for a possible receptor on the host side enabling the sensing of bacterial cell-wall motifs. In *in vitro* assays, the LpWJL purified cell-walls were able to engage TLR2 and NOD2 receptors. We therefore administered LpWJL to undernourished NOD2 KO and MyD88 KO mice. While we observed increased growth in the MyD88 KO mice upon LpWJL administration, this effect was lost in the NOD2 KO mice. Further, by using the tissue specific NOD2 KO mice, we identified that NOD2 receptor expression is necessary in intestinal epithelial cells in order for LpWJL to mediate increased intestinal crypt cell proliferation, type I interferon–regulated gene induction, IGF-1 production, and postnatal growth promotion in malnourished conventional animals (*Schwarzer* et al., 2023) (Figure 1).

CONCLUSIONS

Eukaryotic cells and multicellular organisms have evolved in the world of bacteria. Therefore, during their evolution, they constantly interacted with bacteria and now form symbiotic relationships. In recent years, with advances in sequencing and -omics techniques, we have begun to see the various facets of this symbiotic relationship between host and microbiota.

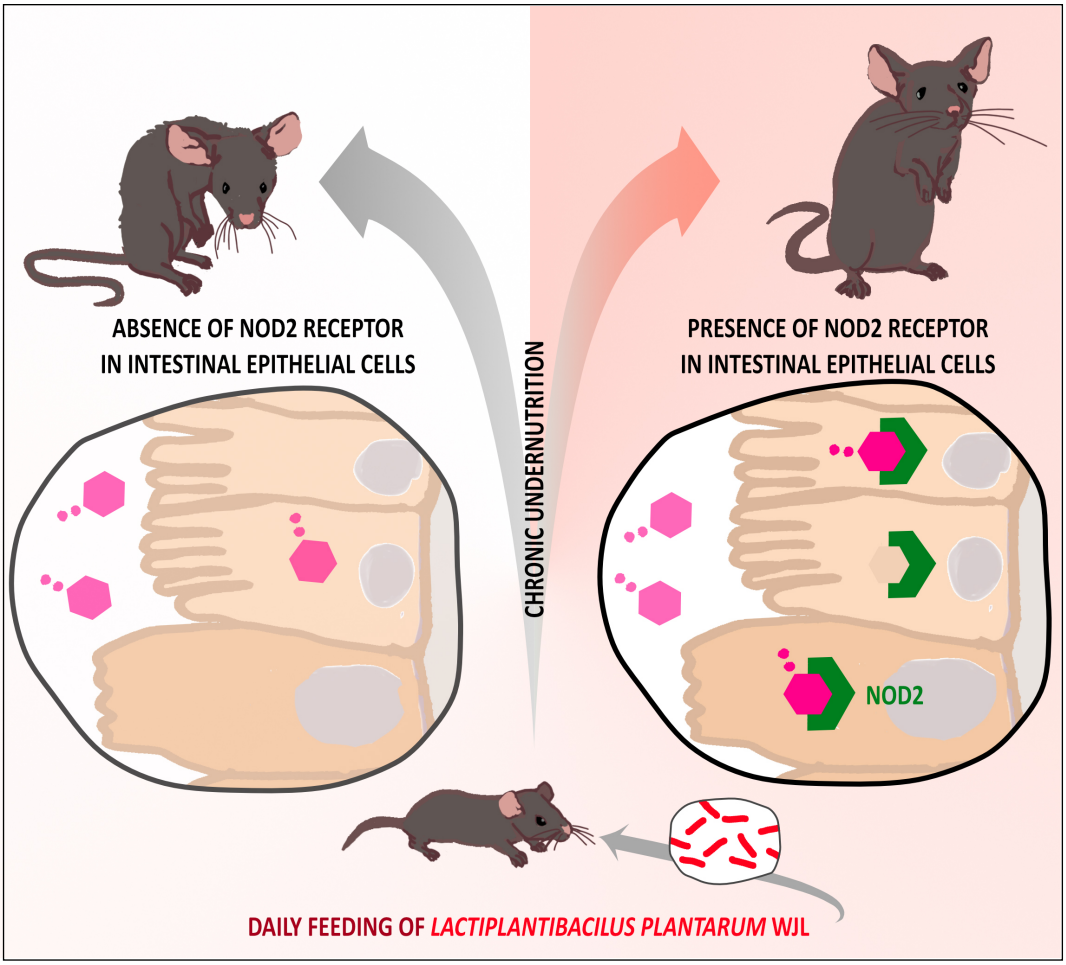

Figure 1: Feeding of *Lactiplantibacillus plantarum* WJL (LpWJL) to chronically undernourished juvenile mice enhance their longitudinal and ponderal growth. LpWJL cell wall motifs are sensed by the pattern recognition receptor NOD2 in the intestinal epithelial cells, enhancing the proliferation of crypt stem cells and sustaining postnatal juvenile growth despite chronic undernutrition. (Illustration created by Petra Schwarzer.)

Ample evidence from both human studies and experimental data from animal models shows that the gut microbiota plays a critical role in the growth of the juvenile host (*Schwarzer* et al., 2018). In a mouse model of chronic undernutrition, we showed that not only microbiota but also a selected strain of *Lactiplantibacillus plantarum* (LpWJL) is able to alleviate the complete stunting observed in germ-free mice. Further, the same LpWJL strain improved linear and ponderal growth in conventional undernourished mice upon daily intervention. The isolated bacterial cell-walls and the NOD2 receptor in the intestinal epithelial cells were both necessary for the observed beneficial effects. In a broad perspective, our findings suggest that certain validated bacterial strains or defined components of their cell wall, together with nutritional therapy, may represent a novel and complementary strategy to buffer the adverse effects of chronic undernutrition on human postnatal growth, which still affects more

than 160 millions of children below 5 years of age in low- and middle-income countries.

Outstanding questions
1) *Lactiplantibacillus plantarum* WJL or NOD2 agonist supplementation supports growth of chronically undernourished mouse model with no small intestinal enteropathy. Would the supplementation be effective also in undernourished models with environmental enteropathy (*i.e*. small intestinal inflammation)?
2) Would *Lactiplantibacillus plantarum* WJL or NOD2 agonist supplementation enhance the efficacy of nutritional interventions in undernourished mice and children?
3) Are other bacteria outside of *Lactobacillaceae* family also able to promote growth of undernourished juvenile animals? And if yes, how?

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