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Review article

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Riboflavin for women's health and emerging microbiome strategies

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Riboflavin (vitamin B2) is an essential water-soluble vitamin that serves as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD are coenzymes involved in key enzymatic reactions in energy metabolism, biosynthesis, detoxification and electron scavenging pathways. Riboflavin deficiency is prevalent worldwide and impacts women's health due to riboflavin demands linked to urogenital and reproductive health, hormonal fluctuations during the menstrual cycle, pregnancy, and breastfeeding. Innovative functional foods and nutraceuticals are increasingly developed to meet women's riboflavin needs to supplement dietary sources. An emerging and particularly promising strategy is the administration of riboflavin-producing lactic acid bacteria, combining the health benefits of riboflavin with those of probiotics and in situ riboflavin production. Specific taxa of lactobacilli are of particular interest for women, because of the crucial role of *Lactobacillus* species in the vagina and the documented health effects of other *Lactobacillaceae* taxa in the gut and on the skin. In this narrative review, we synthesize the underlying molecular mechanisms and clinical benefits of riboflavin intake for women's health, and evaluate the synergistic potential of riboflavin-producing lactobacilli and other microbiota.

Micronutrient status and dietary habits are crucial for human health and quality of life[1–3], especially for women of reproductive age and their children[4]. Riboflavin is an essential water-soluble vitamin that cannot be synthesized in humans and thus requires regular intake[5]. The need for riboflavin is prominent in women, due to their increased riboflavin demands in a variety of life stages and physiological processes[6,7]. For example, riboflavin intake should be increased during pregnancy and lactation, since it is taken up by the placenta and fetus to support growth and prevent birth defects[6] and lost via breast milk to meet the infant's nutritional needs and immune development[7]. To this end, the recommended dietary allowance (RDA) is 1.6 mg/day for adults (both sexes), 1.9 mg/day for pregnant women and 2.0 mg/day for lactating women in Europe[8]. These values are higher than RDA values set for healthy U.S. and Canadian populations, namely 1.1 mg/day for adult women, 1.4 mg/day during pregnancy, and 1.6 mg/day for lactation[9], which can be attributed to corresponding regulatory institutions that define criteria for nutritional adequacy in different ways depending on age, sex, and physiological status[10]. Documented dietary sources of riboflavin include milk and other dairy products, dark-green vegetables, cereals, fatty fish, and organ meat[11]. Yet, between 31%[12] and 92%[13] of women worldwide are reported to have a biochemical riboflavin deficiency. Although the majority of deficiencies occur in developing countries such as in Africa (e.g., Côte d'Ivoire[14]) and Asia (e.g., India[15] and Cambodia[13]), also populations in developed countries can suffer from riboflavin deficiency due to inadequate intake via diet as a result of veganism[16,17], lactose intolerance[18], aging[19], anorexia nervosa[20], or alcoholism[21]. Consequently, both developing and developed countries call for an acquisition of the necessary riboflavin levels by readily available and cost-efficient supplementary means[22].

Dietary intake of riboflavin is associated with health claims defined and evaluated for causality and level of evidence for the general population by the European Food Safety Authority (EFSA)[23] (Fig. 1, indicated in bold). Apart from these, additional health benefits have been suggested by observational and intervention studies (Fig. 1, indicated in light), although the level of evidence is often still limited. Yet, riboflavin supplementation is increasingly explored in specific clinical settings, for example in case of preeclampsia[24], anemia during pregnancy[25], Parkinson's disease and migraine[26], and female[27], and postpartum depression[28], as further discussed below.

Apart from dietary sources and supplements, riboflavin synthesized by microbiota members in the human body forms an additional source that can contribute to the overall riboflavin homeostasis[29]. Bacteria such as

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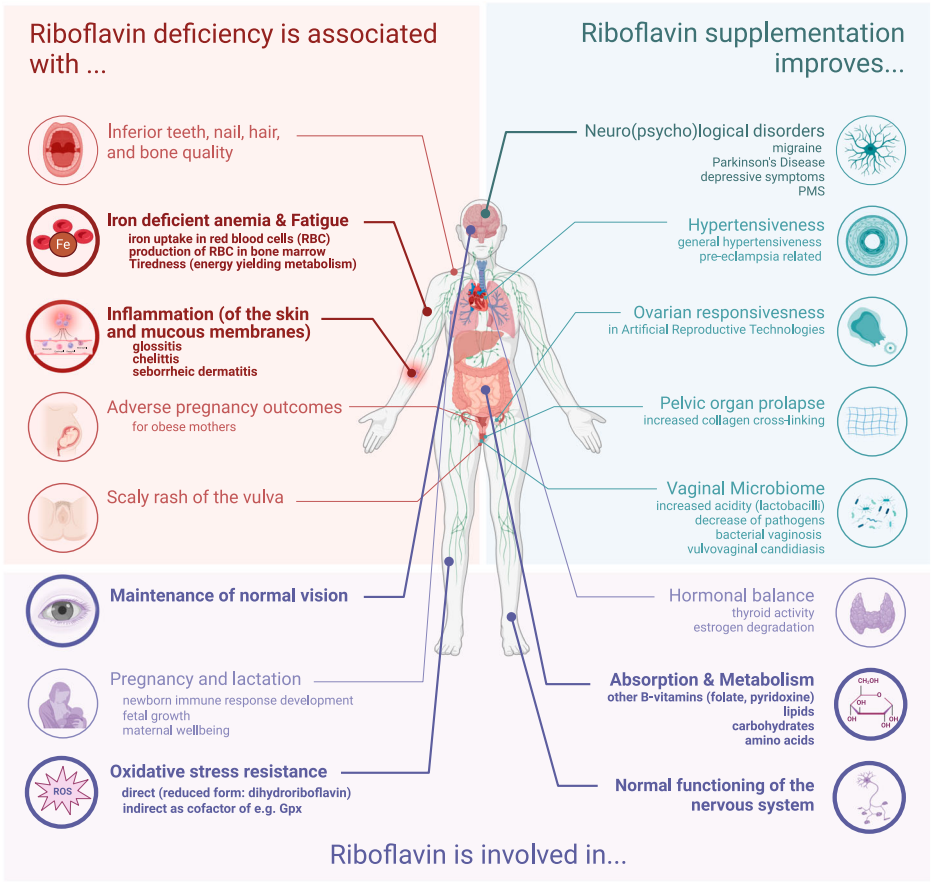

Fig. 1 | Impact of riboflavin deficiency on various aspects of women's health. The different body sites where riboflavin is crucial are indicated. The evidence is categorized based on the available documentation on associations between (1) riboflavin deficiency and specific health problems, (2) riboflavin supplementation and how it improves specific health outcomes and (3) how normal riboflavin levels support and maintain physiological processes. EFSA-approved health claims are indicated in bold. This figure was created with Biorender.com and based on the following key refs. 23–28,42,44,78,87,90,91,96,128,129,215.

Lactobacillaceae and *Bifidobacteriaceae* species have been reported to produce riboflavin that can be used by colonic epithelial cells, but the magnitude of their in situ contribution in the gut is not yet clear[30,31]. Whereas the riboflavin biosynthesis pathway is most extensively studied in *Bacillus subtilis*[32] and *Escherichia coli*[33], which can reside in the gut at different levels, one systematic genome assessment has found that each of the eight B vitamins (B1, B2, B3, B5, B6, B7, B9, and B12) can theoretically be produced by 40–65% of the 256 studied human gut strains[34]. The strains predicted to produce these vitamins belong to dominant gut microbiota genera such as *Bacteroides*, *Prevotella*, *Clostridium, Faecalibacterium* and *Fusobacterium*, and less dominant but prevalent gut taxa such as lactobacilli from the *Limosilactobacillus reuteri, Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* species. Riboflavin (B2) and niacin (B9) were predicted to be synthesized by more than half of the gut microbiota members studied[34]. In addition, the authors validated the genome predictions with experimental data from sixteen human gut microorganisms, published elsewhere, showing that 88% of the predictions matched[34]. Of interest, patients with metabolic diseases, such as obesity and type 2 diabetes[35] have been reported to have a reduction of bacterial riboflavin synthesis genes in their gut microbiome, suggesting that microbially-produced riboflavin could play a role in these diseases, although the causal association remains to be established. Similarly, members of the microbiota at other body sites such as *Lactobacillaceae* in the vagina, have been reported to produce B vitamins in laboratory conditions[36], but—to the best of our knowledge—so far without reference to potential physiological functions for the host. In infants, it is currently common practice to promote vitamin K supplementation to prevent uncontrolled bleeding until the microbiome is sufficiently matured[37]. A similar practice to counterbalance certain microbiome imbalances or deficiencies in infants and/or adults is not yet implemented for B vitamins but could be of interest. In this review, we present several arguments why this is of interest to explore, by providing a mechanistic overview of riboflavin's involvement in women's health based on dedicated molecular studies and clinical trials and associations. We also summarize the current knowledge on riboflavin production by microbiota members and exogenously added probiotics.

Riboflavin's modes of action for women's health

Riboflavin's general properties: antioxidation and anti-inflammation

Riboflavin's health benefits are generally based on the capacity of its active forms flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) to function as coenzymes for ca. 70 human proteins (cfr. the flavoproteome)[38,39] in biochemical reactions related to energy production, macro- and micronutrient metabolism, cell respiration, cell growth and immune responses (Fig. 2)[40,41]. By now, it is common knowledge that riboflavin possesses considerable antioxidative and anti-inflammatory properties[42,43]. The antioxidant effects result from the (in)direct capacity to deactivate reactive oxygen species (ROS), such as O_2^- and H_2O_2, either by riboflavin in its reduced form, as dihydroriboflavin, or as a cofactor of antioxidative enzymes, such as glutathione peroxidase, superoxide dismutase, glutathione reductase, and catalase (as reviewed in refs. 42,44). In this regard, the erythrocyte glutathione reductase activity coefficient (EGRac), defined as the ratio of reductase activity in red blood cells (RBCs) after FAD addition to the activity before addition, is a functional indicator of riboflavin status in the blood and therefore used as golden standard to clinically monitor riboflavin deficiency (EGRac > 1.40)[22]. In general, the higher the EGRac, the less endogenous FAD is available, thus the poorer the blood riboflavin levels[22].

Riboflavin's anti-inflammatory properties have mainly been studied using cell and murine models[43,45], but are not yet well understood. Interference of riboflavin with the generally pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription

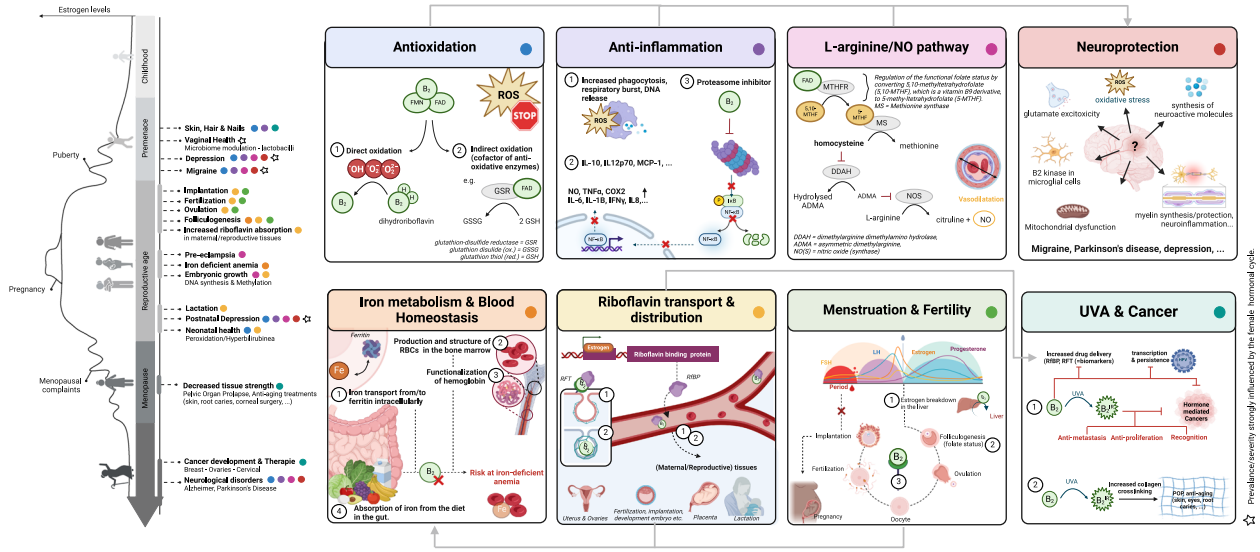

Fig. 2 | Riboflavin mechanisms of action with a focus on women's health (overview). On the left part of the figure, a female's life trajectory is depicted according to five different stages based on associated estrogen levels. Riboflavin's interference in these life stages is indicated, and the involved modes of action of riboflavin (right) are highlighted via colored dots. Starred items are strongly influenced by the female hormonal cycle. Mechanisms affecting each other/acting together are connected with gray arrows. FMN flavin mononucleotide, FAD flavin adenine dinucleotide, IL interleukin, IFN-γ interferon gamma, NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells, NO nitric oxide, TNFα tumor necrosis factor alpha, COX2 cyclooxygenase 2, ROS reactive oxygen species, RfBP riboflavin binding protein, RFT riboflavin transporter, FSH follicle stimulating hormone, LH luteinizing hormone. This figure was created with Biorender.com and based on the following key refs. [22,26,43–45,72–76,88,93,97,101,103,104,118,128,130,140].

factor signaling seems to play a major role[45]. Riboflavin seems to function as a proteasome inhibitor[43,45], resulting in the decreased degradation of the NF-κB inhibitor, phosphorylated-inhibitor kappa (P-IκB), thus preventing nuclear translocation of NF-κB and subsequent pro-inflammatory gene activation[46]. This has been shown to result in decreased production of pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8), and interferon gamma (IFN-γ), and inflammation markers such as cyclooxygenase 2 (COX2)[26,45]. In similar study setups, riboflavin has also been shown to prevent mitochondrial ROS production and DNA release[47]. In addition to inhibiting NF-kB signaling, riboflavin can also inhibit critical components of the non-canonical inflammasomes, such as caspase-1 activity[47], either via the AIM2 cytosolic innate immune receptor, which recognizes double-stranded DNA (dsDNA) released during cellular perturbation, or via the Nod-like receptor NLRC4, which recognizes bacterial ligands such as flagellin and the type three secretion system (T3SS)[47]. This appears to also result in reduction of pro-inflammatory cytokines such as IL-1β and IL-18 in macrophages and mice[48]. Moreover, riboflavin has been shown to directly stimulate the release of anti-inflammatory cytokines (e.g., IL-10, IL-12p70) and immune modulators (i.e., MCP-1, HMGB1, Hsp72, Hsp25)[49,50]. Riboflavin has also been reported to have an indirect role in anti-inflammatory mechanisms via its involvement in vitamin D biosynthesis as a cofactor for flavin-dependent monooxygenases and oxidoreductases (as reviewed in ref. 51).

The antioxidant and anti-inflammatory properties of riboflavin have also been documented in clinical trials. For example, in a 3-week prospective intervention with a high dose of 100 mg riboflavin/day with 70 patients with Crohn's Disease, a reduction in systemic oxidative stress (measured by higher free thiol plasma levels), inflammatory markers (IL-2 and C-reactive protein (CRP)) and clinical symptoms of Crohn's disease (i.e., a reduction in clinical disease activity index and quality of life improvement) were observed[52]. Contrarily, in a shorter placebo-controlled study with healthy individuals, daily supplementation of 50 mg (n = 32) or 100 mg (n = 33) riboflavin showed no reduction in free thiols compared to placebo (n = 34)[53]. In a prospective study with pregnant women and their children (n = 2797 with singleton births), riboflavin levels in the blood were inversely associated with inflammation markers in the blood[54].

Indirect clinical validation of antioxidant and anti-inflammatory effects is linked to roles for riboflavin in the maintenance of healthy skin, nails, hair[55], and good eyesight[56]. However, when this role of riboflavin and associated health claims were evaluated by the EFSA[8] for the general population, the expert panel concluded that a cause-and-effect relationship has only been established for dietary intake of riboflavin in the case of contribution to normal skin and mucous membranes and maintenance of normal vision. For the other indications, the panel concluded that the mechanistic documentation available was too limited. For example, riboflavin has been shown to be involved in the conversion of tryptophan to niacin (vitamin B3)[57], which stimulates the production of collagen (type I, III, and V), elastin and fibrillin (1 and 2), but—to the best of our knowledge—this has only been substantiated ex vivo in dermal fibroblasts[58].

Riboflavin and neurological disorders

Several large observational studies in human cohorts have also associated B vitamins intake (e.g., vitamin B2, B9, and B12) and other dietary habits with mental health, particularly in adolescent girls (Japanese cross-sectional study, n = 3450)[59], and women who suffer from postpartum depression (cross-sectional study, n = 344)[28] or premenstrual syndrome (PMS) (case-control study, n = 3025)[60]. More specifically, the latter nested case-control study (1057 cases and 1968 controls) showed that when women consumed more riboflavin (from fortified cereals, cow milk and/or green vegetables), the risk for PMS was lowered with 35%[60]. In the Japanese cross-sectional study on adolescents (aged 12–15), higher riboflavin in blood were also associated with less depressive symptoms in girls (n = 3450), but not boys (n = 3067)[59]. Another peculiar sex difference was reported in an Iranian observational study (n = 3362) where lower dietary riboflavin levels were associated with anxiety and depression in middle-aged men (n = 1403), and psychological distress in middle-aged women (n = 1959)[61]. However, a recent systematic review of 20 randomized controlled trials (RCTs) (n = 2256)[62] did not substantiate the potential of riboflavin as adjuvant for depressive symptom alleviation. Riboflavin supplementation is also discussed for neurodegenerative diseases such as Parkinson's disease, where women have higher mortality rates and faster disease progression compared

to men[63,64] but the cause of the improved motor capacity of the patients was not solely attributed to riboflavin ($n = 19$)[65].

More clinical evidence exists for the use of riboflavin as prophylaxis against migraine headaches[66]. Women suffer from a three-fold increased risk for migraine symptoms compared to men[67]. This can possibly be explained by a hormonally lowered neuro-excitability threshold for these headache attacks[68]. Strikingly, in a large cross-sectional study ($n = 5725$ females and $n = 1061$ males) menstruation was found as the main trigger factor for migraine episodes in female patients (78% of women)[69], while postmenopausal women appear to have more similar migraine triggers to male migraine patients than fertile female patients concerning hormonal levels[69]. High-dose supplementation (400 mg/day) of riboflavin over a time course of three months was found to significantly reduce the frequency, duration and pain score of migraine attacks in both men and women in a meta-analysis of eight RCTs and one intervention study[66]. Consequently, riboflavin is also included as additional prophylactic therapy in the treatment guidelines of conditions and disorders with co-morbid migraine headaches[70]. For example, the American Neurogastroenterology and Motility Society and the Cyclic Vomiting Association recommend riboflavin for cyclic vomiting, a gastrointestinal and psychological condition characterized by sudden episodes of nausea and vomiting[70].

The neurological mode of action of riboflavin and its active forms FMN and FAD appears to be complex and multifactorial, involving its antioxidant and anti-inflammatory properties, as well as the homocysteine/L-arginine/NO pathway. For example, in a mouse model of lipopolysaccharide-induced neuro-inflammation and Alzheimer's disease, FMN supplementation with specific nanoparticles targeting riboflavin metabolism in the microglia ameliorated cognitive dysfunction, synaptic plasticity, and inflammation[71]. These FMN particles appeared to lower riboflavin kinase expression in the microglia, preventing TNFR1/NF-κB signaling and pro-inflammatory cytokine release[71]. A recent integrative systematic review of 21 (pre)clinical studies (including 8 studies in mice, 12 clinical and one translational) indicated the potential of riboflavin treatment to improve brain damage following oxygen deprivation in children, adults, and elderly people[72]. Apart from these modes of action, riboflavin has also been shown to activate specific neuroactive molecules such as vitamin B_6 (which confers neuroprotection based on a role in serotonin production)[73], homocysteine (which has a double-edged neurological role)[74] and kynurenine involved in glutamate excitotoxicity (i.e., excess of glutamate in neural synapse, resulting in death of neural cells)[75]. Moreover, the synthesis and protection of myelin, which insulates nerve cell axons and increases their electrical pulse rate, require riboflavin[76]. In line with this, riboflavin has been shown to prevent myelin degeneration in murine models of multiple sclerosis by supporting the levels of the protective brain-derived neurotrophic factor[77]. Clinical substantiation remains to be provided in suitable safety and efficacy trials.

Riboflavin and vaginal health

The above reviewed mechanistic and clinical studies with riboflavin have mainly focused on systemic outcomes, with some also highly relevant for women's health. In addition to systemic benefits, as early as 1940, it was observed that riboflavin could also have local benefits: vaginal administration of riboflavin in ten women via a lactose tablet was shown to result in an increased acidity and a favorable impact on vaginal bacteria through increased growth of acid-forming bacilli and decrease of pathogens[78]. Considering the current knowledge on the vaginal microbiome[79,80], these observations suggest an increased activity of lactic acid production by vaginal lactobacilli. To the best of our knowledge, local vaginal applications of riboflavin have not been further explored, so that direct measurements linking vaginal riboflavin supplementation to increased growth of lactobacilli are currently lacking. In contrast, oral supplementation has been explored. In one RCT ($n = 158$), an oral vitamin B complex, including riboflavin sodium phosphate, showed efficacy as adjuvant therapy to fluconazole for women with complicated vulvovaginal candidiasis (VVC)[81]. The B vitamin complex appeared to significantly increase the anti-*Candida* effect, with more women testing negative for hyphae and spore formation[81]. This finding was mechanistically supported by an enhanced antifungal effect of fluconazole in a VVC vaginal epithelial cell model and mouse model when administered together with vitamin B complex injection[81]. In other research, lactobacilli have also been shown to reduce the growth and hyphae formation of *Candida* in vitro[82–84] and in patients with acute VVC[85]. Unfortunately, no data on the impact of riboflavin supplementation on the vaginal microbiome composition or endogenous vaginal lactobacilli have been reported, making it difficult to assess whether an increase in lactobacilli by riboflavin could play a role in these observed enhanced antifungal effects[81].

Riboflavin and reproductive health

Modulation of hormonal fluctuations and impact on fertility. Apart from its role in systemic and vaginal health, riboflavin also seems to impact reproductive health, in part through its reciprocal interaction with estradiol, the most prominent female sex hormone essential for fertility and pregnancy[68,86]. For example, in a longitudinal prospective cohort study with 259 premenopausal women, a secondary analysis showed that higher dietary intake of riboflavin, assessed via a 24h dietary recall, was associated with lower serum levels of estradiol and homocysteine, which are signals required for folliculogenesis[68]. Similar inverse associations between riboflavin intake and blood estradiol levels were found by the Nurses' Health Study II, which is one of the longest running investigations of factors influencing women's health ($n = 116,469$) by combining food-frequency questionnaires (FFQs), health surveys, and biological samples (urine, blood and cheek cell samples)[87]. Likewise, higher intake of riboflavin, vitamin B6 and B12 was also associated with lower incidence of ovulatory infertility[87]. On the one hand, these observations might be explained by the fact that an estrogen drop is required for the onset of ovulation[88], and several flavoproteins (proteins using FMN and FAD as coenzyme) assist estrogen degradation by cytochrome P450[51,89] as they balance electrons from these reactions. On the other hand, the inverse association of riboflavin with plasma homocysteine levels can also be attributed to riboflavin's interaction with methyltetrahydrofolate reductase (MTHFR), another peculiar flavoprotein involved in follicular activity, embryo quality and pregnancy success[90–92]. In short, MTHFR initiates the conversion of folate (vitamin B9) to its functional form 5-methyltetrahydrofolate[93,94], which on its turn acts as a coenzyme of methionine synthase for the formation of methionine from homocysteine[93,94]. Remarkably, elevated homocysteine levels have frequently been associated with poor oocyte maturity, reduced fertilization and poor in vitro embryo quality[95], though direct clinical evidence linking riboflavin, homocysteine and anovulation is sparse. Nevertheless, riboflavin and other B vitamins might assist future subfertility treatments and fertility preservation methods. For example, in a subset of 100 women relying on assisted reproductive technologies (ART), higher preconceptional vitamin B9 and B12 levels in blood were associated with higher live birth rates[96].

Adding to these positive effects of vitamin B9 and B12, higher serum riboflavin levels were associated with increased probabilities of high-quality embryos as well as clinical pregnancy after embryo transfer in a prospective Chinese follow-up study ($n = 216$, age <35)[97]. Moreover, in a mechanistic study in pre-puberty mice, riboflavin, together with vitamin B1 and B6, seemed to stimulate in vitro maturation of follicles through granulocyte proliferation and upregulation of oocyte-specific genes, including genes encoding bone morphogenetic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), zona pellucida glycoprotein 3 (*ZP3*) and estrogen receptor alpha (*ESR1*) and beta (*ESR2*)[98]. This follicle maturation activity is of interest to substantiate in human mechanistic and efficacy studies because perimenopausal women and female cancer survivors relying on fertility preservation methods have typically smaller number of follicles with a reduced probability to mature[98].

Compared to limited evidence for the aforementioned effect of riboflavin on estrogen levels and menstrual cycle, estrogen seems to have a clearer impact on plasma riboflavin levels, as well as its distribution

throughout the female body[68]. In general, riboflavin and FMN are transported across the body by binding to non-specific carrier proteins in the plasma, such as albumin and immunoglobulins (IgA, IgG, IgM), through hydrogen bond formation[99]. As a result of the varying affinity between the flavins and their carrier, the flavins are deposited across the body. Besides these non-specific carriers, there also exist specific riboflavin-binding proteins (RfBPs) in mature females, which are mainly synthesized by the liver under influence of an estrogen-sensitive promoter when becoming sexually mature or when treated with estrogen[100–102]. These RfBPs, highly conserved across mammals and avian species, scavenge riboflavin in blood and transfer the vitamin to specific tissues through receptor-mediated endocytosis by riboflavin transporters (RFTs)[86,100].

Many reproductive and maternal tissues, such as the ovaries, placenta, and mammary glands, make use of estrogen-sensitive RfBPs and RFTs for highly sophisticated riboflavin transport to support fertilization and/or offspring's growth[101,102]. For instance, in rodents and subhuman primates, immunoneutralization of RfBPs has been shown to result in female infertility, peri-implantation embryonic loss and pregnancy termination[103,104]. Evidently, it is more difficult to explore and substantiate such role in humans, but specific intervention studies with riboflavin in relation to female fertility seem worth investigation. The same holds true for the role of other micronutrients, such as folate, vitamin D, and iron, which all depend on the riboflavin status for their activation and which have been positively associated with female fertility, as reviewed in ref. 105. Moreover, it seems of interest to explore the role of riboflavin in male fertility in more detail. For instance, riboflavin seems to be involved in sperm motility and energy generation as well as fertilization (cfr. acrosome reaction) and oxidative stress management, but this has so far only been shown in animal models[106].

We hypothesize that this hormonally induced scavenging of riboflavin might also explain why women taking high-dosed oral contraceptives containing estrogen and progesterone have been reported to be at risk for riboflavin deficiency as measured by the EGRac[107–111]. Similarly, it could clarify that migraine attacks, as side effects of the older generation oral contraceptives, are possibly linked to a reduction in plasma riboflavin[67]. Nevertheless, these mechanisms remain to be substantiated.

Riboflavin, pregnancy, and child's development. Riboflavin is also crucial for the health and well-being of infants during pregnancy and after birth[112]. During pregnancy, estradiol levels are heightened, and riboflavin consumption strongly increases due to a particularly high demand by fetal tissues. In one study of 44 women and their infants, a maternal-fetal riboflavin plasma ratio of 1:4.7 was measured[6]. This high riboflavin demand could be explained by its involvement in DNA synthesis and methylation during embryonic growth[8,105], as well as neural tube formation (cfr. regulation of the functional folate status as mentioned before). The transplacental transport of riboflavin is associated with high RfBP and RFT expression by placental trophoblast cells and appears to be the most intense during the third trimester[6]. To reduce perinatal mortality[113], recommended riboflavin intake is therefore increased for pregnant women to 1.9 mg/day in Europe[8] and 1.4 mg/day in U.S. and Canada[9].

Riboflavin remains essential around birth, with FAD functioning as a crucial cofactor of glutathione, to oppose peroxidation reactions that arise during the rapid change in oxygen concentration in the baby during delivery. After birth, active riboflavin transport by maternal tissues using RfBPs and RFTs and other proteins appears to remain essential, for instance to pump riboflavin into the breast milk and support the child's nutritional needs as shown in mice and humans[101,114]. Therefore, as mentioned before, the recommended daily intake for riboflavin is also increased for breastfeeding women to 2.0 and 1.6 mg/day in Europe (EFSA)[8] and the U.S. and Canada (RDA)[9], respectively.

Riboflavin and iron-deficient anemia: during pregnancy & beyond. Riboflavin is also important for the health of mother during pregnancy and this stems in part from its involvement in iron metabolism. Iron-deficient anemia remains one of the most prevalent medical concerns during pregnancy[115]. Women are specifically at risk for anemia during the first four months of gestation, since hemoglobin levels naturally decrease due to elevated iron demands to support fetal growth and a disproportionate rise of blood plasma volume to RBCs[97]. Iron-deficient anemia has been systematically associated with extreme fatigue and severe pregnancy complications such as postpartum hemorrhage, preterm delivery, stillbirth and reduced offspring birthweight[25]. Besides insufficient iron intake, low riboflavin status, which is more common among women of reproductive age than generally recognized[25,116], appears to be also involved in the development of iron-deficient anemia[22]. For instance, in a large randomized controlled intervention study with 2153 healthy pregnant women in Ireland, 68% of the cohort measured low or deficient blood riboflavin levels[25]. This riboflavin status was found to be a significant determinant of hemoglobin levels[25] and predictor of anemia development at the 12th gestational week[25].

The underlying mechanism of riboflavin in anemia appears multifaceted: flavin-dependent enzymes are involved in the absorption of iron from the diet[117], in the mobilization of iron from/to ferritin (the main intracellular iron storage protein in cells)[118], and the uptake of iron in RBCs[116]. In addition, flavin-dependent enzymes are needed for the functionalization of hemoproteins (cfr. reduction of insoluble Fe^{3+} to soluble Fe^{2+}), as observed for the conversion of inactive methemoglobin into hemoglobin required for oxygen transport[22,119]. Apart from RBC physiology, riboflavin is also involved in RBC structure, by preventing hemolysis through oxidative stress management[120], and RBC generation in the bone marrow, mediated through its interference with corticosteroid metabolism[121], with health implications far beyond pregnancy induced anemia.

Indeed, in an observational study of non-pregnant Malaysian ($n = 210$) and Canadian ($n = 206$) women, it was also shown that riboflavin deficiency (EGRac > 1.40) was a weak, but significant predictive biomarker of hemoglobin and anemia[22]. Altogether, these findings indicate that pregnant women could benefit from preventative riboflavin supplementation as it reduces the risk of iron-deficient anemia[112,122], however it is not yet in treatment guidelines. Other cardiovascular concerns during pregnancy and the postpartum period, such as maternal systemic endothelial dysregulation, intravascular inflammation, and preeclampsia have also been associated with riboflavin deficiency[123]. Especially the latter is of interest, as it affects 3 to 13% of pregnant women, with incidence up to 20% among high-risk women according to the World Health Organization (WHO)[124]. Preeclampsia is a dangerous condition of persistent hypertension, associated with high urinary protein levels or decreased blood platelet development, failure of kidneys, liver or lungs, and neurological complications[123]. In recent years, riboflavin supplementation is increasingly explored to prevent preeclampsia. It is postulated that riboflavin induces NO-mediated vasodilatation, resulting in hypertension relief, through stimulation of the conversion of homocysteine and its subsequent impact on the L-arginine/NO pathway[125] (Fig. 2). In a prospective, randomized, double-blind trial in Tanzania and Venezuela with 455 women, taking riboflavin (15 mg/day) from the 20th week of pregnancy appeared to be associated with prevention of severe cases of preeclampsia[24]. However, compared to anemia, the evidence for riboflavin supplementation to treat preeclampsia is limited, as recently reviewed[112].

Riboflavin, aging and cancer

Pelvic organ prolapse and related issues. One of the most important aging-related conditions for women where riboflavin plays a role is pelvic organ prolapse (POP). POP is a condition with a worldwide prevalence of 9%[126], in which a woman's pelvic muscles and tissues weaken, resulting in bulging of the pelvic organs (uterus, bladder, rectum) into the vagina[127]. Besides vaginal birth and being heavily overweight, one of the causes for POP is diminished vaginal tissue stiffness through the reduction of collagen with age and hormonal changes[128,129]. This loss of collagen and

epithelial stiffness is also suggested to be a cause of other diseases and aging-related complications such as corneal[130], skin[131] and teeth tissue[132] deteroriation. While the latter two have been substantiated with in vitro work[133,134], clinical efficacy has been documented for the treatment of corneal disorders with UVA-activated riboflavin, resulting in its incorporation in routine ophthalmologic procedures[135]. During the exposure of riboflavin to UVA, singlet oxygen molecules are generated, which induces covalent bonding between amino groups of collagen fibrils, and thus strengthens tissue stiffness[136]. Consequently, UVA-activated riboflavin was proposed as potential therapy for POP as well, especially due to its ability to attenuate UVA damage and inhibit necrosis in vaginal cells[128,129]. Until now, this hypothesis has only been substantiated by ex vivo experiments where vaginal tissue strips from POP cases were exposed to riboflavin and subsequent UVA photoactivation cells[128,129]. Dedicated clinical studies are required to substantiate the hypothesized benefits of local and systemic riboflavin application on vaginal health outcomes, including a potential beneficial impact on the vaginal microbiome as a key read-out.

Cancer. For several decades, poor riboflavin intake has been associated with increased risk of cervical cancer in epidemiological studies[137,138]. In addition, a case-control study (n = 257 cases, 133 controls) in 1993 reported that lower riboflavin intake, assessed via 24 h dietary recall, was associated with increased risk of cervical intraepithelial dysplasia, an early stage proceeding invasive cervical cancer[139]. More recently, a Chinese observational study (n = 146) showed that not only plasma, but also tissue riboflavin levels were inversely associated with high-risk human papillomavirus type 16 (HR-HPV16) and HPV18 infection[140]. Moreover, compared to healthy control specimens, plasma and tissue riboflavin levels were decreased in patients with cervical squamous epithelial cancer and cervical intraepithelial dysplasia, respectively. These findings suggest a role for riboflavin in HPV-induced cervical cancer development and progression[140], which is potentially mediated through riboflavin transporter C20orf54[140], and riboflavin's antioxidant properties (see above). Although direct mechanical evidence for riboflavin is lacking, other antioxidants have been reported to reduce HPV transcription and expression in vitro through redox regulation[141,142]. Understanding what drives the progression from precancerous lesions to invasive cervical cancer, which especially impacts HPV-infected women, remains a necessary topic for further research.

Besides cervical cancer, insufficient riboflavin intake has also been associated with breast carcinogenesis[143]. A systematic review and meta-analysis of 21 prospective cohorts and 6 nested case-control studies (n = 49,707 cases and 1,274,060 individuals) indicated that a higher dietary intake of riboflavin, together with folate and vitamin B6, might be associated with a decreased risk of estrogen and progesterone receptor-positive breast cancers[143]. However, other studies report more complicated associations between B vitamins and cancer (as also reviewed in ref. 144). For instance, pharmacokinetic vitamin-drug interaction studies have indicated a decreased uptake of the anticancer drug antifolate methotrexate in cancer cells, and the complexation of methotrexate and doxorubicin C into inactive adducts by riboflavin, thereby reducing the efficacy of these drugs[145]. Care should thus be taken when implementing riboflavin in clinical practice for cancer patients in different disease stages.

Riboflavin-producing microbiota and women's health

The microbiota at different body sites (gut, skin, vagina) also plays an important role in women's health throughout all life stages, although the level of evidence and mechanistic substantiation for its role is fragmented. In the vagina, *Lactobacillus* species are generally dominant in healthy, complaint-free women, such as shown in a pioneering study in the US (n = 396)[146] and a large-scale citizen science cohort study in Belgium (n = 3345)[79]. Nevertheless, more diverse vaginal microbiomes have been observed globally across different healthy populations (North America[146], Scandinavia[147], South Africa[148], and Kenya[149]), but are usually associated with adverse sexual and reproductive outcomes[150]. Systematic reviews have now established that a vaginal composition dominated by *Lactobacillaceae* taxa such as *Lactobacillus crispatus* is linked to protection against conditions such as preterm birth[151], bacterial vaginosis[152] and progression of an HPV infection into cervical cancer[153]. The protective mode of action of lactobacilli in the vagina appears to be mainly due to their capacity to produce lactic acid as antimicrobial factor[80], while a metabolic role for metabolites such as riboflavin is largely underexplored. Research on the gut microbiome in patients (male and female) with metabolic diseases, such as obesity[154] and type 2 diabetes[35,155], has reported a reduction of riboflavin synthesis genes in their gut metagenome. Of interest, in a female-specific gut metagenome study of women with type 2 diabetes (n = 53), impaired glucose tolerance (n = 49), and normal glucose tolerance (n = 43), riboflavin synthesis genes were also more abundant in the normal group[156].

Main documentation is based on in vitro and preclinical data

A variety of microorganisms including bacteria (e.g., *Clostridium difficile*[157]), archaea (e.g., *Methanococcus jannaschii*[158]), fungi (e.g., *Eremothecium ashbyii*[159], *Saccharomyces cerevisiae*[160]) have a documented capacity to produce riboflavin at different levels[40,161], although in general, under physiological conditions the microbial riboflavin production is very low (few µg/L, or less, in culture media). Therefore, some of these microorganisms, such as *Bacillus subtilis, Ashbya gossypii,* and *Candida famata*, have even been genetically, metabolically and/or chemically optimized for industrial-scale riboflavin production[160,162]. For most commercially available dietary supplements with riboflavin, these microorganisms produce riboflavin industrially in bioreactors and the vitamin is extracted to be formulated in supplements[163]. Many of the producing microorganisms do not have an assigned safety status such as 'Generally Recognized as Safe' (GRAS)[164] in the United States and/or 'Qualified Presumption of Safety' (QPS)[165] as evaluated by EFSA for Europe, nor do they comply with the scientific definition of probiotic[166]. This definition states that probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[166]. Consequently, many of the known riboflavin-producing taxa cannot be used live in food products for human consumption[167].

Compared to these traditional industrial-scale producing microorganisms that cannot be consumed, riboflavin-producing *Lactobacillaceae* and *Bifidobacteriaceae* taxa with a GRAS/QPS or related status are of interest, because they can be safely consumed and allow the combination of the benefits of riboflavin with some of the probiotic health benefits for several strains of these taxa (Fig. 3). Probiotic benefits for *Lactobacillaceae* and *Bifidobacteriaceae* evaluated in systemic reviews and meta-analyses include improved gastrointestinal health and prevention of antibiotic-associated diarrhea[168], prevention of relapse of bacterial vaginosis[169], and prevention of respiratory tract infections[170]. It is not yet well-understood how these bacteria can exert these benefits, but documented mechanisms of action include antimicrobial activity against major gastrointestinal[171] and urogenital pathogens[172], barrier-promoting effects at the gut epithelium[173] and other mucosa[174], immunomodulatory effects by stimulating host antimicrobial compounds such as α-defensins[175] and modulating the secretion of cytokines such as IL-10, IL-6, IL-1b, IL-2, TNF-α, and impacting different cell types such as intestinal epithelial cells, dendritic cells, macrophages and regulatory T cells[176–178]. These probiotic mechanisms can be postulated to have direct and indirect physiological effects on women and their health[80] (Fig. 3), but require further validation. A role for (B-)vitamin production in probiotic modes of action is also not well-studied. Yet, when administered, *Lactobacillaceae* and *Bifidobacteriaceae* taxa that have the genetic and biochemical potential[34] might produce riboflavin in situ as a (temporary) part of the gut (and other) microbiota, resulting in altered epithelial morphology as shown in a murine in vivo model[179].

Many *Lactobacillaceae* and *Bifidobacteriaceae* species have such capacity to produce riboflavin, at variable concentrations[36]. For example, *Lactiplantibacillus plantarum* strains M5MA1-B2[180,181] and HY7715[182] have

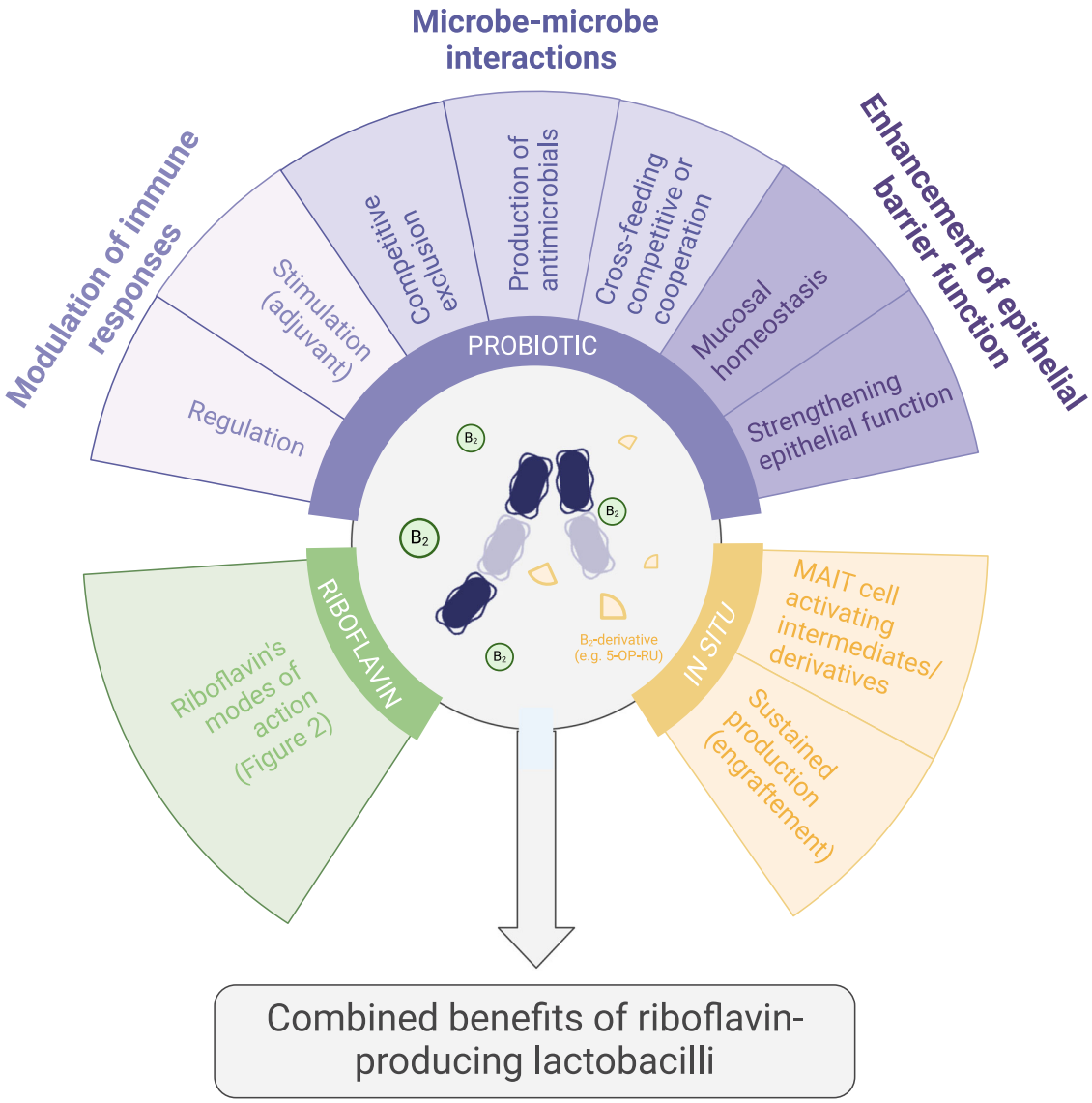

Fig. 3 | The postulated synergistic effects of riboflavin-producing microbiota members such as probiotic lactobacilli and riboflavin. The beneficial properties consist of riboflavin-mediated effects (as previously described in Fig. 2), probiotic and microbiome-promoting effects such as modulation of immune responses, microbe-microbe interactions and enhancement of the epithelial barrier function[178] and effects mediated by unstable riboflavin intermediates/derivatives leading to MR1-dependent activation of Mucosal Associated Invariant T cells (MR1 = Major Histocompatibility complex class 1 related protein)[187]. This figure was created with Biorender.com.

been reported to produce 3–5 µg/mL riboflavin under laboratory conditions. Strain HY7715, isolated from food, could even reach up to 34.5 µg/mL when cultivated in optimized growth media[182]. However, such exceptionally high and industrially relevant riboflavin levels are usually not encountered amongst spontaneous riboflavin-producing lactobacilli[183]. Therefore, a well-established directed evolution method, using the toxic riboflavin analogue roseoflavin, can be applied to enhance riboflavin production in promising probiotic lactobacilli whilst remaining food-grade[184]. Most roseoflavin-resistant strains studied until now carry mutations in the regulatory region upstream of the *rib* operon (more specifically, in the aptamer of the riboswitch), disrupting the negative feedback mechanism, resulting in significantly higher expression[184,185]. Similar nucleotide replacements and deletions are also observed in spontaneous overproducing isolates, including the vaginal isolate *Limosilactobacillus reuteri* AMBV339, that can reach high riboflavin levels of approximately 18.36 µg/mL in laboratory conditions and food matrices[36]. Such high-producing levels are of interest as this could theoretically be sufficient to meet daily needs of 1.6 mg riboflavin with one fermented beverage consumption of 100 mL, as validated by Spacova et al.[36]. However, to the best of our knowledge, no clinical studies in humans have been performed to validate health effects of in situ riboflavin production after administration of riboflavin-producing probiotic strains. Of note, strain *L. reuteri* AMBV339 is currently in clinical evaluation for its impact on the gut and vaginal microbiome and metabolome upon administration as an oral dietary supplement (ClinicalTrials.gov ID NCT06425081)[36].

Colonic epithelial cells are capable of transporting riboflavin basolaterally[186]. They could thus—theoretically—take up microbially produced riboflavin and benefit the physiology of the host. Moreover, riboflavin pathway derivatives, such as 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 6,7-Dimethyl-8-(1-D-ribityl)lumazine (RL-6,7-diME), function as evolutionarily conserved non-peptidic antigens to a subpopulation of innate-like T cells, Mucosal Associated Invariant T (MAIT) cells, particularly enriched at mucosal surfaces of mammals, such as the gut, bronchea, skin and uterus[187–189]. These MAIT cells have a semi-invariant T-cell receptor $TCR\alpha_{(TRAV1\text{-}2\text{–}TRAJ33)}\beta_{(TRBV20\text{-}TRBV6)}$ and can be activated

upon recognition of the riboflavin derivatives 5-OP-RU, 5-OE-RU, or RL-6,7-diMe bound to Major Histocompatibility complex class 1 related (MR1) protein, which is ubiquitously expressed on the cell surface of epithelial cells and immune cells[187]. While researchers traditionally focused on the anti-pathogenic response of MAIT cells, such as their direct cytotoxic activity and/or indirect antimicrobial activity in bacterial[190], fungal[191], and viral infections[192], their protective role in epithelial barrier enforcement was only recently discovered in the context of the immune response to SARS-CoV-2 infections[193–196]. In particular, MAIT cells have been shown to establish commensal-driven tissue homeostasis—tissue maintenance, tissue repair, and wound healing—crucial processes both in absence and presence of infections, as reviewed in refs. 197,198. For instance, the riboflavin derivate 5-OP-RU has been shown to activate MAIT cells and this stimulated the healing of a punch-biopsy induced skin wound in mice[199]. Of interest, early-life exposure to riboflavin-producing commensals has been shown to promote the correct development of the gut and skin MAIT cell response in germ-free murine neonates primed with a synthetic early-life gut microbial community consisting of two *Lactobacillaceae*, two *Enterobacteriaceae* and *Enterococcus faecalis* and the skin commensal *Staphylococcus epidermidis,* respectively[200,201]. In mice, riboflavin-overproducing strains *L. plantarum* ACTT8014[202] and *L. plantarum* CRL2130[203] have also been shown to significantly attenuate pathological changes of chemotherapy-induced mucositis during cancer treatments compared to the non-overproducing *L. plantarum* CRL725 and commercial riboflavin[203]. The same riboflavin-overproducing strain also showed antioxidant and anti-inflammatory mechanisms by attenuating motor deficits and prevented dopaminergic neuronal death in murine models of Parkinson's disease[204]. Yet—to the best of our knowledge—no clinical trial has been initiated or performed linking riboflavin-producing probiotics and impact on host health.

Need for in vivo documentations in humans

The above mentioned studies are mainly based on in vitro and preclinical data, while the field would largely benefit from a more solid documentation of the capacity of riboflavin-producing organisms to increase riboflavin in vivo in humans by for example metagenomic/proteomic/metabolomic studies of the gut and vaginal microbiomes. For example, riboflavin could—theoretically—also support the mutualism between microbiota members, a concept that is increasingly evaluated as (gut) microbiome resilience, also by EFSA[205]. Within microbial communities, riboflavin is produced by prototrophic species (i.e., species that are equipped with a complete and functional pathway for de novo biosynthesis of certain micronutrients) to cross-feed auxotrophic species (i.e., species that lack the corresponding biosynthesis pathways) in exchange for other metabolites, mostly nutrients or energy[206]. For instance, in vitro co-cultures and synthetic gut microbiome communities have shown that riboflavin promotes cross-feeding networks involving butyrate production pathways[207,208]. In addition, riboflavin can stimulate flavin-based extracellular electron transfer (FLEET) by *Lactobacillaceae*, as recently learned from *Lactiplantibacillus plantarum* and vegetable fermentations[209]. In general, these fermentations function as valuable models for studying fundamental microbial interactions, while excluding the host as complicating factor. It is now increasingly understood that FLEET allows respirofermentation in LAB, a hybrid metabolism form that integrates some aspects of (anaerobic) respiration, such as EET, in fermentation (substrate level phosphorylation)[209]. It is suggested that the ability to transfer electrons outside the cell, and thus maintain redox balance during rapid growth, results in a vast fitness advantage, and seemingly more resilient microbial population. However, the role of FLEET in mammalian physiology is still unclear. Yet, FLEET by cecal microbiota has already been observed in mice, rats, and guinea pigs using cyclic voltammetry, while it appears absent in germ-free mice[210]. These findings are in line with the population dynamics hypothesis that cooperation (e.g., cross-feeding) enhances the resilience of microbial communities during ecological disturbances[211,212]. Conformingly, in the gut, where diet is the predominant ecological driver, short-term dietary changes and nutritional shortages do not, according to a systematic review[213], significantly alter the microbiome composition. Conversely, microbiome function is impacted by the diet, for example the production of microbial riboflavin is influenced by a fiber-rich diet[214]. This highlights the potential of modulating the human microbiome in the gut and other body niches through addition of riboflavin-producing microorganisms with multifactorial ecosystem-wide modes of action. This is also one of the core objectives in previous clinical trials such as the observational studies on the association between riboflavin synthesis genes and type 2 diabetes using metagenomics[156] and the ongoing clinical trial with the riboflavin-overproducing strain *L. reuteri* AMBV339 (ClinicalTrials.gov ID NCT06425081). The field would largely benefit from such substantiations of the association between riboflavin-producing probiotics and various aspects of health.

Concluding remarks and future perspectives

In conclusion, riboflavin deficiency could have a large impact on women's health in both developed and developing countries. Due to specific riboflavin demands of women linked to pregnancy, iron deficiency, hormonal homeostasis, contraceptive use, and other physiological and lifestyle aspects, it is crucial to ensure an adequate riboflavin status. Considering that a large proportion of women lack sufficient riboflavin intake, alternative riboflavin sources such as functional foods and nutraceuticals enriched with riboflavin, as well as probiotics, should be considered. Particularly for lactic acid bacteria-based probiotics, women could benefit from the synergistic effect of riboflavin and beneficial bacteria such as lactobacilli with great potential for women's health. However, more research is needed on the underlying mechanisms of the way micronutrient production can shape the female microbiome; the ability to share produced vitamins in microbe-microbe interactions; the beneficial effect of bacterial vitamin production on human and more specifically women's health. Furthermore, formulation, dosage and safety aspects of microbial supplementation should be considered for efficient application of riboflavin-producing bacteria in clinical settings. Ultimately, this research should include large-scale clinical intervention studies in humans with an integrated approach that combines microbiome, multi-omics, metabolomics and immunological readouts, and compares administration formulations and routes. Overall, leveraging riboflavin and riboflavin-producing lactobacilli is a promising avenue with a wide range of potential benefits for women's health.

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Competing interests

This review was written in the framework of the PhD research projects of C.D., I.E., and S.A. However, for transparency, the authors want to declare relationships and interests that could be perceived as a potential conflict of interest. This involves the patent application EP20210606.8

that has been submitted by the host institution (University of Antwerp) on findings related to microbially produced riboflavin in strain *Limosilactobacillus reuteri* AMBV339. S.L., I.S., and S.A. are listed as inventors. Moreover, the authors declare that they have received funding from different probiotic and food supplement companies to perform mechanistic and clinical research related to aspects discussed in this review.

Additional information

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