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Lactic acid bacteria probiotic strains: a review on evaluation criteria and riboflavin production abilities

Henri Sidabéwindin Ouédraogo[1]: PhD student; E-mail henriouedraogo5@gmail.com
Boukaré Kaboré[1:] PhD; E-mail: kaboreboukare27@gmail.com
Oumarou Zongo [1, 2 *]: PhD; E-mail:ozongo25@gmail.com
Ganamé Abasse Ouédraogo[1]: PhD; E-mail: ganamabasse@gmail.com
Prudence Dounia [1, 5:] PhDstudent E-mail : prudencia@gmail.com
Hama Cissé[1]: PhD; E-mail: cissehama70@gmail.com
Moustapha Drabo [1,3:] PhD; E-mail: m.soungalodrabo@gmail.com
Sèmassa Mohamed Ismaël Hoteyi [4]: Senior Researcher ; E-mail : smihot@yahoo.fr
Aly Savadogo[1]: Professor; E-mail: alysavadogo@gmail.com

[1]Laboratoire de Biochimie et Immunologie Appliquées (LaBIA) au Burkina Faso, Université Joseph KI-ZERBO, 03 BP 7021 Ouagadougou 03, Burkina Faso;
[2] Unité de Formation et de Recherche en Science et Techniques (UFR/ST), Université Thomas SANKARA, 12 BP 417 Ouagadougou 12, Burkina Faso;
[3] Centre Universitaire de Gaoua, Burkina Faso
[4] Centre Beninois de Recherche Scientifique et de l'Innovation. 05 BP 1604 Cotonou, Bénin;
[5]Université de N'Djaména, BP 1117 N'Djaména, Tchad
*Corresponding author's Email: ozongo25@gmail.com; **Oumarou Zongo*** Tel.: (00226) 71057944

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Abstract
LABs are powerful fermentation agents and the most used probiotic species in the food processing and health field. Probiotics are live microorganisms whose consumption in adequate quantities improves host health. During food fermentation by LABs, several metabolites are synthesized, among which the water-soluble vitamins essential for the proper functioning of the human organism are of particular interest to researchers. Riboflavin is one of these vitamins whose participation in the proper functioning of the body is observed through its involvement in various processes. Riboflavin is involved in energy reactions and nucleic acid synthesis in humans. Double interest is therefore attached to probiotic LABs producing riboflavin. These microorganisms are used in food biofortification without harmful consequences on the one hand; on the other hand, they improve consumer health. Particular attention is

therefore, paid to the choice of the probiotic LAB-producing riboflavin. The aim of this study was to explore methods of evaluating the probiotic and riboflavin production abilities of lactic acid. Several approaches, including phenotypic and molecular methods, are used to evaluate the suitability of LABs to meet probiotics criteria and their ability to synthesize riboflavin. It also emerged that probiotic LABs are used in various food matrices to biofortify them with riboflavin. This ability to synthesize riboflavin has led to its use in the agri-food industry and healthcare to prevent and treat certain pathologies.

Keywords: Biosynthesis, Criteria, LABs, Probiotic, Riboflavin

1. Introduction

The oldest known use of lactic acid bacteria (LABs) is their involvement in food fermentation. Today, LABs are the focus of much research because of their probiotic ability. Any microorganism whose consumption confers a health benefit to the host is defined as a probiotic. However, LABs are known as the bacterial group with more probiotic species [1]. Probiotic LABs offer several advantages justifying their use in various fields. In the food field, they are used for foodstuffs fermentation. This ability of probiotic LABs improve foods organoleptic properties as well as their shelf life. Authors have reported on the impact of probiotic LAB on organoleptic characteristics such as taste, color, and aroma of fermented foods. Thus, Yang *et al*. [2] reported that the use of probiotic lactic starters such as *Lactobacillus gasseri* BNR17 and *Lactobacillus plantarum* HY7714 improved the aroma, taste, and color of Greek yogurt, which is highly appreciated by consumers. Also, probiotic LABs improve the nutritional quality of foods by degrading certain antinutritional factors such as tannins and mineral-chelating phytates. Probiotic LABs also improve the food matrix's digestibility by hydrolyzing non-digestible oligosaccharides and macromolecules [3]. The literature reports on the choice of lactic acid bacteria as natural starters and probiotics, as well as the ability of these bacteria to produce various vitamins, particularly those in the B group, such as folates and riboflavin. However, little has been known about riboflavin production by probiotic lactic acid bacteria.

Food nutritional quality is also improved through several essential micronutrients such as fat-soluble and water-soluble vitamins synthesized by LAB. This ability to synthesize water-soluble vitamins, in particular those belonging to group B, is sought in LAB probiotics [4].

Group B vitamins are essential for the proper functioning of the human body, but the latter can not synthesize them. Therefore, an exogenous contribution is necessary to meet the body's needs for B vitamins [5]. Thus, plants and fruits are used to meet body needs in folate, riboflavin, thiamine, niacin, biotin, pantothenic acid, and pyridoxine. Mineral and vitamin micronutrient fortification is practiced voluntarily or by imposition in over 149 countries worldwide [6] However, compliance with fortification policy regulations varies according to the economic environment. Manufacturers comply with regulations in high-income countries, but the situation is different in low-income countries, where the majority struggle to monitor compliance. With regard to riboflavin fortification, 62 countries, including the USA, have set standards for this vitamin [6]. These standards apply more to cereal products. In the USA, for example, adding 1.8 mg of riboflavin to a pound of wheat flour has been imposed. The need for group B vitamins constitutes a vast market for the industrialists who produce these vitamins. These are used to fortify certain food products for human consumption in several countries. However, the consumption of high doses of these synthetic vitamins has harmful consequences on the health of consumers. Indeed, several studies have established a correlation between high doses of synthetic vitamins and lung cancer development [7]. Synthetic vitamins are increasingly abandoned in favor of natural ones produced by microorganisms, in particular probiotic LABs. According toTurck *et al*. [8] this micronutrient should be ingested daily in a proportion of 1.0–1.6 mg/day for adults and 0.4–1.4 mg/day for children depending on gender and age. This micronutrient must be ingested daily because the

human organism is unable to store it and excesses are directly excreted in the urine. Pathologies related to riboflavin deficiency have been reported worldwide [9]. Ariboflavinosis affects a high proportion of the population of Western countries. In some European countries, vitamin B2 deficiency is estimated to affect 5 to 75% of the population [10]. Riboflavin production is estimated at more than 10,000 tonnes per year, 70% of which enters the livestock sector compared to 30% in the pharmaceutical industry and human food [11]. This study highlights the advantages of using probiotic lactic acid bacteria as natural riboflavin synthesis factories. It discusses the importance of probiotic lactic acid bacteria in the riboflavin biofortification strategy for foods. Also, the current trend of researchers is to find probiotic LABs as B2 vitamin biofortifying agents during food fermentation. These bacteria offer consumers a dual advantage. On the one hand, they have a direct positive impact on health, and on the other, they produce essential micronutrients indispensable to the proper functioning of consumer organisms.

2. LABs as probiotics

Lactobacilli, with over 183 species known today, has the largest number of probiotic LABs. It is also the dominant type of LABs in animals' and humans' gastrointestinal and digestive systems. Other probiotic LABs species belonging to *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc* genera have been identified. Probiotic LABs originate from several ecological niches, such as milk, dairy products, and fermented products of plant origin. LABs are used in food processing to enrich certain food products, such as bread, pasta, and soy milk, with riboflavin [12,13]. *Lactobacillus plantarum* CRL 2130 and *Lactobacillus fermentum* can synthesize riboflavin from these food matrices [14,15]. To qualify LAB as probiotic, LABs must meet the criteria established by the FAO and WHO [16]. According to these organisations, the probiotic must have the ability to resist gastrointestinal hostile conditions of the digestive tract, and it must be able to adhere to epithelial and mucosal cells of the digestive tract. The probiotic must have antipathogenic activity and be safe and suitable for clinical applications.

2.1. Phenotypic and genetic screening of LAB probiotic abilities

Several phenotypic techniques are performed in the evaluation of probiotic properties. Thus, resistance to hostile environmental conditions of the gastrointestinal tract, in particular pH acidity, bile salts, and gastrointestinal juices, are sought *in vitro*. All these tests are carried out in medium mimicking digestive tract conditions. Thus, for the first two, either HCl or Oxgall is added to a PBS solution to assess low pH and bile salts tolerance, respectively. Kim *et al*. [17] evaluated *Limosilactobacillus fermentum* KGC1601 ability to resist acid pH and bile salts by inoculating 10^8 CFU/mL of the strains in PBS adjusted to pH 2.5 with HCl and 0.1% oxgall, respectively. After 3 hours of incubation, the strains were plated on MRS agar, and the strains that tolerated low pH and bile salts were counted. The technique is certainly simple but has the disadvantage of eliminating certain strains capable of resisting low pH and bile salts. Although, neither pH nor bile salts are stable *in vivo*, the pH for example can reach a magnitude between 4 and 5 during food remains time in the digestive tract [18].

To assess gastric and intestinal juices resistance, Iyer *et al*. [19] used a solution composed of 3 g/L pepsin from porcine stomach mucosa (Sigma) pH 1.5, pH 2.0, and 3.0 and another solution whose composition was 1 g/L pancreatin from porcine pancreas (Sigma), pH 1.5, pH 2.0 and 3.0 with 0%, 0.5%, 1%, 2% bile) for gastric and intestinal juice respectively. *Streptococcus thermophilus* strains at 7.0 log CFU/mL concentration were inoculated into these solutions at 37°C, and strain resistance was evaluated at 0, 30, 60, and 120 min corresponding to transit time in the stomach and the intestine. In addition, the ability of strains to adhere is an important criterion in probiotic strain selection. This property is evaluated *in vitro* through various tests including autoaggregation, hydrophobicity, and the use of mammalian epithelial cells.

[20,21] evaluated the adhesion capacity of LAB through autoaggregation and hydrophobicity. Thus, for autoaggregation, the cells were washed twice in PBS and resuspended in PBS or saline (0.85% w/v) solution, and the OD_{600} was adjusted to 0.5 or 0.4. After 2 hours of incubation, 1 mL of the supernatant was taken to read the absorbance. The strain-free broth was used as a control. The autoaggregation capacity was expressed as a percentage.

As for the adhesion capacity by cellular hydrophobicity, it can be carried out by tests on hydrocarbons, in particular xylene or n-hexane. To do this, 3 mL of bacterial suspension was added to 600 µL of hexane or xylene, then vortexed and left to incubate at 37°C for 1 hour. The aqueous phase was carefully recovered, and its absorbance is measured at 600 nm. Also, strain adhesion capacity can be evaluated using mammalian epithelial cells, particularly HT-29 (a human colorectal adenocarcinoma cell line) and Caco-2 (Human colonic adenocarcinoma cell line) cells. According to Zuo *et al*. [22], the adhesive activity of bacterial strains to HT-29 and Caco-2 cells is assessed by seeding 5.10^5 cells/well in 24-well culture plates. They were incubated at 37°C under a humidified atmosphere with 5 and 95 % CO_2. After 24 hours, a confluent monolayer formed on the wells of the plate. These monolayers were rinsed twice in PBS and 10^8 CFU/mL concentration of test strain was added to the plate wells. After 24 hours of incubation, the wells were washed six times in PBS (pH 7.4). Thus, strains unable to adhere to HT-29 and Caco-2 cells were discarded, and the monolayers were fixed with methanol, stained following Gram protocol, and then observed under the microscope.

In addition to human cells, other cells, such as those from pigs and chicken cells, were used to test the adhesion ability of strains with probiotic potential. Dowarah *et al*. [23] collected pig and chicken intestines and washed them three times with PBS at 4°C for 30 min. A suspension of 10^9 CFU/mL of the test strain was incubated with an intestinal sample (1 cm^2) for 30 min at 37°C. After incubation, the intestinal sample was fixed with 10% formalin, dehydrated, and embedded in kerosene. Serial sections (5 µm) were made and stained with hematoxylin and eosin. Slides were observed at 100× and 400× magnifications and photographed.

In addition to the above-mentioned criteria, antipathogenic activity is also an essential criterion in the search for probiotics. This property is exerted against pathogens through antimicrobial metabolites (ribosomal and non-ribosomal peptides) and coaggregation. Several authors have highlighted the antipathogenic activity of probiotic LABs. Parlindungan *et al*. [21] have demonstrated the ability of probiotic candidates to synthesize antimicrobials against pathogens. These authors used the agar diffusion technique to prove the ability of LAB as probiotic candidates to produce ribosomal peptides, particularly bacteriocins, as antimicrobials. Thus, after 24 hours of growth in broth, potential probiotics were centrifuged at 4000 g for 10 min. The cell supernatant without bacterial cells was collected and filtered using a 0.20 µm pore membrane. The pH of the filtrate was neutralized using 5 M NaOH. And to ensure that the pH is not an inhibiting factor and to eliminate the activity of other antimicrobials such as H_20_2 5 mg/mL catalase was added to the filtrate. Then, fresh strains indicator (pathogenic) were mixed with semi-solid medium (0.8% w/v) agar. After solidification, 8 mm diameter wells were made to contain 100 uL of cell-free supernatant. The antipathogenic activity of ribosomal origin was evaluated after 24 hours of incubation by measuring the inhibition zone.

Also, probiotic LABs exert their antipathogenic activity by coaggregation of pathogenic strains. This property was researched by de souza *et al*. [24]. The latter grew pathogens and LAB with probiotic potential in their specific growth media. The strains were then washed with a 0.85 % saline solution at an OD_{660} of 0.3. The coaggregation capacity was evaluated by bringing into contact the same volume (500 µL LAB cells in combination with 500 µL) of pathogenic strains as of LAB strains in a cuvette. After 1 hour of incubation, the suspension

was vortexed and the OD_{660} of the supernatant was measured. As for competition with pathogens, the test can be carried out according to previous studies. The latter made a mixture containing an equal volume (10 µL) of individual probiotic and pathogenic strains (OD_{600}=0.5, ca. $\backsimeq 10^7$ CFU/mL) were added in the ratio 1: 1in a plate at 12 wells each containing ten synchronized ages of probiotic strains or probiotic candidate.

The plates were incubated at 20°C for 7-10 days. Physiological, morphological, and survival changes of the candidate probiotic strain were recorded at regular intervals. In addition to the previously mentioned criteria, the probiotic strain must demonstrate safety. This last property is evaluated through the search for undesirable activities such as hemolytic capacity, enterotoxin production, virulence, and antibiotic resistance. These activities have been phenotypically evaluated during several studies. The search for hemolytic activity was most often carried out on blood agar. Ficoseco *et al*. [25] evaluated the hemolytic activity of a lactic strain with probiotic potential by inoculating it on Columbia blood agar containing 5 % defibrinogenated horse blood after 48 hours of incubation at 37°C under aerobic and anaerobic conditions. The appearance of a clear zone around the colonies signified the production of β-hemolysin. To ensure the absence of hemolytic activity, potential probiotics should not produce enterotoxins. Several studies have investigated the presence of the genes responsible for the production of enterotoxins. The techniques used in the search for these genes are essentially PCR and *in silico* analysis. Table 1 summarizes the genes commonly sought in evaluating strains qualify as probiotics.

Table 1: Genes sought in the evaluation of the probiotic abilities of the strains

Genes	Proteins	General function	Reference
	Probiotic markers with a desired activity		
Ir 1516	Putative esterase	Low pH and bile tolerance	[47]
clpL	clpATPase (chaperon)		[48]
gtf	glycosyltransferase		[48]
bsh	Bile salt hydrolase	Bile salt resistance	[49]
LBA1679	ABC transporter		[50]
LBA1446	Multidrug resistance protein		[50]
LBA1429	Major facilitator superfamily permease		[51]
LBA0552	Major facilitator superfamily permease		[51]
lr1584	Major facilitator superfamily permease		[52]
aguA	Agmatine deiminase	Resistance to low pH	[53]
groEL	Heat shock protein 60		[53]
dltD	D-Alanine transfer protein		[53]
LBA1272	Cyclopropane FA synthase		[53]
La57	Amino acid antiporter		[50]
apf	aggregation-promoting factor	Auto-aggregation and Aggregation	[54]
Inu	Inulosucrase		[55]
mub	mucus-binding protein		[24]
fbp	fibronectin-binding protein		[20]

eno A1	Fibronectin binding/ alfa-enolase 1	Adhesion to intestinal mucus	[56]
msa	mannose adhesin		[56]
Mub-1	Mucin binding factor		[51]
map A	mucus adhesion promoting protein		[57]
srtA	Mucin binding factor		[18]
genes with exclusion activity			
ace	adhesion collagen protein	Virulence	[58]
esp	Enterococcal surface protein		[59]
agg	Aggregation protein involved in adherence to eukaryotic cells		[58]
gelE	Gelatinase		[59]
asa	Aggregation substance		[58]
cylA	Cytolysin		[24]
uidA	b-glucuronidase		[59]
sprE	Serine protease		[24]
hyl	Hyaluronidase		[24]
hdc	Histidine decarboxylase	Biogenic amines	[27]
tdc	Tyrosine decarboxylase		[27]
odc	Ornithine decarboxylase		[27]

2.2. Antibiotic resistance: a major criterion for probiotic exclusion

Antimicrobial resistance linked to probiotic consumption was first reported byEuropean Food Safety Authority [26]. This led to the inclusion of this aspect in the criteria for assessing the *GRAS* (generally recognized as safe) status of probiotics in 2012 [27]. The evaluation of antibiotic resistance remains one of the most important criteria in probiotic selection. Susceptibility to antibiotics is commonly carried out by the phenotypic method through the determination of the minimum inhibitory concentration. However, it is limited by the fact that the expression of resistance is linked to the environment and the presence of resistance genes.

Therefore, a molecular technique is necessary to search for the genetic determinants that could be at the origin of any form of resistance. Several researchers have investigated LABs genetic material when evaluating their probiotic potential. Still, the presence of a resistance gene in LABs does not automatically exclude it from being probiotic; rather the location of this gene is a determining factor of exclusion. A gene located on the bacterial chromosome is not transferable, and in this case, we speak of intrinsic resistance reported by several authors. For example, [28,29] reported that *Lactobacillus* species exhibit high intrinsic resistance to vancomycin.

In these species, the Vanc gene, responsible for resistance to vancomycin, is located on the chromosome. On the other hand, when the gene responsible for antibiotic resistance is located on a mobile element such as integrons, transposons, and plasmids of the bacterium, a safety problem arises about probiotic considerations. Indeed, resistance genes located on mobile genetic elements such as plasmids and transposons are likely to be transferred to pathogenic microorganisms in the intestine, compromising antibiotic therapy. In a review, Jose *et al*. [30] have been reported the presence and transfer of tetracycline tet (M) and erythromycin erm (B) resistance genes from probiotic LAB *L. plantarum* plasmid to *E. faecalis.* This is the main cause of healthcare-associated infections. Antibiotic resistance types in probiotic LAB, particularly in Lactobacilli are shown in Figure 1.

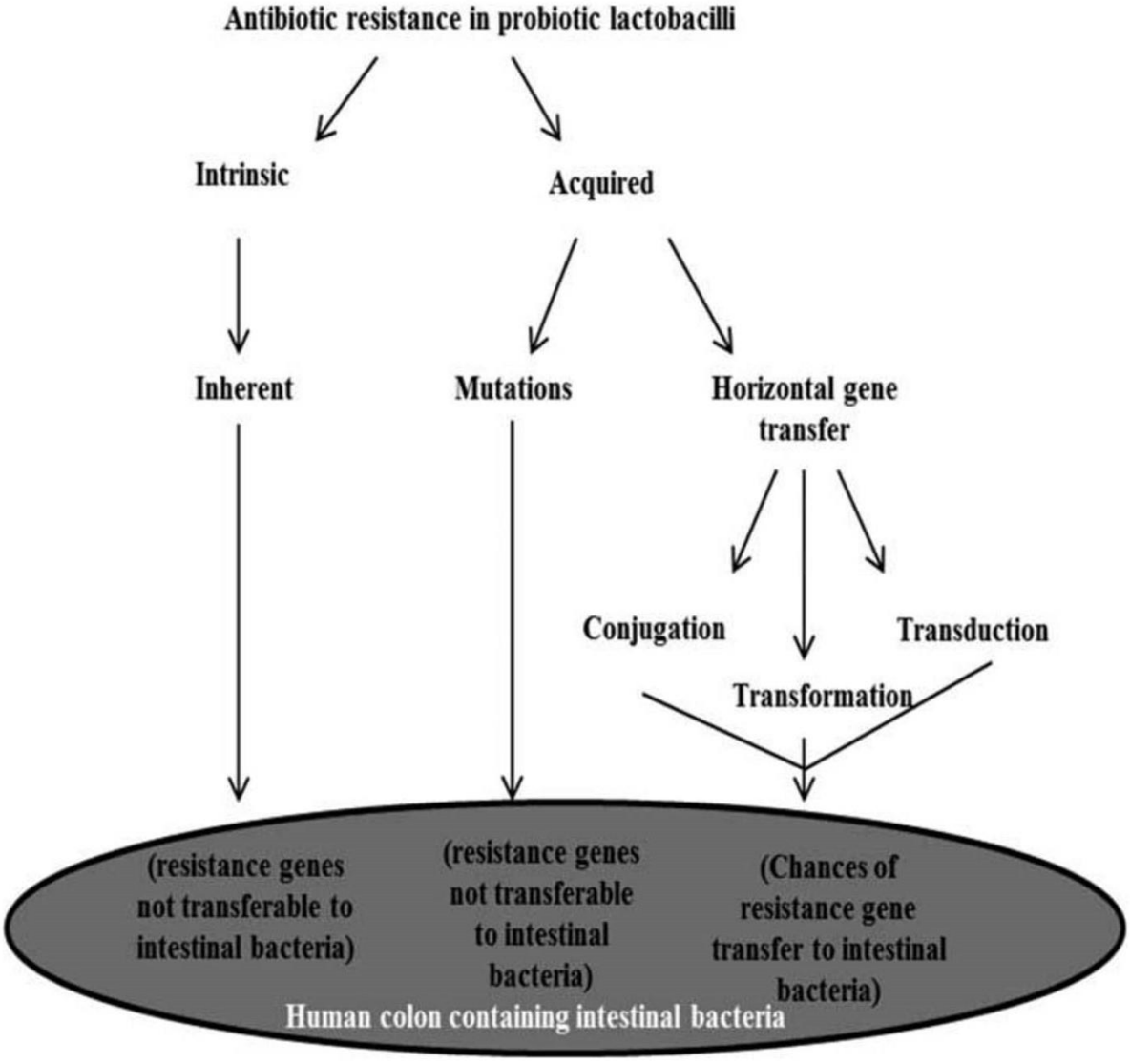

Figure 1 : The types of antibiotic resistance and classification of the resistance mechanisms resulting in transferable or nontransferable resistance genes according to Jose *et al*. [30]

To be qualified as a LAB probiotic, the lactic acid strain must demonstrate an efficacy *in*

vitro or in vivo clinical trials. In line with WHO recommendations, several clinical trials have been reported in the literature to assess the probiotic effect of lactic acid bacteria (Table 2).

Table 2: Some clinical trials of probiotic LABs according to the WHO recommendation

Probiotic LABs	Different health impacts according to designated references	References
	Overweigth and Obesity	
Enterococcus faecium, *Streptococcus thermophilus*	Body weigth, systolic Blood pressure LDL-C (Low-Density Lipoprotein Cholesterol) reduction, and fibrinogen levels increasing.	[60]
Lactobacillus gasseri SBT2055	Reduction in body mass index (BMI), waist and hip circumference and visceral abdominal fat (VFA).	[61]
Lactobacillus salivarius Ls-33	Ratios of Bacteroides, Prevotellae, and Porphyyromonas increase.	[62]
Lactobacillus gasseri SBT2055	Decrease in BMI and blood pressure measurements	[61]
Lactobacillus lantarum	Decreased BMI and blood arterial pressure.	[63]
Lactobacillus paracasei N19	No effects have been noticed.	[64]
	Non-Insulin-Dependent Diabetes Mellitus (NIDDM) and hyperlipidemia	
Lactobacillus olantarum A7	Reduction in the methylation process, SOD (Superoxide dismutase).	[65]
Lactobacillus acidophilus La-5, *Lactobacillus animalis* BB-12	Average changes in HbA1c, TC and LDL-C (Low density lipoprotein cholesterol) were significant between groups.	[66]
Lactobacillus acidophilus, *Lactobacillus reuteri* NCIMB	Lower LDL-C (Low density lipoprotein cholesterol) levels.	[67]
Lactobacillus acidophilus	A significant drop in LDL was observed.	[68]
Lactobacillus reuteri NCIMB 30242	In adults with hypercholesterolemia, low-density lipoprotein cholesterol and total cholesterol were reduced by 11.64% and 9.14% respectively.	[69]
	Antibiotic-Associated Diarrhea, Diarrheas, Colic, Ulcerative colitis	
Lactobacillus reuteri ATCC 55730	Suppression of symptoms and pain directly, related to intestinal colic.	[70]
Lactobacillus GG	The period of severe diarrhea in children and infants can be reduced by 24 hours by using probiotics.	[71]
Lactobacillus reuteri	Daily crying time was reduced by 25.4 minutes in infants treated against colic.	[72]
	Lactose intolerance attenuation	
Streptococcus lactis,	Promotes lactose assimilation and	[73]

Streptococcus plantarum, *Streptococcus cremoris*, *Streptococcus casei*, *Streptococcus diacetylactis*, *Streptococcus florentinus*	reduction of its intolerance.	
Lactobacillus delbrueckii sub sp. bulgaricus and *Streptococcus thermophilus*	Consumption of yogurt containing an average of 10^8 CFU per gram improves lactose digestion in people suffering from lactose indigestion.	[74]
Seasonal rhinitis		
Streptococcus paracasei-33	Nasal decongestion improvement, itching, and runny nose reduction.	[75]
Lactobacillus paracasei-33	Immunological and clinical benefits of probiotics in the treatment of allergic rhinitis	[76]
Cancer and related side effects		
Lactobacillus rhamnosus 573	Significant reduction in hospital care, abdominal discomfort, and number of chemotherapy doses.	[77]
Lactobacillus casei shirota (LcS)	Proven efficacy in preventing colorectal cancer.	[78]
Lactobacillus casei ATCC 393	In vivo apoptotic effects in colon carcinoma cells.	[79]
Lactobacillus Paracasei	Anticancer activity	[80]
Bacterial Vaginosis		
Lactobacillus rhamnosus	Vaginally administered probiotic strain balances vaginal flora and significantly reduces the incidence of bacterial vaginosis.	[81]
Lactcobacillus gasseri LN40, *Lactobacillus fermentum* LN99, *Lactobacillus casei* LN113, *Pediococcus acidilactici* LN23	LN strain is highly concentrated in the bacterial flora of the vagina Women with bacterial vaginosis treated with LN strain were cured within 2 to 3 days.	[82]
Lactobacillus acidophilus La-14® and *Lactobacillus Rahmnosus* HN001®	Probiotic strains La-14® and HN001® used in combination with bovine lactoferrin as part of an antibiotic treatment considerably reduced Bacterial Vaginosis symptoms. Fewer recurrences were recorded with the combination than with antibiotics alone.	[83]
Lactobacillus crispatus CTV-05	In 228 women suffering from recurrent BV and treated with 2 billion CFU of *L. crispatus* CTV-05, recurrence was reduced by 30% and 45% respectively in the intervention and placebo groups after 6 months.	[84]

3. History of riboflavin synthesis and Search for LABs with enhanced riboflavin productivity

In 1933, Merck developed an industrial process for a purely chemical synthesis of riboflavin. Fifty (50) years later, this process was still used to synthesize commercial riboflavin. This chemical synthesis of riboflavin was made from glucose through oxidation, conversion, esterification of the lactone, hydrogenation, and cyclization [31]. It was in 1980 that a semi-synthetic method was developed. It combines chemical enzymes and D-glucose from fermentation. Riboflavin was then produced following processes of crystallization purification, a cycle of condensation, crystallization, and drying [32]. This method was certainly better than the first; however, both were energy-intensive and polluting for the environment. The first riboflavin-producing microorganisms were used in the 1940s [32]. Since then, the synthesis of riboflavin at the industrial level has been ensured by microorganisms due to their multiple advantages, such as time savings, low energy use, and the substantial reduction in waste to be managed after production. Since, several screening techniques are used to monitor LAB-producing riboflavin. They were based on the LAB's ability to grow in a riboflavin-deprived environment. Thus, the strains are cultivated in a liquid or solid medium devoid of riboflavin. In the first situation, the LABs are cultured in a liquid minimum medium for 24 hours at 37°C. And the evaluation of bacterial growth was made by measuring turbidity [4].

For screening on a solid medium, the strains were inoculated into a modified chemical minimum medium. Then, 100 µL of the bacterial inoculum were cultured on an agar medium without riboflavin and incubated at 37°C. After 48 h, only strains capable of growing were selected as riboflavin producers [17]. However, improving the yield of riboflavin production in LAB can be done naturally by using toxic riboflavin analogs, which is by far the oldest strategy. In this technique, strains are put in contact with roseoflavin, and strains resistant to this molecule increase their riboflavin yield. Indeed, roseoflavin causes a spontaneous mutation in the regulatory region genes of probiotic LAB rib operon of probiotic LABs, resulting in high productivity of riboflavin [14]. Resoflavin-resistant LABs have been shown as probiotics with a high capacity to synthesize riboflavin in certain food products lacking this vitamin [13].

3.1. Importance of riboflavin in humans

In living organisms, adenine flavin dinucleotide (FAD) and flavin mononucleotide (FMN) are the two possible forms in which riboflavin can exist [33]. Through these forms, riboflavin participates in oxidation reactions at the tissue level and the electron transport of the respiratory chain. These coenzymes are necessary for all flavoproteins, such as glutathione reductase, which protects the cell against the harmful effects of reactive oxygen species (ROS). The latter oxidize nucleic acids (DNA), lipids, and proteins. In its FAD form, riboflavin is involved in the folate synthesis cycle by acting as a cofactor for methylenetetrahydrofolate reductase (MTHFR). Malabsorption and deficiency of riboflavin in the metabolism are characterized by low levels of FAD and FMN in the cells. This leads to cardiovascular pathologies, hypertension, mitochondrial, gastrointestinal, and brain dysfunction [34]. Acyl-CoA dehydrogenase deficiency can also be induced by poor riboflavin metabolism.

3.2. Biosynthesis and regulation of riboflavin in probiotics LABs

The biosynthesis of riboflavin takes place in LABs and can be done by two metabolic pathways, namely the purine pathway and the pentose phosphate pathway (Figure 2A). The purine pathway is a major pathway in cell function. This pathway participates in the synthesis of nucleic acids (DNA, RNA) and other metabolites, such as amino acids and vitamins essential to cell life [35]. Guanosine Triphosphate (GTP) and D-ribose-5phosphate undergo seven-step enzymatic reactions during riboflavin biosynthesis in probiotic LABs and other Gram-positive bacteria. The first step in riboflavin biosynthesis is catalyzed by the enzymatic activity of GTP cyclohydrolase II. GTP is encoded by the Rib A gene of the rib operon.

This gene encodes another enzymatic function that involves in the synthesis of a four-carbon unit from ribulose-5-phosphate [36]. The deamination of the pyrimidine ring and ribosyl side chain shortening, constituting respectively the second and third enzymatic steps, are controlled by the rib G gene [36].

The penultimate step in riboflavin synthesis is catalyzed by lumazine, an enzyme the rib H gene produces. Riboflavin synthetase regulates the last step in the pathway and is encoded by the rib H gene.[37]. At the LAB level, there are four proteins: Rib G, Rib B, Rib A, and Rib H, whose coding genes constitute the rib operon (Figure 2B).

The regulation of expression of the latter is done by transcriptional attenuation by the riboswitch FMN or RFN located in the 5' region of the untranslated rib mRNA. The riboswitch contains an aptamer binding the FMN. The behavior of the aptamer-FMN complex depends on the FMN cytosolic concentration. Indeed, once the level of the latter reaches the level required for its cofactor function, the complex binds to the riboswitch [38,39]. This results in the formation of a hairpin in the riboswitch and repression of transcription, inhibiting riboflavin biosynthesis (Figure 2C).

However, in the absence of cytosolic FMN, transcription of the operon occurs [40]. This regulatory mechanism is conserved in some species of lactic acid bacteria, such as*Lactococcus lactis*, *Leuconostoc mesenteroide*, and *Lactiplantibacillus plantarum* [9].

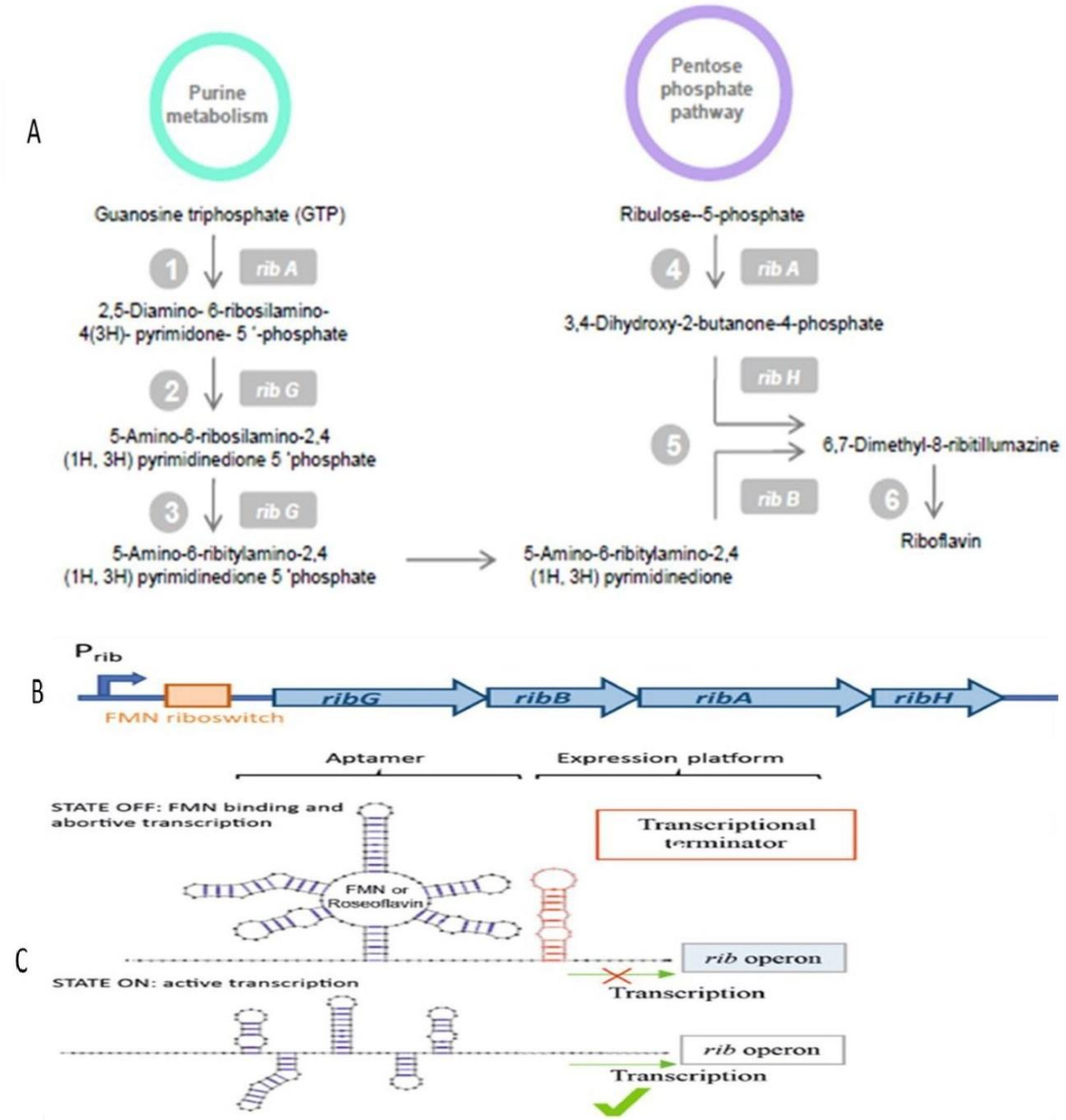

Figure 2: Mechanism of biosynthesis and regulation of riboflavin production in probiotic lactic acid bacteria. Adapted from Martín-loarte *et al*. [40] and Levit *et al*. [85]

Legend: (A): biosynthesis pathways of riboflavin (1: Cyclohydrolase II; 2: Desaminase; 3: Reductase; 4: 3,4-dihydroxy-2butanone-4-phosphate synthase; 5 Lumazine synthase 6: Riboflavin synthase); (B): Riboflavin operon (rib operon) and regulatory regions (P promoter and FMN riboswitch) and structural genes (ribG, ribB, ribA, and rib H); (C): Riboswitch of the rib operon with the FMN-binding recognition aptamer and the expression platform. Dual-conformation regulatory domain. The "off" state is responsible for the formation of a terminator and the "on" state is responsible for the formation of an anti-terminator structure.

4. Use of riboflavin-producing probiotic LABs in clinical care and the food sector

The therapeutic use of riboflavin-producing LAB probiotics is particularly focused on inflammatory bowel disease (IBD). IBD represent a group of chronic disorders in which inflammation of the gastrointestinal tract is the main manifestation [41]. Riboflavin-producing probiotic LABs have been proven effective in the treatment and prevention of IBD. These strains act on the immune system by regulating it. They also act against certain phenomena, such as oxidation. In the case of colitis, probiotic LABs exerted anti-inflammatory activity through riboflavin [42]. Thus, in different models of IBD, riboflavin-producing LABs probiotics (*Lactobacillus plantarum* CRL 2130) attenuated inflammation in inflamed rat colitis. This property is related to the bacterium's ability to prevent the anarchic increase of pro-inflammatory cytokines [17]. The immunological aspect revealed that the administration of riboflavin-producing LAB probiotic strains induced a fall in the concentration of pro-inflammatory markers in subjects suffering from inflammation, in contrast to anti- inflammatory markers, whose concentration increased in serum. A similar anti-inflammatory effect was reported by Levit *et al*. [43]. Regardless of whether the administered riboflavin- producing probiotic bacteria are incorporated into a food matrix or in bacterial suspension, their anti-inflammatory effect is evident *in vivo*.

The administration of vitamin-producing LABs to animals suffering from colitis has improved their health status. Among other things, certain disorders such as weight loss, large intestinal lesions, and microbial translocation have been reduced. The therapeutic potential of riboflavin-producing probiotic LABs has been demonstrated in 5-fluorouracil (5-FU) induced mucus inflammation. Following the administration of riboflavin-producing probiotic LABs, a reduction in diarrhea and an increase in liver mass were observed in animals [42]. LABs capable of reducing intestinal mucus colitis could be used as adjuvants in cancer chemotherapy [44].

In the food processing sector, riboflavin-producing probiotic LABs were used to improve nutritional quality and as an alternative to fortification with synthetic vitamins. In the dairy and baking industry, several studies have mentioned the use of riboflavin-synthesizing LABs probiotics. Zhu *et al*. [45] reported an enhancement of riboflavin levels in soymilk from 0.2 to 3.8 μg/mL and 1.9 μg/mL by *Lactobacillus fermentum* UFG169 and *Lactobacillus plantarum* UFG10, respectively. Those strains are suitable for the development of functional foods

enriched with riboflavin *in situ* and would provide a health benefit related to probiotics properties [46].

5. Perspectives

The present study focuses on tests to evaluate the probiotic criteria of lactic acid bacteria and the capacity of the latter to synthesize riboflavin. It appears that several approaches are used, notably phenotypic and molecular, in evaluating probiotic criteria. The absence of transferable resistance and virulence genes are among the most important safety criteria in the choice of probiotics. Indeed, the ideal probiotic is devoid of intrinsic or acquired resistance. However, authors have reported the presence of antibiotic resistance genes as well as the presence of elements transferable mobile genetics within commercially available probiotic lactic acid bacteria. Also, the presence of virulence genes has been reported in probiotic strains [30]. The probiotic bacteria that have been studied include *Enterococcus faecalis, Enterococcus faecim,* and *Streptococcus thermophilus.* These data raise questions about the effectiveness of multiple means of evaluating probiotic criteria. The methods used to identify the existence of elements compromising the health of the host within probiotics are comparative proteomics, functional genomics, and whole genome sequencing. We must therefore emphasize screening techniques for genes responsible for adverse effects during the probiotic selection process.
From a nutritional point of view, *in situ* biofortification with riboflavin-producing probiotic LABs would be an alternative to fortification with synthetic vitamins. Various food matrices have been subject to conclusive experiments of biofortification with riboflavin synthesized by LABs. These are soy milk, watermelon juice, and dairy products. LABs currently used for this purpose are *Lactobacillus fermentum* and *Lactobacillus plantarum* [45]. It appears from all of the above that the techniques used during the evaluation of probiotic criteria are decisive for the choice of a probiotic. We also note that the use of probiotic lactic acid bacteria as leavens in fermentation could be a source of functional foods. However, it would be interesting to find probiotic lactic acid bacteria having a stable genome devoid of elements transferable mobile genetics to prevent these strains which present themselves as hope in the fight against antimicrobials from becoming sources of failure therapeutics, which would amplify public health problems.

6. Conclusion

Riboflavin-producing probiotic LABs could be used in food fortification instead of synthetic vitamins. The use of LABs probiotic synthesizing riboflavin is an opportunity for manufacturers from an economic and environmental point of view. This strategy consumes less energy and generates little waste. Probiotic LABs synthesizing riboflavin are safe and offer several advantages justifying their use in food processing for micronutrient-enriched and multifunctional foods. In the health field, these LABs are used to prevent and treat pathologies. Several studies have demonstrated the efficacy of riboflavin-producing LABs in the treatment of diarrheal and inflammatory diseases *in vivo* in rats and *in vitro*. Using riboflavin-producing probiotic LABs as starters is advantageous from economic and health perspective.

Authors Contributions

Conceptualization, A.S and S.M.I.H; writing-original draft preparation, M.D., B.K and H.C; writing-review and editing, H.S.O., P.D., O.Z. and G.A.O.

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