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Biotechnological Production of Vitamin B2-Enriched Bread and Pasta

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ABSTRACT: Lactic acid bacteria (LAB) were obtained from durum wheat flour samples and screened for roseoflavin-resistant variants to isolate natural riboflavin-overproducing strains. Two riboflavin-overproducing strains of *Lactobacillus plantarum* isolated as described above were used for the preparation of bread (by means of sourdough fermentation) and pasta (using a prefermentation step) to enhance their vitamin B2 content. Pasta was produced from a monovarietal semolina obtained from the durum wheat cultivar PR22D89 and, for experimental purposes, from a commercial remilled semolina. Several samples were collected during the pasta-making process (dough, extruded, dried, and cooked pasta) and tested for their riboflavin content by a high-performance liquid chromatography method. The applied approaches resulted in a considerable increase of vitamin B2 content (about 2- and 3-fold increases in pasta and bread, respectively), thus representing a convenient and efficient food-grade biotechnological application for the production of vitamin B2-enriched bread and pasta. This methodology may be extended to a wide range of cereal-based foods, feed, and beverages. Additionally, this work exemplifies the production of a functional food by a novel biotechnological exploitation of LAB in pasta-making.

KEYWORDS: bread, pasta, lactic acid bacteria, riboflavin

INTRODUCTION

Vitamins are minor but essential constituents of food. Riboflavin, also known as vitamin B2, is a water-soluble vitamin found in a range of nutritional sources. Riboflavin is a central and crucial component of cellular metabolism as it is the precursor of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), two coenzymes that play a central role in metabolism, acting as hydrogen carriers in biological redox reactions. In humans, vitamin B2 supports optimal body growth and erythrocyte production and aids in performing energy-yielding metabolic pathways (for reviews see refs 1 and 2). Humans require the presence of vitamin B2 in their diet because they lack a riboflavin biosynthesis ability, which is commonly present in many plants, fungi, and bacteria. Dietary riboflavin is present in liver, egg yolk, milk, and meat, whereas the vitamin is commercially synthesized for nutritional use in the fortification of various food products such as bread and breakfast cereals.[3] Besides dietary sources, riboflavin is also produced by certain components of the microbiota of the large intestine.[4,5] The Recommended Daily Intake (RDI) for riboflavin is 1.3 mg/day for men and 1.1 mg/day for women,[6] whereas the European Union riboflavin Recommended Daily Amount (EU RDA) is 1.6 mg/day.[7] The ingestion of excess quantities of riboflavin results in its renal clearance; consequently, sufficient amounts of riboflavin need to be regularly ingested. In the developing world malnutrition can lead to dietary deficiencies, mainly in vitamins, which may provoke a variety of health disorders. Growing health consciousness among the general public determines food choices and purchase behavior. Additionally, the incidence of inadequate vitamin intake is common in developing countries but is also found in many industrialized countries. In the developed world consumers are increasingly aware of their nutritional requirements and, even if vitamin requirement is usually well supplied by a balanced diet, significant subgroups are still subjected to the risks associated with low micronutrient intakes.[8]

Wheat-derived foods (bread, pasta, noodles, and other food products) represent a primary component in the diet of many populations. Wheat contains various essential nutrients including the B group of vitamins.[9−12] Like other B vitamins, riboflavin is present in the germ (26% of the total grain riboflavin content) and in the aleurone layer (42%); however, also the endosperm contains a significant concentration of this vitamin (32%).[13] In response to the consumption of refined products, fortified food products have received increasing attention. Riboflavin is stable when exposed to high temperature, oxygen, and acid, but becomes unstable following alkali and light exposure.[14−16] Vitamin B2 belongs to a wide range of vitamins and minerals that can be safely added to foods at nutritionally relevant levels to supplement the normal diet.[8] For instance, 40% of the French population was marginally deficient with respect to the recommended intake of dietary riboflavin, whereas 6−15% of the population was receiving less than two-thirds of the RDI for the B vitamins.[17]

As an attractive alternative to the chemical synthesis of riboflavin, specific biotechnological processes for vitamin inclusion in foods have been developed.[18] Biotechnological approaches are

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more environmentally friendly, include the use of renewable sources, and lead to a yield of equal or superior quality.[19] Some bacteria and fungi are capable of riboflavin overproduction.[2] In bacteria this trait can be achieved either by metabolic engineering[20] or by exposure to purine analogues and/or the toxic riboflavin analogue roseoflavin.[21,22] Particularly, in *Lactococcus lactis* both of these approaches have been used with success.[3] Recent studies have reported on the selection of riboflavin-overproducing strains for potential food applications, for example, the manufacture of vitamin B2-enriched dairy products,[23] which were found to improve the riboflavin status of deficient rats.[24] The metabolic exploitation of such virtuous microbes has a relevant importance in also several foods prepared from cereals. From this point of view, sourdough represents an ideal environment as the microbiota of the traditional wheat sourdoughs has well been characterized, consisting of specifically adapted lactic acid bacteria (LAB), mostly lactobacilli, as well as yeasts. In particular, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paralimentarius*, *Lactobacillus rossiae*, and *Lactobacillus sanfranciscensis*, all characterized by low incubation temperatures, dominate the sourdough fermentation process.[25]

The present study reports on the selection of food-grade, riboflavin-overproducing LAB isolated from durum wheat flour and on their exploitation in bread- and pasta-making for the production of vitamin B2-enriched bread and pasta.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The LAB strains included in this study were routinely cultured on de Man−Rogosa−Sharp (MRS) medium. The pH of the medium was adjusted to pH 5.8 with 1 N KOH before sterilization for 15 min at 121 °C. Samples from three natural sourdoughs were aseptically collected from an artisanal manufacturer located in Apulia (Italy). For microbiological analyses, 10 g of each sample was homogenized in a stomacher bag with 10 mL of a saline−peptone water for 4 min, after which serial dilutions were prepared. For the isolation of LAB, MRS agar containing 100 mg/L cycloheximide was used, and the plates were incubated under anaerobic conditions (BBL, GasPack-System) at 30 °C for 72 h. Isolates were identified as putative LAB by positive Gram staining and negative catalase assay. All strains were stored at −80 °C in MRS supplemented with glycerol (20% v/v).

Genomic DNA of putative LAB strains was isolated using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's procedure. Then, 20 ng of DNA was added to a 50 μL PCR mixture and amplified with GoTaq (Promega, Milan, Italy). The primer pair ribF 5′ CTT CRG GGC AGG GTG 3′ and ribR 5′ GGR AAD ABR TGN CCN GG 3′ was used for the rapid selection of candidate riboflavin-producing strains using the GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Monza, Italy). Primer design was performed on the basis of a specific *rib* operon sequence alignment as reported under Results. The temperature profile was 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 120 s for 35 cycles. PCR reactions were terminated by performing a final elongation step (72 °C for 5 min), and then the amplicons were analyzed by electrophoresis. A 1 kb ladder (Promega) was used as molecular weight marker. As a positive control DNA from strain CB300, a roseoflavin-resistant derivative of *L. plantarum* NCDO1752, was used.[23] Multiple alignments for primer design were performed with the Clustal W2 program available on the European Bioinformatics Institute (EBI) Website.[26] The chemically defined medium (CDM) described by Wegkamp et al.[27] was modified for the selection of roseoflavin-resistant derivative (adapted by removal of riboflavin and nucleotides).

16S rDNA Sequence Analysis. Genomic DNA of putative *Lactobacillus* spp. roseoflavin-resistant derivative was isolated using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's procedure. The isolated strains were identify by partially sequencing the 16S rRNA-encoding gene. Most of the gene encoding the 16S rRNA was amplified by PCR using primers pA (5′ AGAGTTTGATCCTGGCTCAG 3′) and pH (5′ AAGGAGGTGATCCAGCCGCA 3′) according to the method of Edwards et al.[28] The amplicons were purified using a GenElute PCR Clean-Up Kit (Sigma, Dorset, U.K.) and sequenced. The resulting sequences were compared with sequences available at the NCBI database (GenBank) using the standard nucleotide−nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

Isolation of Roseoflavin-Resistant Strains. Spontaneous roseoflavin-resistant derivatives of *L. plantarum* isolates were obtained by plating mid logarithmic phase cells on CDM containing 100 mg/L roseoflavin (Santa Cruz Biotechnology, Santa Cruz, CA). After 5 days of incubation at 30 °C, roseoflavin-resistant colonies were isolated from the plates and further cultured on liquid CDM supplemented with 50 mg/L roseoflavin. Fully grown cultures were subsequently diluted in fresh medium and exposed to a roseoflavin concentration that was stepwise increased from 100 to 200 mg/L. From the final culture were isolated single colonies, which were analyzed for riboflavin production. Riboflavin-overproducing derivatives were subcultured in liquid CDM lacking roseoflavin for 60 generations, and extracellular riboflavin levels were measured every 10 generations to determine the stability of the riboflavin-overproducing phenotype.

Bread-Making Process. The commercial wheat flour (type "0" Manitoba) used in the baking process was supplied by Lo Conte − IPAFOOD (Ariano Irpino, Italy). Breads were prepared according to the procedure described in AACC 10-10B[29] with minor modifications in accordance with the procedure described by Borghi.[30] The reference water level was based on the farinographic water absorption of 57.6 mL. Standard bread-making was performed using commercial yeast starter cultures (*Saccharomyces cerevisiae*). An additional control bread was made using yeast starter cultures supplemented with *L. plantarum* WCFS1 to a final concentration of 2×10^8 CFU/g. In the in situ riboflavin bioproducing trial, together with yeasts, two selected spontaneous roseoflavin-resistant derivatives of *L. plantarum* isolates were added to a final concentration of 10^8 CFU/g for each of the employed strains.

Pasta-Making Process. Pasta was produced with two sources of semolina: a monovarietal semolina from the durum wheat cultivar PR22D89 and a commercial remilled semolina (De Cecco high-quality semolina, Fara San Martino, Italy). Semolina from PR22D89 was milled in a laboratory mill (MLU 202, Buhler, Uzwill, Switzerland) following overnight moistening of a grain sample (10 kg) by exposure to 16.5% humidity. The pasta-making process consisted of 16 h of semolina prefermentation with or without microbial starter, followed by kneading (about 10 min), extrusion (pressure range of 9.1−12.1 MPa and vacuum of 700 Torr), and drying at low temperature (50 °C for 18 h) in a pilot plant (Giussani, Fara D'Adda, Bergamo, Italy). In the in situ riboflavin bioproducing trials, to the two selected spontaneous roseoflavin-resistant variants of *L. plantarum* were added up to 10^7 CFU/g for each employed strain. The prefermentation at 30 °C was performed at two water content levels: 42 and 60%. For 60% water content, only the necessary amount of semolina was added to the final water volume to achieve the desired water content; after 16 h of prefermentation, the remaining semolina fraction was added to perform a classical pasta-making procedure. The dough was processed into spaghetti with a diameter of 1.7 mm. To assess pasta cooking quality, each type of spaghetti (100 g) was cooked in 1 L of boiling tap water, and the optimal cooking time was taken when the white core in the strands disappeared after squeezing them between two glass plates.

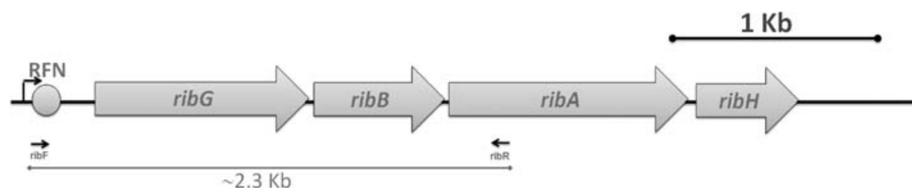

Figure 1. Schematic diagram of the *rib* operon for riboflavin biosynthesis in *L. plantarum* NCDO 1752.[3] Primer positions are indicated by arrows.

Table 1. Presence of Riboflavin Biosynthesis Genes among Various LAB Strains (Adapted from Burgess et al.[3])

Strain	*ribG*	*ribB*	*ribA*	*ribH*
Lactococcus lactis subsp. *cremoris* SK11	■	■	■	■
Lactococcus lactis subsp. *lactis* IL1403	■	■	■	■
Streptococcus pneumoniae TIGR4	■	■	■	■
Streptococcus pneumoniae R6	■	■	■	■
Streptococcus agalactiae 2603V/R	■	■	■	■
Pediococcus pentosaceus ATCC 25745	■	■	■	■
Lactobacillus brevis ATCC 367	■	■	■	■
Lactobacillus plantarum WCFS1			■	■
Lactobacillus gasseri ATCC 33323				■
Lactobacillus casei ATCC 334				
Lactobacillus bulgaricus ATCC BAA365				
Streptococcus thermophilus LMD-9				
Streptococcus pyogenes MGAS8232				
Streptococcus mitis				
Oenococcus oeni PSU1				
Enterococcus faecalis V583				
Bacillus subtilis				

Quantitative Analysis of Riboflavin. Samples were ground to homogeneity using a Tecator 1093 Cyclotec (Foss Italia, Padova, Italy) using a 1 mm sieve. Extraction conditions were the same as described by Leporati et al.[31] Briefly, samples were ground to homogeneity, using a domestic blender. Then, 1 g of sample was weighed out and quantitatively transferred into a 50 mL brown glass flask and hydrolyzed with 25 mL of 0.1 M HCl in a boiling bath for 30 min (with frequent mixing); alternatively, the mixture was treated in an autoclave for 30 min at 121−123 °C. After cooling, the sample was adjusted to 50 mL with 2 M ammonium acetate, shaken on a vortex, and centrifuged at 12000*g* for 10 min. About 1 mL of sample was filtered on 0.45 μm membranes, and 20 μL was then injected onto the LC column.

Pasta samples were taken at different steps of the process (kneading, extrusion, drying) and after cooking. Chromatographic conditions were the same as proposed by Batifoulier et al.,[12] with minor modifications. The determinations were carried out with a HPLC equipped with an UV detector (HPLC-1200 Agilent, Waldbronn, Germany). The separation was accomplished with a C18 column (250 mm × 4.6 mm, 5 μm Zorbax SB-C18 column, Agilent); the mobile phase (isocratic conditions) consisted of 0.05 M sodium acetate/methanol (30:70, v/v; pH 6.0). The separation was performed at 35 °C at a flow rate of 0.8 mL min^{-1} and an injection volume of 20 μL. The range of stock solutions used for the standard curve encompassed the entire range of classical riboflavin concentrations in cereal-derived food products. The correlation coefficient of the standard curve was 0.996. All quantifications were based on the peak area of riboflavin standard (Sigma-Aldrich). Three replicates were considered in all analyses. Vitamin concentrations were expressed as micrograms of riboflavin per gram of food material.

RESULTS

Isolation of Roseoflavin-Resistant LAB from a Sourdough Environment. Samples from three natural sourdoughs were collected in an artisanal pasta manufactory. A total of 120 microbial isolates were randomly recovered from the analyzed samples using selective MRS medium. Following a preliminary Gram stain and catalase reaction, 60 isolates (20 for each sourdough) considered as presumptive LAB (i.e., being Gram-positive and catalase negative) were selected. These strains were genotypically screened by PCR for the presence of the *rib* operon, which contains the genes involved in vitamin B2 biosynthesis in bacteria (Figure 1), using primers ribF and ribR so as to amplify the proximal part of this operon. In fact, bioinformatic analysis had previously shown that when LAB contain an incomplete *rib* operon, the first genes are absent from the genome[3] (Table 1). Primer design was performed on the basis of the ClustalW alignment of *rib* operon sequences selected among complete sequenced LAB genomes (*Lactobacillus brevis* ATCC 367, *Lactobacillus fermentum* IFO 3956, *Lactobacillus reuteri* DSM 20016, *Lactobacillus reuteri* JCM 1112, *Lactococcus lactis* subsp. *cremoris* MG1363, *Lactococcus lactis* subsp. *cremoris* SK11, *Lactococcus lactis* subsp. *lactis* Il1403, *Leuconostoc citreum* KM20, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293, *Pediococcus pentosaceus* ATCC 25745, *Bacillus subtilis* subsp. *subtilis* str. 168). The forward primer was designed to anneal to the regulatory region of the operon,[32] whereas the reverse primer corresponded to the *ribA* gene, amplifying a region of about 2.3 kb (Figure 1). Two strains were found positive after this preliminary PCR-based screening (data not shown), suggesting possible *rib* operon conservation. After 16S rDNA sequence analysis, the isolates were identified as strains belonging to *L. plantarum* and were consequently named *L. plantarum* UNIFG1 and *L. plantarum* UNIFG2. They were found to grow in the absence of vitamin B2, indicating a functional riboflavin biosynthetic capability. Spontaneous bacterial resistance to the toxic riboflavin analogue roseoflavin has previously been shown to lead to a riboflavin-overproducing phenotype.[2,3,23] To determine whether this method was also applicable to the LAB isolates from the sourdough environment, the two strains were plated at mid-exponential growth phase on CDM containing 100 mg/L roseoflavin. Following this strategy, a total of 17 roseoflavin-resistant variants were isolated (Figure 2). These isolates were grown in CDM and, after the entry in the stationary phase, the supernatant was analyzed for

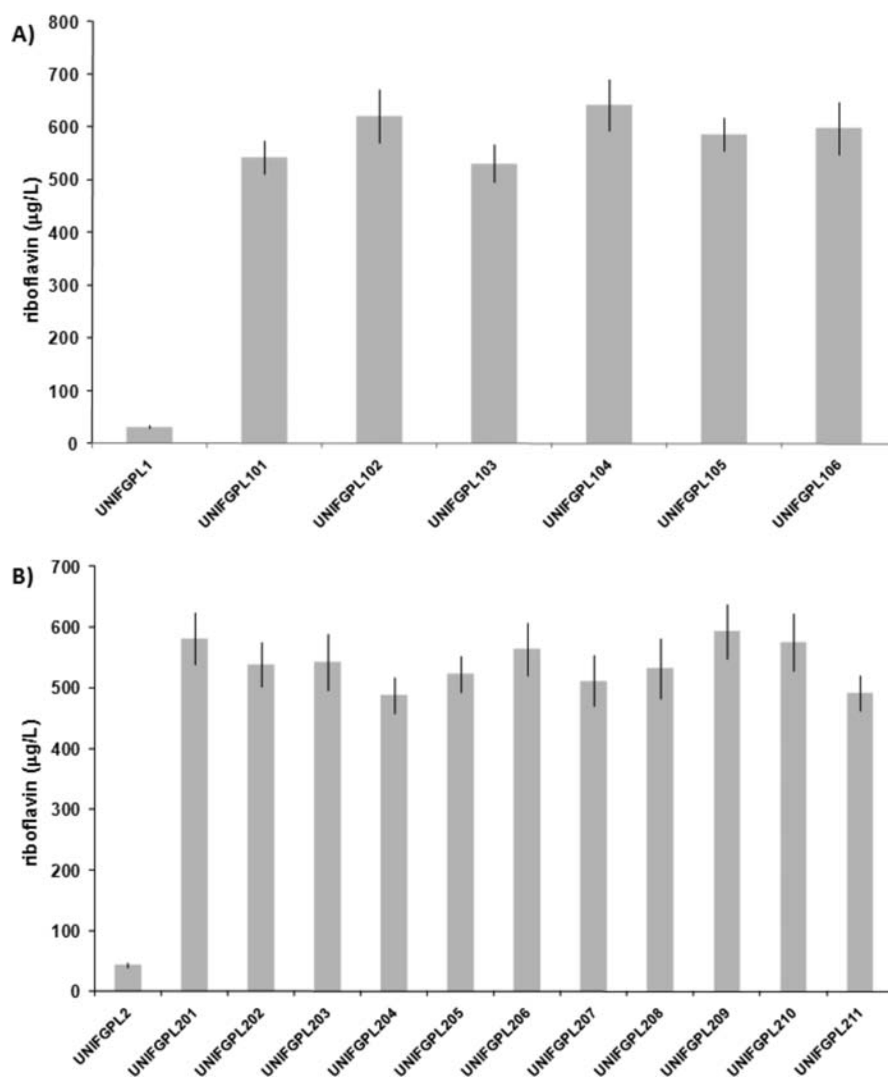

Figure 2. (A) Riboflavin produced by various roseoflavin-resistant *L. plantarum* UNIFG1 isolates; (B) riboflavin produced by various roseoflavin-resistant *L. plantarum* UNIFG2 isolates. The riboflavin concentration was determined in the cell-free supernatant as cells entered stationary phase. Data are the mean ± standard deviations, for $n = 3$.

riboflavin content. All isolates were found to accumulate vitamin B2 into the medium to different extents, from 488 to 642 μg/L (Figure 2). For each strain we selected a roseoflavin-resistant variant (UNIFGPL104 and UNIFGPL209) that exhibited the highest level of riboflavin production (Figure 2). To assess if the riboflavin-overproducing phenotype is stably maintained, the strains UNIFGPL104 and UNIFGPL209 were subcultured for 60 generations in the absence of selective pressure (i.e., in the absence of roseoflavin); the results showed that this phenotype is stably maintained for at least 60 generations (data not shown).

Bread Production Using Selected Riboflavin-Overproducing Strains. During a standard bread-making procedure, each of the two riboflavin-overproducing strains (UNIFGPL104 and UNIFGPL209) was added to the dough, together with baking yeasts, up to a final concentration of 10^8 CFU/g. The wheat flour used in these trials was found to contain 0.92 μg/g vitamin B2. A control assay was performed by adding only commercial baking yeast culture, whereas an additional control was set with *L. plantarum* WCFS1, which is unable to biosynthesize vitamin B2 due to an incomplete *rib* operon.[3,23] The contribution of baking yeast led to an increase in the riboflavin content in the final product of up to 2.41 μg/g (Figure 3). No relevant differences were detected when *L. plantarum* WCFS1 biomass was added (Figure 3). In contrast, we found a noticeable enhancement in vitamin B2 content (6.81 μg/g) of the final product when the two selected riboflavin-overproducing derivatives were added to the dough (Figure 3).

Pasta Production Using Selected Riboflavin-Overproducing Strains. The exploitation of LAB bioproductions in pasta-making represents an innovative biotechnological perspective. In our experimental design, we included a prefermentation step of 16 h at 30 °C with two different water contents: 33 and 60%. The former represents the water content of a typical pasta-making process, whereas 60% is the water content normally used for dough prepared in bread-making. After the prefermentation phase, the dough was directly mixed, extruded, and dried, following a classical pasta-making process. In semolina from PR22D89 we detected 0.85 μg/g vitamin B2. In the trial with no microbial addition and 42% water content, the samples collected after kneading, extrusion, drying, and cooking showed riboflavin

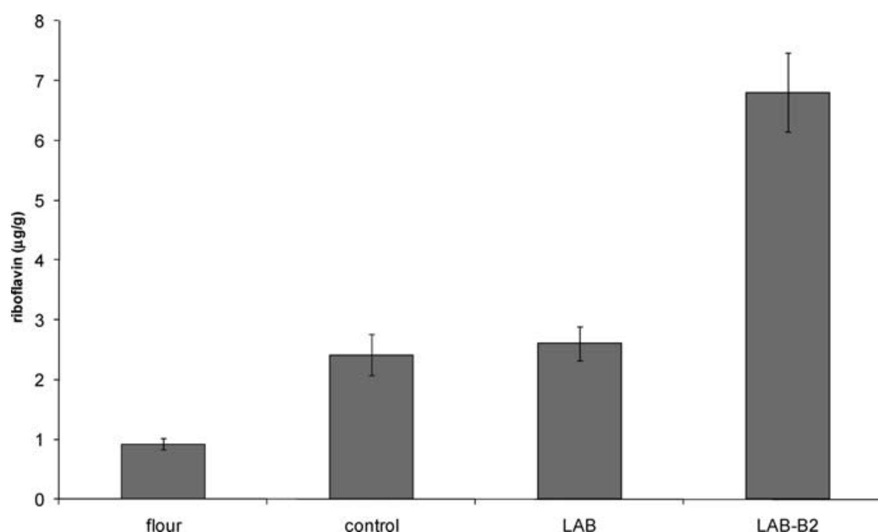

Figure 3. Riboflavin levels in the wheat flour and in bread inoculated with baking yeast cultures (control), with *L. plantarum* WCFS1 (LAB), and with selected riboflavin-overproducing derivatives (LAB-B2). Data are the mean ± standard deviations for three replicates. Riboflavin concentration was expressed as micrograms per gram of food material.

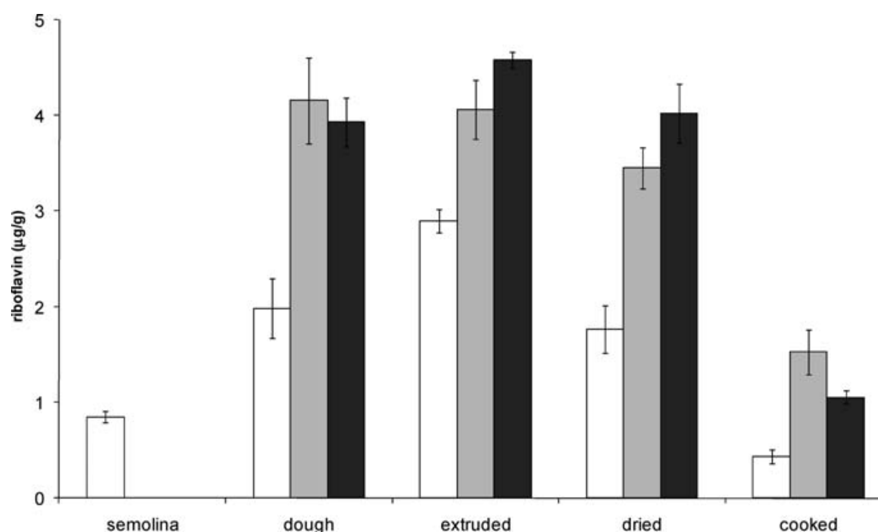

Figure 4. Vitamin B2 content is presented for PR22D89 semolina and for samples taken after kneading (dough), extrusion (extruded), drying (dried), and cooking (cooked). All trials were subjected to a prefermentation step of 16 h. White bars indicate the dough humidity at 42%, without microbial inoculation; gray bars and black bars indicate the dough inoculated with selected riboflavin-overproducing derivatives, with respectively 42 and 60% humidity levels. Data are the mean ± standard deviations for three replicates. Riboflavin concentration was expressed as micrograms per gram of food material.

contents of 1.98, 2.90, 1.77, and 0.44 μg/g, respectively (Figure 4). When the two selected riboflavin-overproducing derivatives were added to the prefermentation step, a significant increase of vitamin B2 was observed in all steps analyzed with respect to the noninoculated trial (Figure 4). In particular, vitamin B2 content doubled in cooked pasta (Figure 4). When the selected roseoflavin-resistant derivative was added to the prefermentation step at the high water content (60%), again, an increase of vitamin B2 was observed in all steps analyzed (Figure 4).

In addition to a monovarietal semolina from the durum wheat cultivar PR22D89, commercial remilled semolina was used for experimental pourpose. Commercial remilled semolina was found to contain 1.13 μg/g riboflavin. In the trial with no microbial addition and humidity levels set at 42% water content, the samples collected after kneading, extrusion, drying, and cooking showed riboflavin contents of 2.19, 3.20, 1.97, and 0.46 μg/g, respectively (Figure 5). When the two selected riboflavin-overproducing derivatives were added to the prefermentation step, the vitamin B2 profile changed as follows: 4.01 (dough), 5.91 (extruded), 4.59 (dried), and 2.89 (cooked) μg/g (Figure 5). When the two selected roseoflavin-resistant derivatives were added to the prefermentation step at higher water content (60%), the detected vitamin B2 contents were 4.37 (dough), 6.11 (extruded), 4.41 (dried), and 2.48 (cooked) μg/g (Figure 5). Furthermore, we observed a better performance of selected riboflavin-overproducing derivatives in remilled semolina, suggesting the importance of nutrient availability for the bacteria in the food matrix. In fact, after

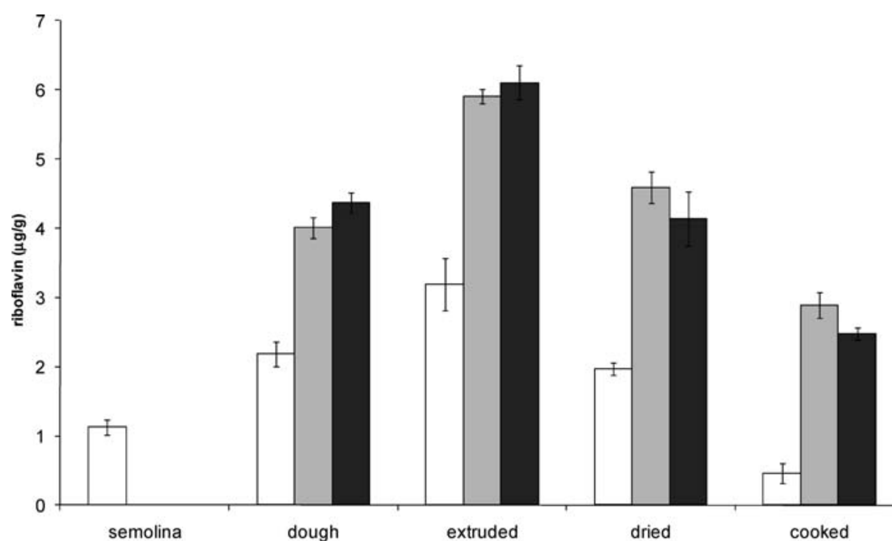

Figure 5. Vitamin B2 content is reported for commercial remilled semolina and for samples taken after kneading (dough), extrusion (extruded), drying (dried), and cooking (cooked). All trials were subjected to a prefermentation step of 16 h. White bars indicate the dough humidity at 42%, without microbial inoculation; gray bars and black bars indicate the dough inoculated with selected riboflavin-overproducing derivatives, with 42 and 60% humidity levels, respectively. Data are the mean ± standard deviations for three replicates. Riboflavin concentration was expressed as micrograms per gram of food material.

the remilling process, a higher percentage of damaged starch is generally released which, furthermore, may be susceptible to additional enzymatic activity.[33]

DISCUSSION

The concept of in situ fortification by bacterial fermentation provides the basis to enhance the nutritional value of food products and their commercial value. In recent years a number of biotechnological processes have been explored to perform a more economical and sustainable vitamin production than that obtained via chemical synthesis.[2] In fact, the biotechnological approach (i) is less expensive, (ii) is of equal or superior quality, and (iii) includes the use of renewable sources.[2]

Roseofavin-mediated riboflavin overproduction has been found to be associated with nucleotide changes and deletions in the RFN regulatory element.[2,3,23] The leading idea in this microbially based in situ vitamin B2 bioproduction was well elucidated by Burgess et al.,[2] who suggested replacing riboflavin-consuming strains used in traditional food fermentation processes with riboflavin-producing counterparts, thereby increasing riboflavin bioavailability in the food product and introducing an added health benefit.[2] Following this principle, we applied this strategy with two major wheat-derived products.

Batifoulier et al.[11] analyzed 10 wheat cultivars selected as representative of normal variability: riboflavin white flour content was 0.49 ± 0.01 μg/g, ranging from 0.43 to 0.58 μg/g. Corresponding bread products contained 1.10 ± 0.03 μg/g, with a range from 0.90 to 1.26 μg/g. The authors found an increase of about 2−3 times due to yeast fermentation, whereas no changes in riboflavin content were detected after the use of a sourdough starter culture. In accordance, we detected an increase of 2.6 times using only yeasts (when compared to the flour), confirming a contribution by baker's yeast metabolism. The addition of selected riboflavin-overproducing derivatives led to an increase of 7.39 times with respect to flour (2.82 times, if related to the addition of only yeast culture). One hundred grams of the obtained riboflavin-enriched bread contains 0.68 mg of vitamin B2 (52.3% of the RDI for men, 61.8% of the RDI for women, and 42.5% of the EU RDA), whereas 100 g of the corresponding uninoculated bread contains 0.24 mg (18.5% of the RDI for men, 21.8% of the RDI for women, and 15.0% of the EU RDA). The proposed approach is suitable for application in other sourdough-based products, tailoring the fermentation conditions on the basis of the product peculiarities.

Pasta is not a fermented product; nevertheless, the present study offers new interesting insights. To the best of our knowledge, this is the first report on the direct exploitation of the biotechnological potential of LAB in pasta-making. Indeed, although LAB were already used in a pasta-making process by di Cagno et al.,[34] in that case, semolina was processed by a pool of selected LAB; the final product was freeze-dried and, after that, used as a base to produce pasta. Spaghetti was generally found to contain 0.36−0.55 μg/g vitamin B2, with percentage loss in riboflavin during process and cooking of 36.4−52.8%.[10] Data reported by Bui and Small[35] provide a general idea about riboflavin in commercial fortified pasta: vitamin B2 contents in enriched spaghetti and in protein-fortified spaghetti were 0.50 ± 0.01 and 0.52 ± 0.02 μg/g, respectively (with losses during processing and cooking of 41.8 and 23.6%, respectively). Our study leads to a maximal content, after cooking, of 2.48 μg/g, corresponding to a contribution of a single 100 g serving dose of this pasta to the EU RDA of 15.6% (19.2% of the RDI for men, 22.7% of the RDI for women). In contrast, 100 g of the non-enriched pasta provides just 2.8% of the EU RDA, 3.5% of the RDI for men, and 4.2% of the RDI for women. In all pasta-making trials reported in this study, the riboflavin content follows the same profile, indicating a clear influence of the different processing steps on the biotechnological process. First, we detected an increase in vitamin B2 concentration between the kneading and the extrusion. Even if Russo et al.[36] noted an increase of LAB population from kneading to extrusion (suggesting a further contamination due to the kneading process), it appears difficult to address vitamin augmentation to differences in microbial population, because of the limited fermentation time (about 15

min between kneading and extrusion). We argue that the high pressures achieved during extrusion provoke bacterial lysis with consequent riboflavin release in the food matrix. The next two steps (drying and cooking) led to a consistent decrease of riboflavin content. During the drying process, spaghetti was noted to exude a lot of water, and we hypothesize that with water the product also lost a portion of the water-soluble vitamin B2. Finally, during the cooking process a wide mass transfer takes place between spaghetti and water, which therefore negatively affects the riboflavin content. In comparison with semolina, after 16 h of incubation, we found an increase in vitamin B2 content also in non-inoculated pasta. This observation indicates that autochthonous microbial consortia (yeasts and bacteria) are able to produce riboflavin in the dough at a low level. Additionally, we observed a better performance of selected riboflavin-overproducing derivatives in remilled semolina, and this result emphasizes the importance of nutrient availability for the bacteria in the food matrix. As a result of the remilling process a higher percentage of damaged starch is generally released.[33] Consequently, higher concentrations of soluble sugars may become available and promote microbial growth.

In conclusion, this paper presents a convenient and efficient food-grade biotechnological approach for the production of vitamin B2-enriched bread and pasta. The applied biotechnological strategy reported may be extended to a wide range of cereal-based traditional foods. Furthermore, our work presents a novel and direct exploitation of LAB with biotechnological potential in pasta-making.

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