frontiers

Supplementary Material

Weissella cibaria riboflavin-overproducing and dextran-producing strains useful for the development of functional bread

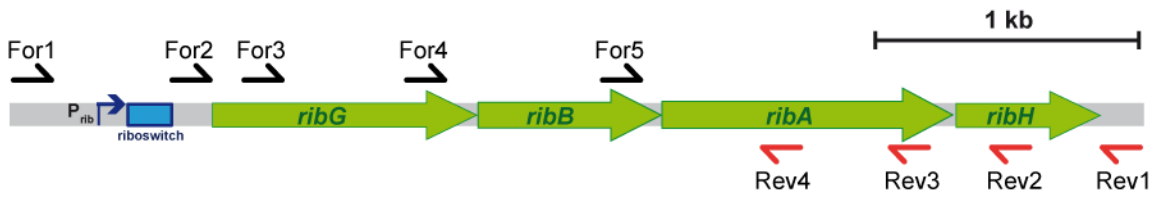
Annel M. Hernández-Alcántara[1], Rosana Chiva[2], Maria Luz Mohedano[1], Pasquale Russo[3], José Angel Ruiz-Maso[1], Gloria del Solar[1], Giuseppe Spano[3], Mercedes Tamame[2], Paloma López[1]*

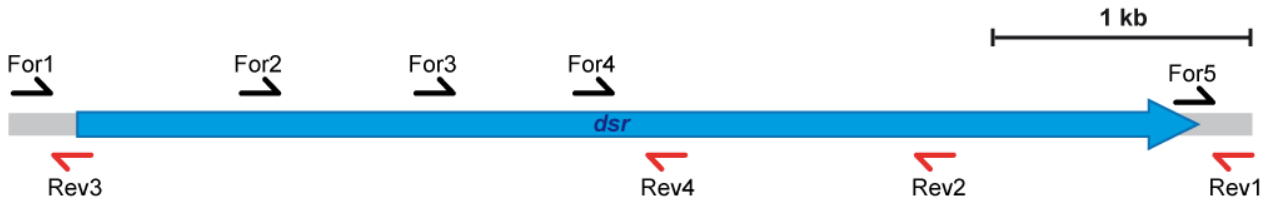
[1] Centro de Investigaciones Biológicas Margarita Salas (CIB), CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain

[2] Instituto de Biología Funcional y Genómica (IBFG), CSIC-Universidad de Salamanca, Zacarias González 2, 37007 Salamanca, Spain

[3] Department of Agriculture Food Natural Science Engineering, University of Foggia, , via Napoli 25, 71122 Foggia, Italy

Supplementary Table S1. Description and location of oligonucleotides used for DNA amplification and sequencing of *rib* operon and *dsr* genes

Amplification and sequencing of the *rib* operon		
Amplicon size (bp)	**Primers for amplification (5'-3') Length (nt)**	**Primers for sequencing (5'-3') Length (nt)**
4045 bp	For1: TGGCCTTGCGTGATATTTCG 20 Rev1: AGCATTGTACATCCCCTCAAA 21	For2: TTTTGGCCCCTTTACGCAG 19 For3: TATCAAGCCGCGCAACAAG 19 For4: TCCCAATCACACACCAACAAC 21 For5: CTAGTGCGACGGCGTTTGTGATT 23 Rev2: GTGGAATTTCAAATGCGCCG 20 Rev3: CTTCAATACCGTGCTTGGCT 19 Rev4: TTCACGTGCCATCCGACCATC 21
Primer walking hybridization map		
		
Amplification and sequencing of the *dsr* gene		
Amplicon size (bp)	**Primers for amplification (5'-3') Length (nt)**	**Primers for sequencing (5'-3') Length (nt)**
4546 bp	For1: GAAAGATTATGCCCGCGTTA 20 Rev1: GCCATATAACAGACTCCTCAAA 22	For2: TGGCGTGAAAGTGATGGTAA 20 For3: TTGAAAATAACGGCGACACA 20

		For4: TGGGTTAATGCCTACGGAAG	20
		For5: CCTGCCAAATGGTATTGCTT	20
		Rev2: AAAGCTTGATTGCGGACAAC	20
		Rev3: CGTTGCTTACCCGTTACCAT	20
		Rev4: CTACCGCACTTGCACTGTCA	20
Primer walking hybridization map			
			

Supplementary Table S2. Detection of LAB survival after dough fermentation and prior to the baking process

***W. cibaria* strain**	**CFU/g**
Uninoculated LAB	4.37×10^6
BAL3C-5	2.26×10^9
BAL3C-5 B2	1.83×10^9
BAL3C-7	1.82×10^9
BAL3C-7 B2	9.15×10^8
BAL3C-22	2.09×10^9
BAL3C-22 B2	2.81×10^9

Doughs were independently inoculated with cells of each BAL3C strain at a concentration of 1×10^9 CFU/g, and after 16 h of fermentation prior baking, the level of LAB CFU/g for the inoculated doughs was determined by plating on MRS agar medium.

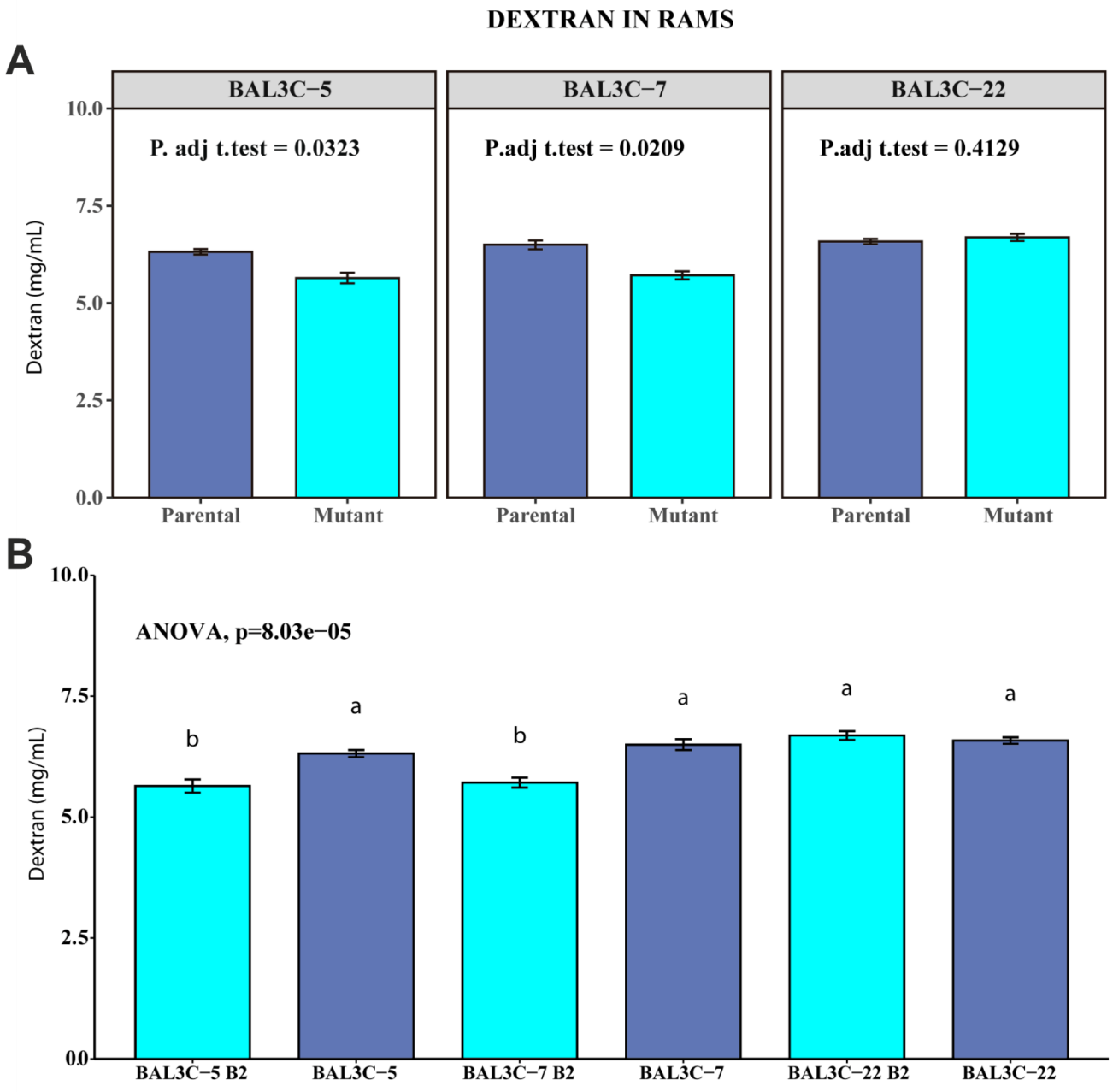
To that end, 10 g samples of dough (prepared as described in Materials and Methods) were homogenized in 90 mL of sterile peptone water (1 g/L peptone, 8.5 g/L NaCl) in 250 mL flasks and incubated at 28 °C for 1 h with shaking (200 rpm). For CFU/g quantification, samples of 1 mL were collected by centrifugation at 10 000 rpm and ten-fold dilutions of the supernatants were spread as 0.1 mL aliquots on MRS agar plates that were incubated at 30 ºC for ~ 48 h.

In the spontaneously fermented uninoculated dough, low concentrations of LAB (4.37×10^6 CFU/g), likely endogenous to the white wheat flour, were detected. In addition, values $>1 \times 10^9$ CFU/g were detected in all doughs inoculated with each BAL3C strain.

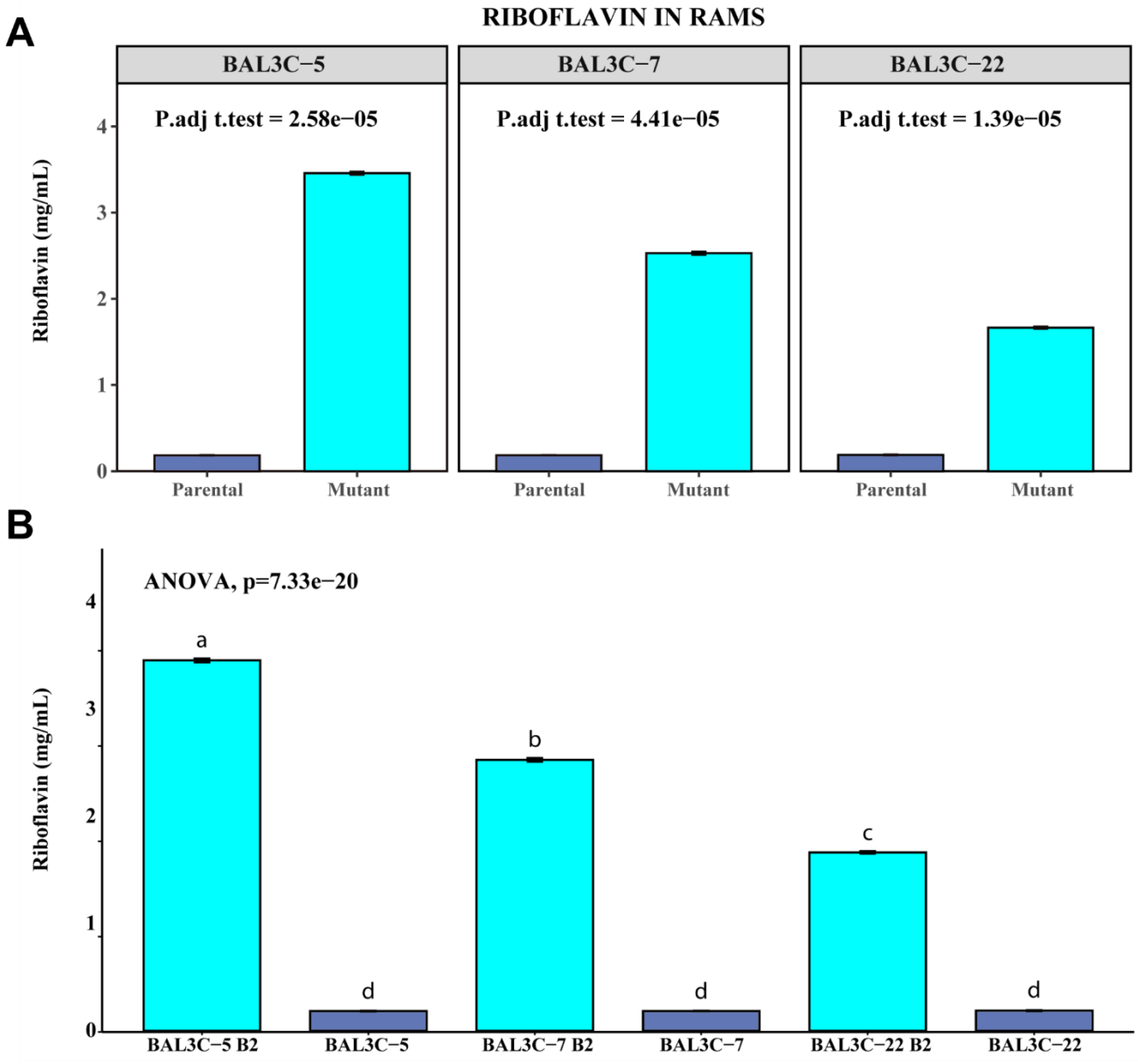
Supplementary Table S3. Comparison of flavin levels present in the experimental breads quantified by direct fluorescence measurement (direct determination) or after HPLC analysis (HPLC)

***W. cibaria* strain**	**Direct determination (mg/100 g of bread)**	**HPLC (mg/100 g of bread)**	**Ratio Direct/HPLC**
Without BAL	0.20±0.01	0.09±0.01	2.17
BAL3C-5	0.24±0.02	0.08±0.02	2.90
BAL3C-5 B2	0.45±0.04	0.56±0.02	0.81
BAL3C-7	0.25±0.00	0.16±0.02	1.50
BAL3C-7 B2	0.48±0.01	0.61±0.08	0.78
BAL3C-22	0.25±0.01	0.13±0.02	1.95
BAL3C-22 B2	0.43±0.06	0.46±0.06	0.93

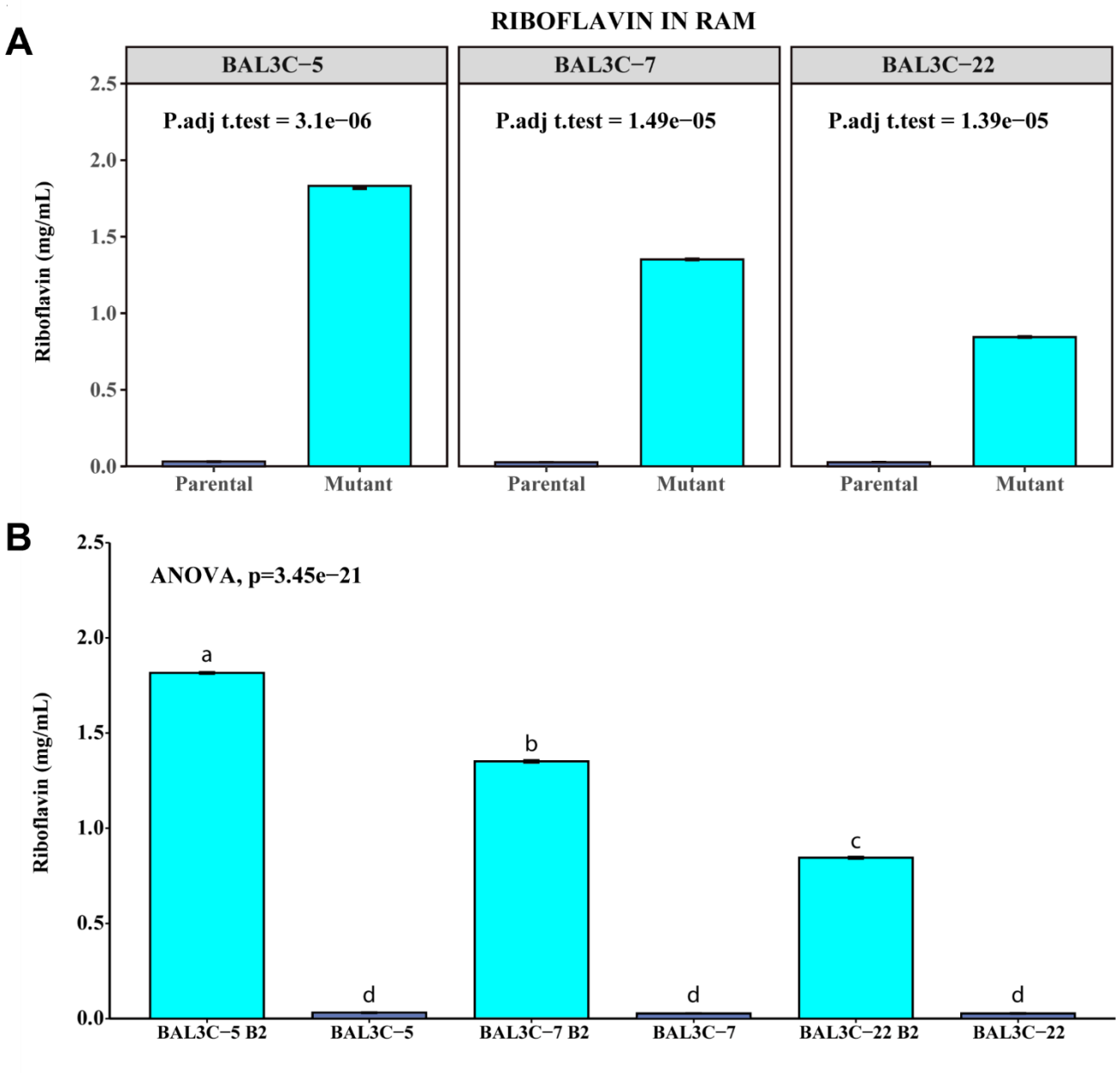
*Bread samples were subjected to acidic and thermal treatment to convert flavins into riboflavin prior to measurement of fluorescence.



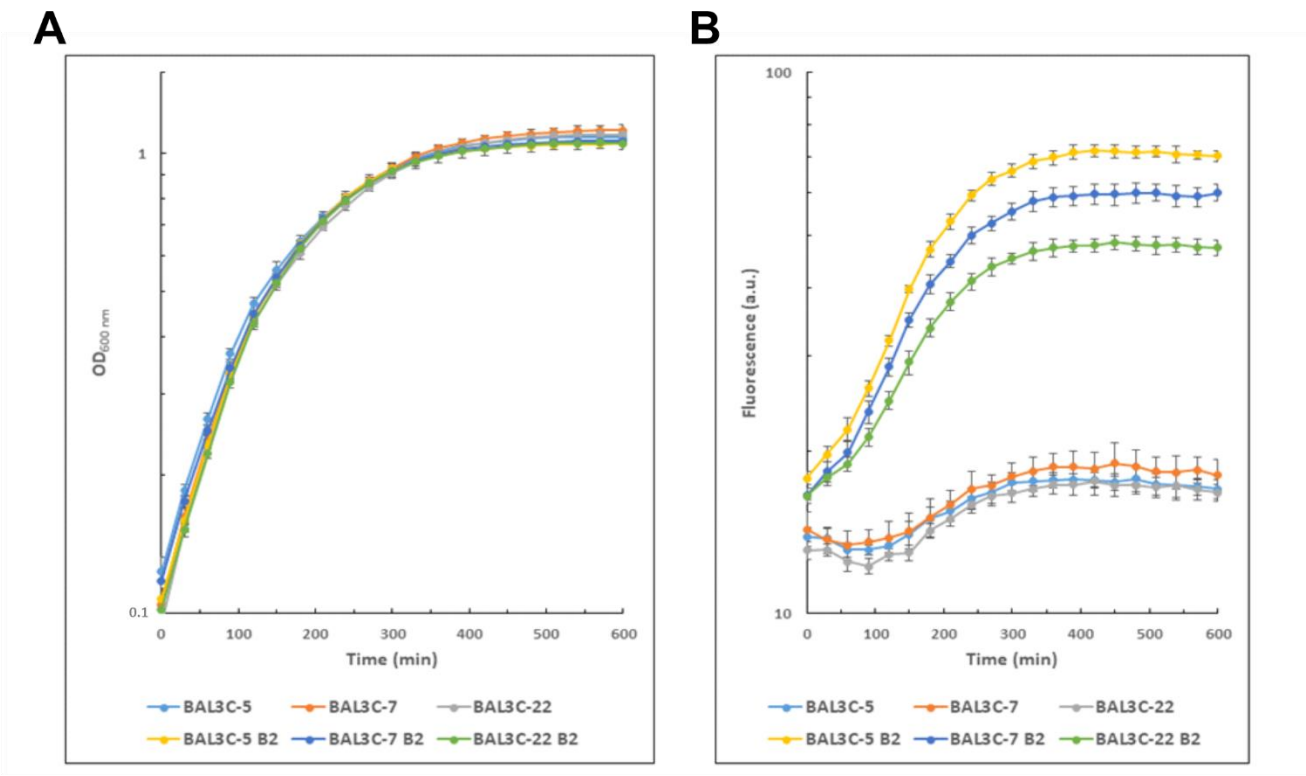
Supplementary Figure S1. Dextran levels produced by *W. cibaria* strains grown in RAMS medium. Values are represented as mean ± standard deviation of three independent experiments. Statistical analyses were carried out by t-test to determine if parental and mutant dextran levels were significantly different (A), or by one-way Anova to establish differences in dextran production between groups (B). In both cases a p value ≤ 0.05 was considered significant.



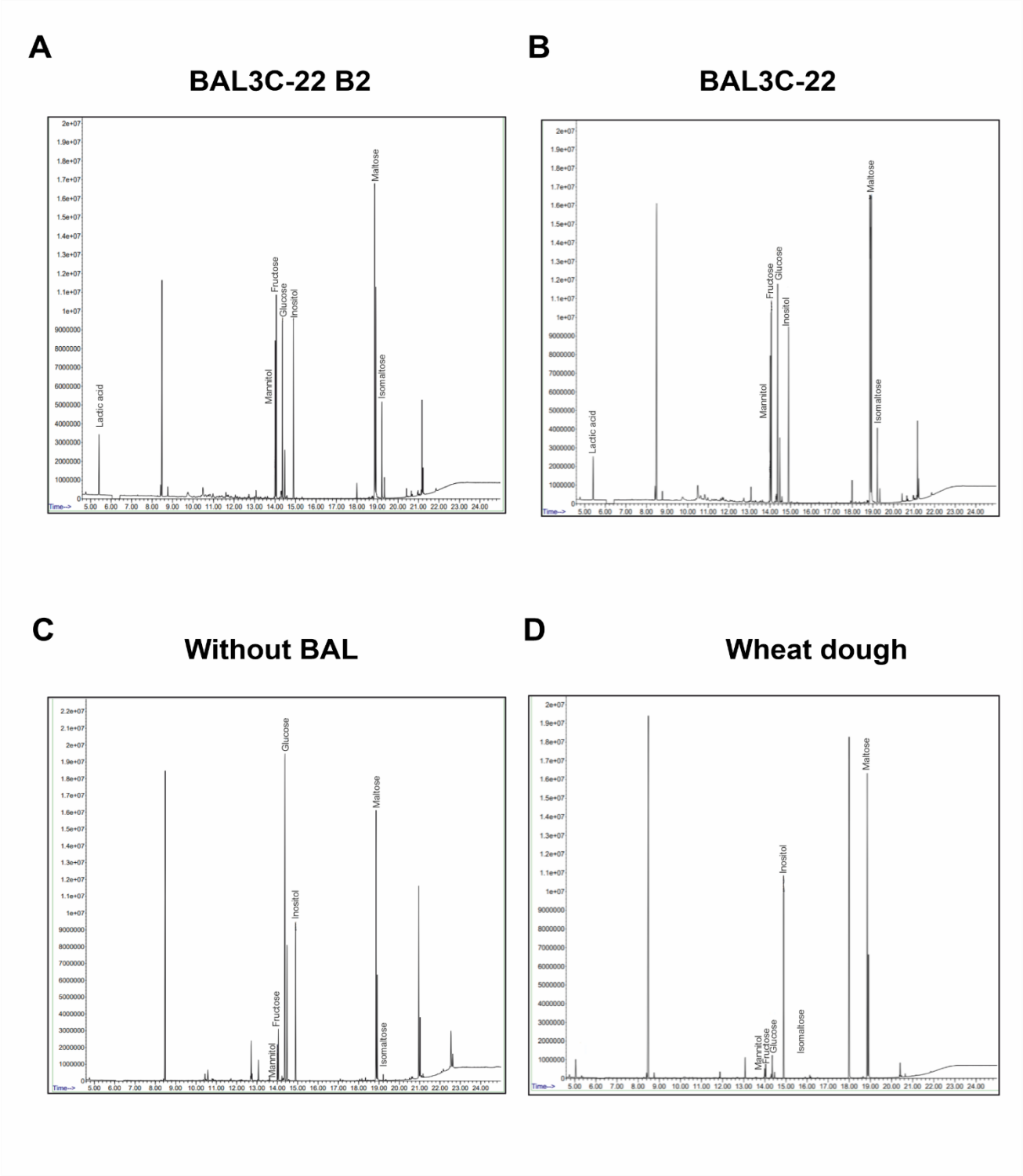
Supplementary Figure S2. Riboflavin produced by *W. cibaria* strains grown in RAMS medium. Values are represented as mean ± standard deviation of three independent experiments. Statistical analyses were carried out by t-test to determine if parental and mutant riboflavin levels were significantly different (A), or by one-way Anova to establish differences in riboflavin production between groups (B), in both cases a p value ≤ 0.05 was considered significant.



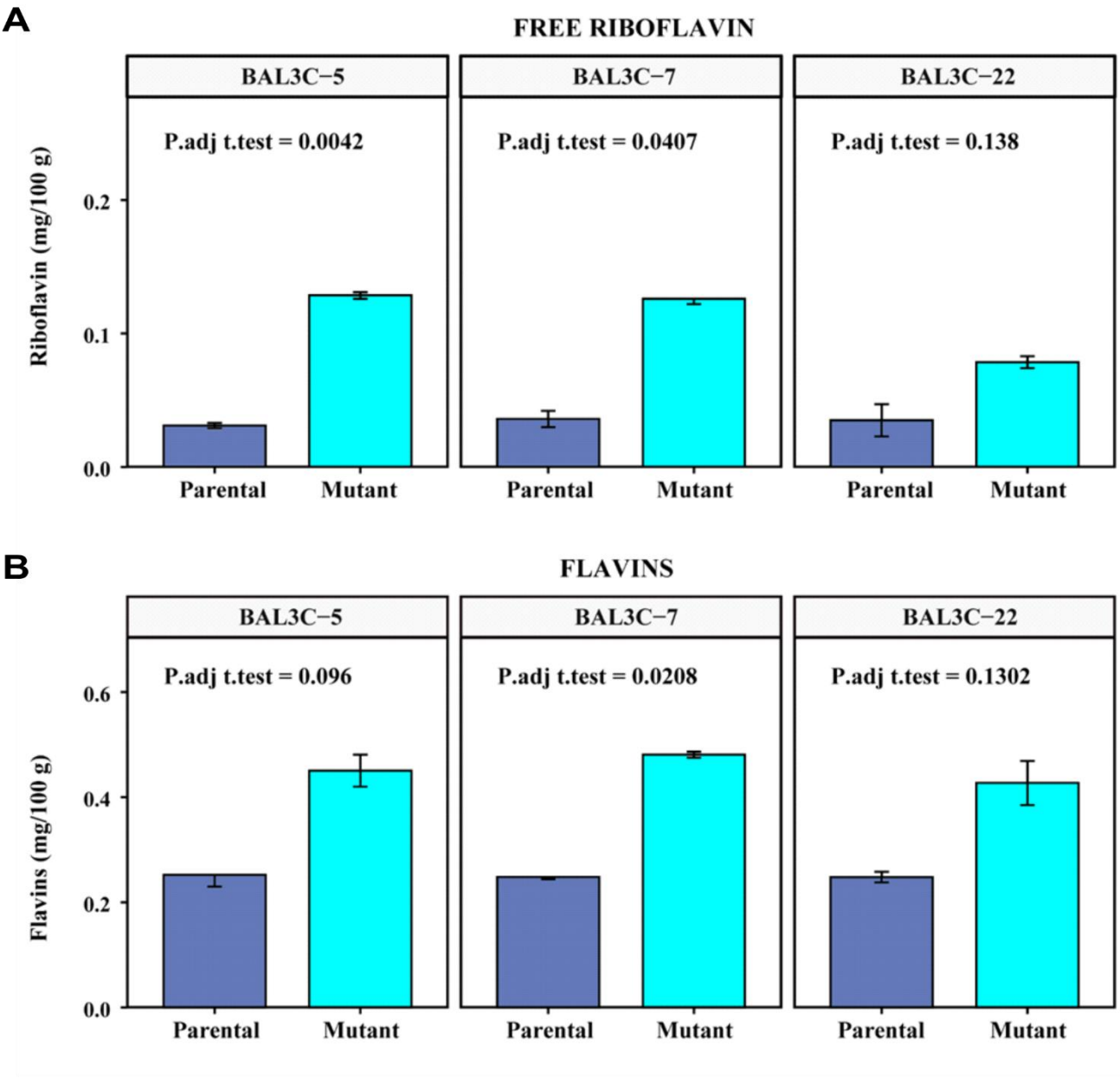
Supplementary Figure S3. Riboflavin produced by *W. cibaria* strains growth in RAM medium. Values are represented as the mean ± standard deviation of three independent experiments. Statistical analyses were carried out by t-test to determine if parental and mutant riboflavin levels were significantly different (A), and by one-way Anova to establish differences in riboflavin production between groups (B) in both cases a p value ≤ 0.05 was considered significant.



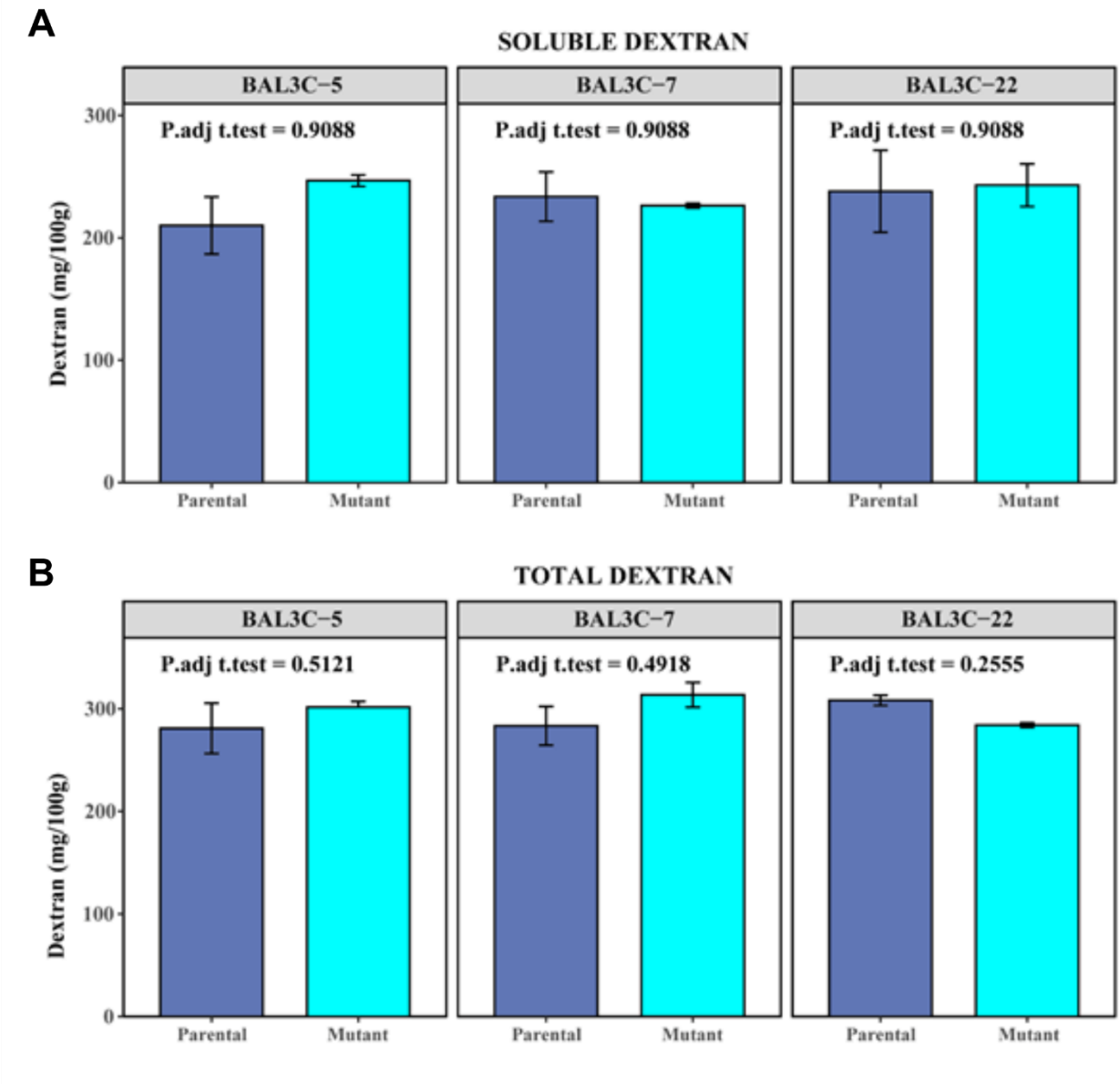
Supplementary Figure S4. Real time analysis of the influence of FMN on riboflavin production by *W. cibaria* strains. The bacteria were grown in RAMS medium supplemented with 3 μM FMN in a Varioskan Flask System. The growth was estimated by measurement of the $OD_{600\ nm}$ (A) and flavin fluorescence (B) was measured upon excitation at a wavelength of 440 nm and detection of emission at a wavelength of 520 nm.



Supplementary Figure S5. GC-MS analysis of breads for detection of soluble dextran hydrolyzed to isomaltose. Chromatograms of breads produced by fermentation with BAL3C-22 B2 (A), BAL3C-22 B2 (B), with only the dough microbiota (without LAB) (C) and of wheat dough (D) are depicted. The samples were resuspended in H_2O and treated with the *Chaetomium erraticum* dextranase at 30 °C for 18 h, as described in Material and Methods, prior to the GC-MS analysis.



Supplementary Figure S6. Statistical analysis of free riboflavin (A) and flavins (B) levels in experimental breads produced with *W. cibaria* strains. Values are represented as mean ± standard deviation of three independent technical replicates. Statistical analyses were carried out by t-test to determine if levels of riboflavin and flavins synthesized by parental and mutant strains were significantly different ($p \leq 0.05$).



Supplementary Figure S7. Determination of soluble (A) and total (B) dextran levels in experimental breads produced with *W. cibaria* strains. Values are represented as mean ± standard deviation of three independent technical replicates. Statistical analyses were carried out by t-test to determine if levels of dextran produced by parental and mutant strains were significantly different ($p \leq 0.05$).