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Deliverable 3.2. Report on the capability to produce folate for dairy-origin LAB strains (M18)

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1. Aim of the work and summary

Modern consumers ask now more about natural and functional products that can satisfy at the same time the nutritional demand together with the prevention of health problems. Folate uptake by humans is one of the issues that could be solved through the consumption of biofortified foods rich in folates, where these vitamins can be synthetised through fermentation by lactic acid bacteria. Certain lactic acid bacteria (LAB) are known to produce folate during fermentation, with THF and methyl-tetrahydrofolate (MTHF) being the main forms (Meucci et al., 2018). LAB generate folate within their cells for growth, but some of it is also released into the surrounding medium, thereby boosting its folate content (Greppi et al., 2017).

This study analyzed the potential of different lactic acid bacteria (LAB) strains, of human origin or isolated from different food sources, to produce folates.

As a first step, a microbiological assay was used for the initial screening of selected strains. Subsequently, molecular biology analyses were performed on the producing strains to investigate differences in the expression of key genes involved in folate biosynthesis.

The ability to synthesize folates within a milk matrix was preliminarily assessed by using the most promising *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains, selected on the results of the microbiological assay combined with folate genes analysis. To better improve the folate detection method used so far by applying the microbiological assay, we set an untargeted approach using ultra-high-performance liquid chromatography combined with high-resolution mass spectrometry (UHPLC-HRMS) with the support of an Orbitrap spectrometer to identify the most efficient biotypes.

The best folate-producer strains, selected on the base of UHPLC analysis were used in yogurt fermentation, and extracellular folate levels were monitored throughout the product's entire shelf life using UHPLC-HRMS analysis at different time points (T0, 7d, 14d).

The primary goal of the study was to identify LAB strains with a high capacity for folate production to be used as functional cultures for dairy biofortification, enhancing their nutritional value.

2. Report on the activities of Task 3.2. “In vitro screening for dairy-origin LAB strains with efficient folate-producing ability”

2.1. Materials and Methods

2.1.1 Strains and culture conditions

A total of 194 lactic acid bacteria strains, derived from both dairy products and human sources, were tested for their ability to produce folates. The bacterial cultures, part of the collections at

Università Cattolica del Sacro Cuore, were reactivated by inoculating them twice in a fresh selective medium. *Lactobacillus* and *Pediococcus* species were cultured in MRS broth at 37°C overnight, *Enterococcus* strains in BHI broth at 37°C overnight, and *S. thermophilus* in M17 and/or LAPT with 2% and 1% lactose, respectively, at 42°C overnight.

Table 1 summarizes the bacterial strains and the growth conditions used for the initial screening.

	Bacterial strain	***ID***	***Origin***	***Growth conditions***	
1	*L. brevis*	UC10249	human	MRS	37°C
2	*L. casei*	UC10138	human	MRS	37°C
3	*L. curvatus*	UC8798	food	MRS	37°C
4	*L. fermentum*	UC10250	food	MRS	37°C
5	*L. fermentum*	UC10251	food	MRS	37°C
6	*L. fermentum*	UC10252	food	MRS	37°C
7	*L. fermentum*	UC10253	food	MRS	37°C
8	*L. fermentum*	UC10254	food	MRS	37°C
9	*L. fermentum*	UC10255	food	MRS	37°C
10	*L. fermentum*	UC10256	human	MRS	37°C
11	*L. gasseri*	UC90011	human	MRS	37°C
12	*L. parabuchneri*	UC10257	human	MRS	37°C
13	*L. paracasei*	UC10258	human	MRS	37°C
14	*L. paracasei*	UC10259	human	MRS	37°C
15	*L. paracasei*	UC10260	human	MRS	37°C
16	*L. plantarum*	UC8805	human	MRS	37°C
17	*L. reuteri*	UC9036	human	MRS	37°C
18	*L. reuteri*	UC9037	human	MRS	37°C
19	*L. rhamnosus*	UC8812	human	MRS	37°C
20	*L. rhamnosus*	UC90012	human	MRS	37°C
21	*L. delbrueckii subsp. bulgaricus*	UC8085	food	MRS	37°C
22	*Lactobacillus spp*	ILF3011(2)1	human	MRS	37°C
23	*Lactobacillus spp*	ILF301(2)2	human	MRS	37°C
24	*Lactobacillus spp*	ILAM831A(1)5	human	MRS	37°C
25	*Lactobacillus spp*	R10FR5	human	MRS	37°C
26	*Lactobacillus spp*	ILAM8417(1)1	human	MRS	37°C
27	*Lactobacillus spp*	ILF225(3)2	human	MRS	37°C
28	*Lactobacillus spp*	ILF305(2)3	human	MRS	37°C
29	*Lactobacillus spp*	ILF303(1)3	human	MRS	37°C
30	*Lactobacillus spp*	ILF305(1)2	human	MRS	37°C
31	*Lactobacillus spp*	ILAM835(2)1	human	MRS	37°C
32	*Lactobacillus spp*	ILAM8413(2)2	human	MRS	37°C
33	*Lactobacillus spp*	ILAM8417(2)1	human	MRS	37°C
34	*Lactobacillus spp*	IL3202(2)11	human	MRS	37°C
35	*Lactobacillus spp*	ILF225(1)2	human	MRS	37°C
36	*Lactobacillus spp*	ILF225(2)2	human	MRS	37°C
37	*Lactobacillus spp*	ILF301(1)3	human	MRS	37°C
38	*Lactobacillus spp*	ILF301(2)3	human	MRS	37°C
39	*Lactobacillus spp*	ILF303(1)2	human	MRS	37°C
40	*Lactobacillus spp*	ILF305(2)1	human	MRS	37°C
41	*Lactobacillus spp*	IVG1202RV4.3	human	MRS	37°C

42	*Lactobacillus spp*	DEAG204	human	MRS	37°C
43	*Lactobacillus spp*	IVG1202RV4.10	human	MRS	37°C
44	*Lactobacillus spp*	ILF201(2)1	human	MRS	37°C
45	*Lactobacillus spp*	ILF3011(2)3	human	MRS	37°C
46	*Lactobacillus spp*	ILF309(1)1	human	MRS	37°C
47	*Lactobacillus spp*	ILF3011(2)1	human	MRS	37°C
48	*Lactobacillus spp*	ILAM8414(3)2	human	MRS	37°C
49	*Lactobacillus spp*	ILAM831(3)1	human	MRS	37°C
50	*Lactobacillus spp*	ILAM831a(1)5	human	MRS	37°C
51	*Lactobacillus spp*	ILAM8417(1)1	human	MRS	37°C
52	*Lactobacillus spp*	ILF225(3)2	human	MRS	37°C
53	*Lactobacillus spp*	ILF305(2)3	human	MRS	37°C
54	*Lactobacillus spp*	ILF303(5)1	human	MRS	37°C
55	*Lactobacillus spp*	ILAM844(2)1	human	MRS	37°C
56	*Lactobacillus spp*	ILF225(2)1	human	MRS	37°C
57	*Lactobacillus spp*	ILF304(2)1	human	MRS	37°C
58	*Lactobacillus spp*	ILAM835(3)1	human	MRS	37°C
59	*Lactobacillus spp*	ILAM844((3)2	human	MRS	37°C
60	*Lactobacillus spp*	ILAM844(2)2	human	MRS	37°C
61	*Lactobacillus spp*	ILA2216C(1)3	human	MRS	37°C
62	*Lactobacillus spp*	ILAM835(2)1	human	MRS	37°C
63	*Lactobacillus spp*	ILF225(1)2	human	MRS	37°C
64	*Lactobacillus spp*	ILF225(1)1	human	MRS	37°C
65	*Lactobacillus spp*	ILAM8414(3)1	human	MRS	37°C
66	*Lactobacillus spp*	ILAM8417(3)1	human	MRS	37°C
67	*Lactobacillus spp*	ILAM831(1)5	human	MRS	37°C
68	*Lactobacillus spp*	ILAM836(2)1	human	MRS	37°C
69	*Lactobacillus spp*	3549PMV	human	MRS	37°C
70	*S. thermophilus*	ST07	food	M17 lactose	42°C
71	*S. thermophilus*	ST292	food	M17 lactose	42°C
72	*S. thermophilus*	ST366	food	M17 lactose	42°C
73	*S. thermophilus*	ST383	food	M17 lactose	42°C
74	*S. thermophilus*	ST407	food	M17 lactose	42°C
75	*S. thermophilus*	ST503	food	M17 lactose	42°C
76	*S. thermophilus*	ST581	food	M17 lactose	42°C
77	*S. thermophilus*	ST609	food	M17 lactose	42°C
78	*S. thermophilus*	ST658	food	M17 lactose	42°C
79	*S. thermophilus*	ST679	food	M17 lactose	42°C
80	*S. thermophilus*	UC90013	food	M17 lactose	42°C
81	*S. thermophilus*	UC90014	food	M17 lactose	42°C
82	*S. thermophilus*	UC90015	food	M17 lactose	42°C
83	*S. thermophilus*	UC90016	food	M17 lactose	42°C
84	*S. thermophilus*	UC90017	food	M17 lactose	42°C
85	*S. thermophilus*	UC90018	food	M17 lactose	42°C
86	*S. thermophilus*	UC90019	food	M17 lactose	42°C
87	*S. thermophilus*	UC90020	food	M17 lactose	42°C
88	*S. thermophilus*	UC90021	food	M17 lactose	42°C
89	*S. thermophilus*	UC90022	food	M17 lactose	42°C
90	*S. thermophilus*	UC90023	food	M17 lactose	42°C

91	*S. thermophilus*	UC90024	food	M17 lactose	42°C
92	*S. thermophilus*	UC90025	food	M17 lactose	42°C
93	*S. thermophilus*	UC90026	food	M17 lactose	42°C
94	*S. thermophilus*	UC90027	food	M17 lactose	42°C
95	*S. thermophilus*	UC8542	food	M17 lactose	42°C
96	*S. thermophilus*	UC90028	food	M17 lactose	42°C
97	*S. thermophilus*	UC90029	food	M17 lactose	42°C
98	*S. thermophilus*	UC90030	food	M17 lactose	42°C
99	*S. thermophilus*	UC90031	food	M17 lactose	42°C
100	*S. thermophilus*	UC90032	food	M17 lactose	42°C
101	*S. thermophilus*	UC90033	food	M17 lactose	42°C
102	*S. thermophilus*	UC90034	food	M17 lactose	42°C
103	*S. thermophilus*	UC90035	food	M17 lactose	42°C
104	*S. thermophilus*	UC90036	food	M17 lactose	42°C
105	*S. thermophilus*	UC90037	food	M17 lactose	42°C
106	*S. thermophilus*	UC90038	food	M17 lactose	42°C
107	*S. thermophilus*	UC90039	food	M17 lactose	42°C
108	*S. thermophilus*	UC90040	food	M17 lactose	42°C
109	*S. thermophilus*	UC90041	food	M17 lactose	42°C
110	*S. thermophilus*	UC90042	food	M17 lactose	42°C
111	*S. thermophilus*	UC90043	food	M17 lactose	42°C
112	*S. thermophilus*	UC90044	food	M17 lactose	42°C
113	*S. thermophilus*	UC90045	food	M17 lactose	42°C
114	*S. thermophilus*	UC90046	food	M17 lactose	42°C
115	*S. thermophilus*	UC90047	food	M17 lactose	42°C
116	*S. thermophilus*	UC90048	food	M17 lactose	42°C
117	*S. thermophilus*	UC90049	food	M17 lactose	42°C
118	*S. thermophilus*	UC90050	food	M17 lactose	42°C
119	*S. thermophilus*	UC90051	food	M17 lactose	42°C
120	*S. thermophilus*	UC90052	food	M17 lactose	42°C
121	*S. thermophilus*	UC90053	food	M17 lactose	42°C
122	*S. thermophilus*	UC90054	food	M17 lactose	42°C
123	*S. thermophilus*	UC90055	food	M17 lactose	42°C
124	*S. thermophilus*	UC90056	food	M17 lactose	42°C
125	*S. thermophilus*	UC90057	food	M17 lactose	42°C
126	*S. thermophilus*	UC8055	food	M17 lactose	42°C
127	*S. thermophilus*	UC8056	food	M17 lactose	42°C
128	*S. thermophilus*	UC8057	food	M17 lactose	42°C
129	*S. thermophilus*	UC8072	food	M17 lactose	42°C
130	*S. thermophilus*	UC8073	food	M17 lactose	42°C
131	*S. thermophilus*	UC8074	food	M17 lactose	42°C
132	*S. thermophilus*	UC8075	food	M17 lactose	42°C
133	*S. thermophilus*	UC8076	food	M17 lactose	42°C
134	*S. thermophilus*	UC8078	food	M17 lactose	42°C
135	*S. thermophilus*	UC8079	food	M17 lactose	42°C
136	*S. thermophilus*	UC8080	food	M17 lactose	42°C
137	*S. thermophilus*	UC8435	food	M17 lactose	42°C
138	*S. thermophilus*	UC8436	food	M17 lactose	42°C
139	*S. thermophilus*	UC8523	food	M17 lactose	42°C

140	*S. thermophilus*	UC8525	food	M17 lactose	42°C
141	*S. thermophilus*	UC8528	food	M17 lactose	42°C
142	*S. thermophilus*	UC8529	food	M17 lactose	42°C
143	*S. thermophilus*	UC8531	food	M17 lactose	42°C
144	*S. thermophilus*	UC8532	food	M17 lactose	42°C
145	*S. thermophilus*	UC8533	food	M17 lactose	42°C
146	*S. thermophilus*	UC8537	food	M17 lactose	42°C
147	*S. thermophilus*	UC8539	food	M17 lactose	42°C
148	*S. thermophilus*	UC8540	food	M17 lactose	42°C
149	*S. thermophilus*	UC8545	food	M17 lactose	42°C
150	*S. thermophilus*	UC8546	food	M17 lactose	42°C
151	*S. thermophilus*	UC8551	food	M17 lactose	42°C
152	*S. thermophilus*	UC8552	food	M17 lactose	42°C
153	*L. sakei*	UC8437	food	MRS	37°C
154	*L. sakei*	UC8438	food	MRS	37°C
155	*L. sakei*	UC8706	food	MRS	37°C
156	*L. sakei*	UC8439	food	MRS	37°C
157	*L. sakei*	UC8705	food	MRS	37°C
158	*L. sakei*	UC8440	food	MRS	37°C
159	*L. sakei*	UC90058	food	MRS	37°C
160	*L. sakei*	UC90059	food	MRS	37°C
161	*L. sakei*	UC90060	food	MRS	37°C
162	*L. sakei*	UC90061	food	MRS	37°C
163	*L. sakei*	UC90062	food	MRS	37°C
164	*L. sakei*	UC90063	food	MRS	37°C
165	*L. sakei*	UC8708	food	MRS	37°C
166	*L. sakei*	UC 8572	food	MRS	37°C
167	*L. sakei*	UC 8574	food	MRS	37°C
168	*L. sakei*	UC 8576	food	MRS	37°C
169	*L. sakei*	UC 8577	food	MRS	37°C
170	*L. sakei*	UC 8578	food	MRS	37°C
171	*L. sakei*	UC 8579	food	MRS	37°C
172	*L. sakei*	UC 8580	food	MRS	37°C
173	*L. sakei*	UC 8585	food	MRS	37°C
174	*L. sakei*	UC 8590	food	MRS	37°C
175	*L. plantarum*	UC10117	food	MRS	37°C
176	*L. plantarum*	UC10116	food	MRS	37°C
177	*L. plantarum*	UC10119	food	MRS	37°C
178	*L. plantarum*	UC90067	food	MRS	37°C
179	*L. plantarum*	UC90066	food	MRS	37°C
180	*L. plantarum*	M1212	food	MRS	37°C
181	*P. acidilactici*	UC8817	food	MRS	37°C
182	*L. curvatus*	UC8704	food	MRS	37°C
183	*L. curvatus*	UC90068	food	MRS	37°C
184	*L. curvatus*	UC8442	food	MRS	37°C
185	*L. curvatus*	UC8472	food	MRS	37°C
186	*L. curvatus*	UC90069	food	MRS	37°C
187	*L. curvatus*	UC8998	food	MRS	37°C
188	*L. curvatus*	UC90065	food	MRS	37°C

189	*L. curvatus*	UC8707	food	MRS	37°C
190	*L. pentosus*	UC90064	food	MRS	37°C
191	*E. faecium*	UC8719	food	MRS	37°C
192	*E. faecium*	UC8733	food	MRS	37°C
193	*E. faecium*	UC8714	food	MRS	37°C
194	*E. faecium*	UC8813	food	MRS	37°C

Tab. 1 *List of strains tested for extracellular folate production, origin, and growth conditions.*

2.1.2. *In vitro* screening of bacterial strains for folate production in skim milk: Microbiological assay (MA) for folate production

The determination of folate production was performed through the microbiological assay (MA) (S. Hugenschmidt, 2010) using *Lacticaseibacillus rhamnosus* ATCC® 7469, auxotrophic strain for folate, as the indicator strain.

After an overnight culture at 37º C, cells of indicator strains were harvested by centrifugation, washed with saline solution, and inoculated (2%v/v) in double concentrated Folic Acid Casei Medium (Difco® Laboratories, Becton Dickinson and Company, Sparks, Maryland) and loaded in triplicates into the 96-well microplate prepared as follows.

500 µL of fermented milk samples were collected after fermentation and added to 500 µL of saline solution. After mixing and boiling the samples at 100°C for 5 minutes, they were centrifuged at 10,000 rpm for 6 minutes. The supernatant was collected and diluted 1:40 in a Sodium Phosphate Buffer solution. The samples were added in triplicate to the 96-well microplate.

A standard curve was realized using a standard solution of folic acid diluted in a range from 0.0078 to 10 ng/ mL, for a total of 12 standard wells in order to obtain values within the range of the standard curve.

Plates were incubated at 37°C for 24 hours under anaerobic conditions. After this incubation period, the growth of indicator strain was measured with optical density (OD) at 620 nm microplate reader. Total folate concentration was computed according to the linear regression of the function given by the standard curve. The final concentration of folate produced by tested strains was expressed in µg/ml.

2.1.3 DNA extraction and molecular identification of folate-producing genes.

The presence of the four genes (Tab. 2) responsible for the production of folate was investigated (*folA, folC, folk, folP*).

The chromosomal DNA was extracted from overnight cultures through microLYSIS® – Plus DNA (Microzone, UK), following the manufacturer's recommended protocol.

The PCR assay was performed in a final volume of 25 µl, including Master Mix PCR 2X (Pomega, Germany), 1µM forward primer, 1µM reverse primer, and 2µl of bacterial DNA. The

reaction was conducted with the following thermal conditions: 5 min at 94°C; 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s; and 72°C for 7 min.

The amplification products were observed on a 1% agarose gel with SYBR® Safe (Invitrogen Corporation Life technologies). Both negative and positive controls were included in each reaction.

Genes	Primers	Sequence (5'→ 3')	Dimension
fol A	folAf	AGCTACGTTTGGGCAGAAGA	489 bp
	folAr	CGGTGGGCTTCACTCTTTAC	
fol C	folCf	GTATTTTGCCGAACAGCGGG	1338 bp
	folCr	TCAACAAATGCGCTGATGCC	
fol K	folKf	GTATTTTGCCGAACAGCGGG	1074 bp
	folKr	GAAAGTTCGCGCTGCTGATT	
fol P	folPf	ACATTTAGCGGCAACGTCAC	1101 bp
	folPr	CTTTTTCAAGCCCAACGCCT	

Tab. 2 Lists of primers and conditions used in PCR test for identification of folate-producing genes

2.1.4 Fermented milk preparation

Based on previous analysis three strains of *S. thermophilus* and one strain of *L. delbrueckii* subsp. *bulgaricus* (Tab. 3) were selected for the highest folate production and were used in three different blend combinations as starter cultures for biofortified yoghurts production. The yoghurts were prepared from UHT cow's milk from a local supermarket.

Name of the blend	*S. thermophilus*	*L. bulgaricus*	Ratio	% inoculated
YCB1	ST 07	UC 8085	2:1	8%:4%
YCB2	ST 292	UC 8085	2:1	8%:4%
YCB3	ST 658	UC 8085	2:1	8%:4%

Tab. 3 Microbial blend conditions to be used for yoghurt fermentation and folate measurement

Bacterial strains were grown as previously described and, for each formulation (table 3) they were inoculated in 25 ml of UHT cow milk. Samples were incubated at 42°C until reaching approximately a pH of 4.6. The yoghurts, prepared in duplicates, were stored at a temperature of 4°C for 14 days.

2.1.5 Viability of lactic acid bacteria during storage

The viability of the lactic acid bacteria strains inoculated into the milk samples was monitored by microbiological counts on agar plates at 3 different storage times: initial time point (T0), after 7 days (T7), and after 14 days (T14). For each sample, serial dilutions were prepared and spread on MRS and M17 agar supplemented with lactose (2% v/v) for the counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, respectively. MRS and M17 plates were incubated anaerobically for 48 hours at 37°C and 42°C, respectively. Microbiological counts for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were also performed on a commercial yoghurt containing *L. bulgaricus*, *S. thermophilus*, *Bifidobacterium* spp., and *L. casei* as a control sample.

2.1.6 Extraction and semi-quantitative analysis of folates based on UHPLC-HRMS

A total of 200 µL of each milk sample was combined into a 2 mL-eppendorf tube with 400 µL of an extracting solution containing 80% methanol and 3% formic acid (v/v). The mixture was subjected to an ultrasound-assisted extracton to promote the extraction of folates, working at 25°C, for 10 min at 120 watts. Afterwards, the extracted samples were centrifuged at 14,000 rpm, at 4°C for 15 min, and then incubated at -18°C overnight, to promote protein precipitation. Thereafter, supernatants were filtered through 0.22 µm syringe filters, and transferred into UHPLC vials until further instrumental analysis.

Folate production by the 11 LAB strains was assessed through a semi-quantitative approach based on ultra-high performance liquid chromatography (UHPLC) combined with high-resolution mass spectrometry (HRMS) data analysis. The semi-quantification of folates was done in both skim milk samples and yogurts. A Q-Exactive™Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, USA) equipped with a heated electrospray ionization (HESI) probe was used. The mobile phase consisted of water acidified with 0.1% (v/v) formic acid (phase A) and methanol acidified with 0.1% (v/v) formic acid (phase B). Each injection lasts for 20 minutes at a constant flow rate of 0.2 mL/min. The gradient elution program was as follows: time 2 min, 90/10 A/B; time 8 min, 50/50 A/B; time 8.1 min, 5/95 A/B; time 8.5 min, 5/95 A/B; time 8.6 min, 90/10. The mass spectrometer worked in positive polarity (HESI +) and SCAN mode, with a range from 150 to 600 m/z, which corresponds to the molecular weight of folates targeted in this work, working with a nominal resolution at 70,000 FWHM. The HESI source parameters were as follows: sheath gas flow rate of 40, auxiliary gas flow rate of 20, temperature of HESI probe equal to 320°C. The standard compounds used for the semi-quantitative analysis of the different folates were folic acid (Sigma-Aldrich, USA) and 5-methyltetrahydrofolic acid (Sigma-Aldrich, USA). Standard solutions of these compounds were prepared dissolving 5 mg of FA and 5-MTHF powder in a buffer solution composed of 1g/L aqueous ascorbic acid, with the addition of 4% (w/v) NH_4OH for 5-MTHF. Calibration curves were then built for the two standards, considering coefficients of determination higher than 97% and working in the calibration range 5-500 µg/L.

2.2 Results

2.2.1 Selection of producing strains

Folate production in the supernatants of lactic acid bacteria was determined using a microbiological microplate assay with *L. rhamnosus* ATCC 7469 as the indicator strain. The growth of this microorganism was measured by optical density readings at 600 nm using a microplate reader. Figure 1 presents the results of the initial screening, which includes 172 strains, for folate production.

The folate concentration in the samples, expressed in ng/mL, was calculated using a folic acid standard curve while accounting for the folate present in the culture medium. Results ranged from approximately -6 ng/mL to 1500 ng/mL, with negative values indicating folate consumption.

Lactic acid bacteria belonging to the species *Limosilactobacillus reuteri*, *Limosilactobacillus fermentum*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus casei*, *Lentilactobacillus parabuchneri*, and *Streptococcus thermophilus* exhibited high levels of production (>200 ng/mL), with a maximum value of 1400 ng/mL observed in *Limosilactobacillus reuteri* UC9036.

Many studies report *S. thermophilus* as a dominant species in folate production (Crittenden et al., n.d.; Iyer et al., 2011; Meucci et al., 2018), achieving yields higher than those of other lactic acid bacteria. The values obtained in this study, consistently positive and in some cases exceeding 800 ng/mL, further confirmed its importance in this role.

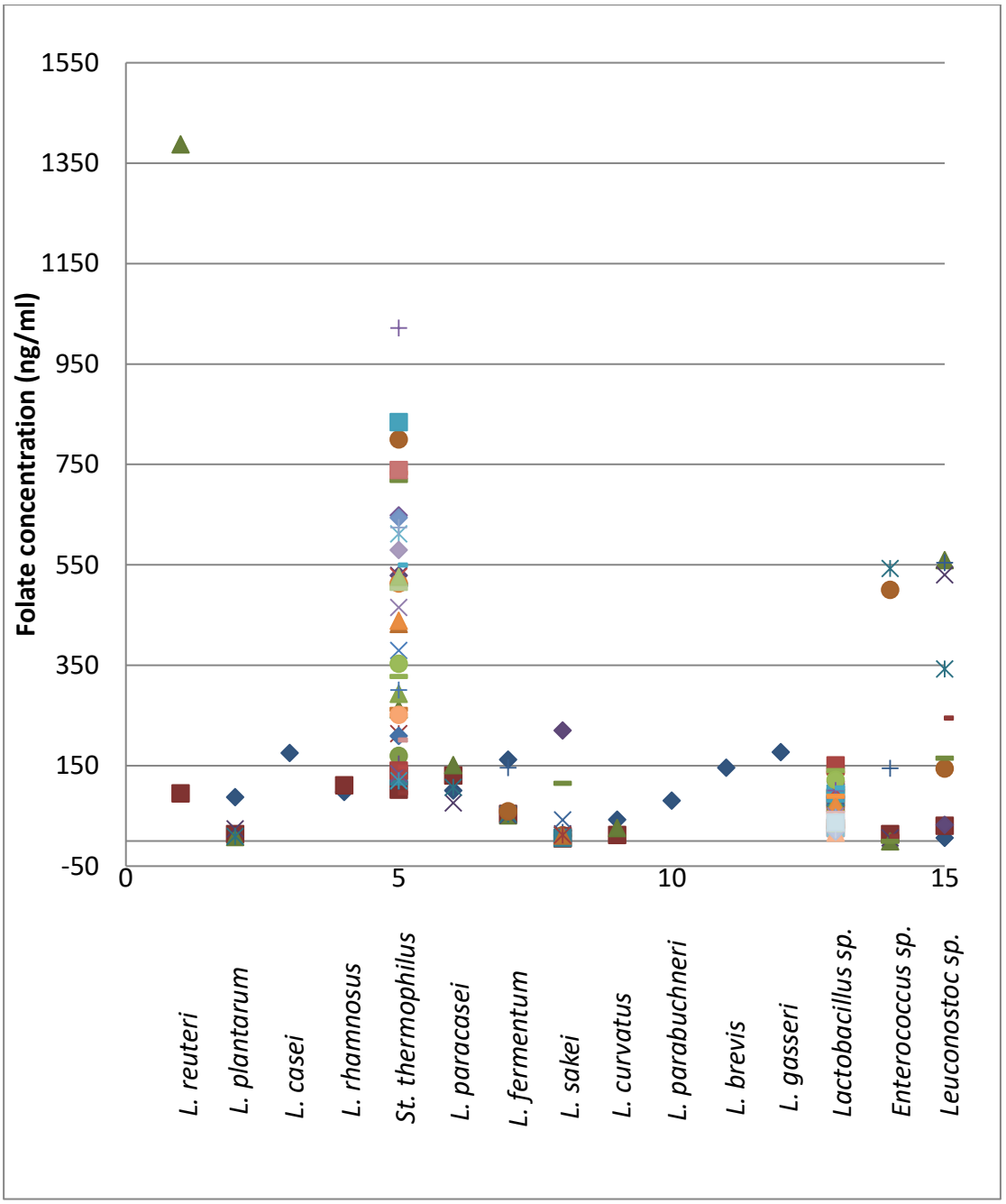

Fig. 1. Screening for folate production in the supernatants of lactic acid bacteria cultures. The results are grouped by bacterial species. Each point represents the folate concentration obtained for the different strains analyzed and measured in ng/ml.

2.2.2 Identification of folate-producing genes

PCR analysis was performed for the identification of folate-producing genes (folA, folC, folK, folP) for all the 194 selected strains. More specifically, the gene *folA* encodes the dihydrofolate reductase, the gene *folC* is responsible for encoding folate synthetase/folyl polyglutamate synthetase, the gene *folK* encodes the 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase; and finally, the gene *folP* encodes the dihydropteroate synthase. The table 4 shows the results regarding the presence of genes involved in folate biosynthesis. Out of the 194 strains tested, only 12 tested positive for the presence of the target genes.

Strain	ID	*folA*	*folC*	*folP*	*folK*
S. thermophilus	ST 07	√	√	√	√
S. thermophilus	ST 292	√	√	√	√
S. thermophilus	ST 366	√	√	√	√
S. thermophilus	ST 407	√	√	√	√
S. thermophilus	ST 503	√	√	√	√
S. thermophilus	ST 581	√	√	√	√
S. thermophilus	ST 609	√	√	√	√
S. thermophilus	ST 658	√	√	√	√
S. thermophilus	ST 679	√	√	√	√
L. bulgaricus	UC 8085	√	√	√	√
L. reuteri	UC 9036	X	√	√	√
L. reuteri	UC 9037	X	√	√	√

Tab. 4 Strains that tested positive for the presence of genes involved in folate biosynthesis.

For the subsequent phases, it was decided to proceed exclusively with the *S. thermophilus* and *L. bulgaricus* species. This decision was made as a preliminary approach, as the ultimate goal is to produce a biofortified yogurt, thereby maintaining the characteristic strains used in traditional yogurt production. The *L. reuteri* strains that showed good performance will be considered at a later stage, once the technique is refined and the study is expanded to include the introduction of other LAB for different fermented milks production (*i.e.* mesophilic fermented milks or acid-alcoholic ones, like kefir).

2.2.3. Quantification of folates produced by *S. thermophilus* strains through an UHPLC-HRMS approach

With the aim to measure folate production as a result of biofortification activity by LAB in milk matrix, we have selected among *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, the most efficient strains, for a first test of yogurt fermentation and folate assessment in the dairy product during the shelf-life. In table 5 the strains used for the study are listed.

To bypass the hurdle of a semi-quantitative detection method that refers the extracellular folate quantification on a microbiological activity, we decided to test a new detection method based on UHPLC-HMRS. A preliminary screening to assess the ability of *S. thermophilus* strains to produce folates was performed. The production of folic acid by *S. thermophilus* in skim milk stored at 42°C for 24 hours was analysed and the results obtained are presented in Table 6.

Bacterial strain	ID
Lb.delbrueckii subsp. *bulgaricus*	UC 8085
S. thermophilus	ST 07
S. thermophilus	ST 292
S. thermophilus	ST 366
S. thermophilus	ST 407
S. thermophilus	ST 503
S. thermophilus	ST 581
S. thermophilus	ST 609
S. thermophilus	ST 658
S. thermophilus	ST 679

Tab. 5 Strains with the highest folate production selected for further analyses.

Strain	Folic acid (ng/L)	Total folic acid metabolites (µg/L)
UC 8085		398.57 ± 51.5
ST 07	123.0 ± 29.7	211.02 ± 4.20
ST 292	94.5 ± 19.1	140.83 ± 28.10
ST 366	75.0 ± 21.2	128.82 ± 1.90
ST 407	76.5 ± 2.1	71.60 ± 12.30
ST 503	72.0 ± 4.2	132.68 ± 3.20
ST 581	49.5 ± 6.4	58.14 ± 5.04
ST 609	24.0 ± 4.2	72.34 ± 2.50
ST 658	84.0 ± 25.5	227.99 ± 9.30
ST 679	57.0 ± 8.5	89.02 ± 9.50

Tab. 6 Folic acid and total folic acid metabolites produced by S. thermophilus strains analysed by UHPLC-HRMS.

The instrumental analysis showed that those skim milk samples characterized by the highest content of folic acid were those inoculated with the strains ST 07, ST 292 and ST 658. Their production reached values equal to 123 ng/L, 94.5 ng/L and 84 ng/L respectively. On the contrary, those showing the lowest quantity were represented by ST 609, ST 581, and ST 679. Although the investigation produced positive results, the concentrations of folic acid in the

sample analysed were extremely low. For this reason, an untargeted metabolomics approach based on a semi-quantitative analysis of the main folate metabolites was exploited. Particularly, 6 folate metabolites, mainly folate precursors and products, were identified and semi-quantified as total folate metabolites. The metabolites considered were dihydrofolate, tetrahydrofolate (THF), 10-formyl-THF, 5,10-methenylTHF, 5,10-methylene-THF, and 5-methyl-THF. The semi-quantification of folate metabolites was done against the calibration curve of 5-Methyl-THF. As a general cosideration, definitely higher semi-quantitative values were obtained when compared to results of the only folic acid. Particularly, the ST 658 was found as the best producer of total folates, with 227.99 µg/L, followed by ST 07 (211.02 µg/L) and ST 292 (140.83 µg/L). The *L. bulgaricus* strain also yielded a good result, reaching a value of 398.57 µg/L.

2.2.4 Shelf-life study on yoghurt

The previously described analysis allowed to define the best candidate strains to be used as starter cultures for yoghurt production. The *S. thermophilus* strains ST 658, ST 292 and ST 07 exhibited the most favourable results with regard to total folate metabolites, as determined by HPLC-MS/MS analysis. They were therefore selected for the formulation of 3 mixtures to be inoculated into cow milk, together with *L. bulgaricus* UC 8085, the only *L. delbrueckii* strains studied in this work. The fermentation process for the production of yoghurt took a total of 8 hours, with a final pH range between 4.68 and 4.58. The initial inoculum of the selected bacterial strains was 8 log CFU/ml. The growth of the bacteria was monitored throughout a shelf-life of 14-day. The microbiological counts performed at time intervals 0, 7 and 14 days are presented in the Fig. 2.

Blend	**Time**	***L. bulgaricus***	***S. thermophilus***
YCB1	0	8.29 ± 0.48	9.21 ± 0.24
(ST07 + UC8085)	7	7.97 ± 0.52	8.96 ± 0.03
	14	8.08 ± 0.05	8.28 ± 0.00
YCB2	0	7.83 ± 0.18	9,15 ± 0.04
(ST292 + UC8085)	7	8.20 ± 0.12	8.98 ± 0.07
	14	8.03 ± 0.47	8.49 ± 0.15
YCB3	0	8.17 ± 0.18	9.00 ± 0.06
(ST658 + UC8085)	7	8.02 ± 0.09	9.04 ± 0.04
	14	8.20 ± 0.08	8.76 ± 0.12

Tab. 7. Bacterial counts (log CFU/ml ± standard deviation) of microbiological species in yoghurt samples produced with the three blends at T0, T7 and T14.

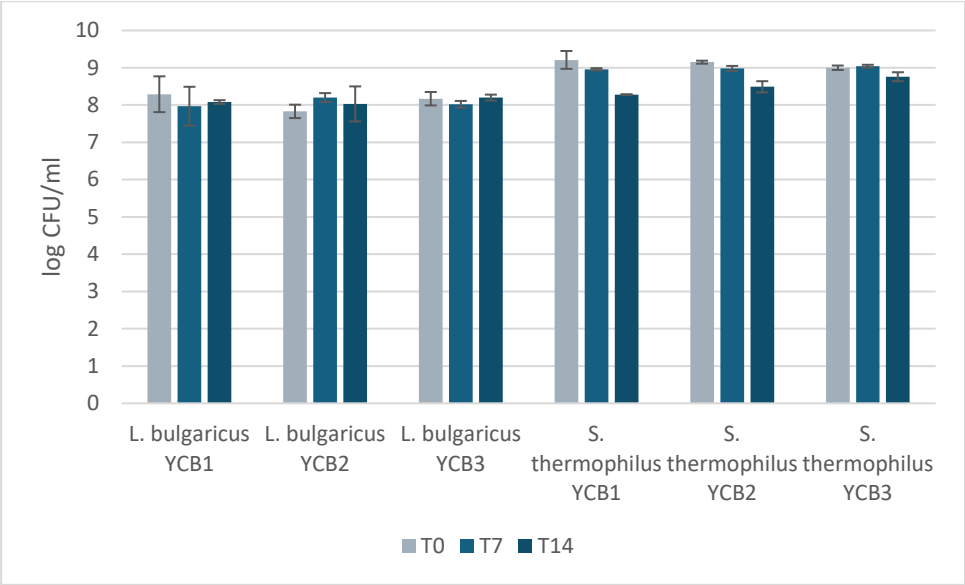

Fig. 1. Bacterial counts (log CFU/ml ± standard deviation) of L. bulgaricus UC8085 and S. thermophilus spp. in yoghurt during storage at 4°C after 0 (T0), 7 (T7), and 14 (T14) days.

After fermentation, it was observed that the concentration of *S. thermophilus* strains was greater than that of *L. bulgaricus* UC 8085 in all three mixtures. Following a 14-day storage period, the mixture containing ST 658 and UC 8085 (YCB3) exhibited the highest concentration of bacteria (Fig. 2). The values for the *Lactobacillus spp.* were 8.20 log CFU/ml, while those for *Streptococcus spp.* were 8.76 log CFU/ml.

Folic acid and total folate metabolites were then assessed in the yoghurt samples at T0, aand during the shelf-life at T7 and T14 days. The results are shown in the table 8.

Blend	Time	Folic acid (µg/L)	Total folate metabolites (µg/L)	Folate intake (µg/125 ml)
YCB1	0	0.16 ± 0.06^{a}	170.68 ± 2.79^{a}	21.34
	7	0.35 ± 0.01^{b}	373.83 ± 23.13^{b}	46.73
	14	2.82 ± 0.03^{c}	432.08 ± 1.63^{c}	54.01
YCB2	0	0.42 ± 0.08^{a}	169.53 ± 3.96^{a}	21.19
	7	1.18 ± 0.04^{b}	405.70 ± 2.46^{b}	50.71
	14	2.14 ± 0.14^{c}	421.74 ± 6.96^{c}	52.72
YCB3	0	0.10 ± 0.03^{a}	178.23 ± 0.78^{a}	22.28
	7	1.05 ± 0.03^{c}	400.30 ± 17.96^{b}	50.04
	14	0.90 ± 0.02^{b}	424.41 ± 30.97^{b}	53.05

Tab. 8 Production of folic acid and total folate metabolites (µg/L) in yoghurts inoculated with different bacterial mixes at 0, 7, and 14 days, and estimated intake per 125 mL serving. Values are presented as mean ± standard deviation. Superscript letters (a, b, c) denote significant differences between time points within each bacterial mix, as determined by Duncan's post hoc test.

The results from one-way ANOVA and Duncan's post hoc test revealed significant variations in folic acid production across all three bacterial mixtures at the three time points analyzed. Both YCB1 and YCB2 mixtures exhibited a consistent increase in folic acid production at days 0, 7, and 14. In contrast, the YCB3 mixture displayed a distinct pattern, with a significant peak in folic acid production at day 7, followed by a decrease at day 14. However, the folic acid levels at day 14 remained significantly higher than at day 0.

Overall, the data indicate that the folic acid content in the yoghurt samples was significantly greater than in the skim milk. The combination of the two bacterial strains resulted in a higher yield of folate than when the strains were cultivated separately.

For both YCB1 and YCB2, the production of folate metabolites significantly increased at each time point (0, 7, and 14 days). Duncan's post hoc analysis confirmed that day 14 consistently exhibited the highest folate concentrations, significantly surpassing both days 0 and 7 (Tab. 7). At the end of the estimated shelf-life, the mixture with the highest level was YCB1, with 432.08 µg/L. In contrast, the YCB3 mixture followed a different trend. Significant differences were observed between days 0 and 7, as well as between days 0 and 14; however, no significant difference was found between days 7 and 14. This suggests that the folate production in mix YCB3 leveled off after 7 days, with no further significant increase in folate content between 7 and 14 days.

The data, presented in Table 7, are also expressed as the hypothetical intake of 125 mL of yoghurt, the typical serving size. This provides an estimate of the folate intake that could result from consuming a single serving. The highest folate intake, 54.01 µg/125 mL, corresponds to yoghurt produced with the YCB1 mixture at the end of its shelf-life.

Even though it has been noticed that LAB considerably increases the folate level in milk

fermented food product, the values are still not adequate to meet daily needs.

The National Institutes of Health's folate guidelines recommend that all women and adolescents who could become pregnant consume 400 µg of folic acid daily through supplements, fortified foods, or a combination of both, in addition to the folate obtained from a healthy diet.

Different solutions could be taken in consideration for increasing the folate content during the second part of the project. One approach could be to better study the best conditions of growth to promote higher folate levels by the selected strains; on the other hand, a different extraction method and an improved HPLC-MS/MS analysis will be investigated to measure also intracellular folate metabolites, contributing to quantify total folate production by LAB strains. In addition, different LAB species (mesophilic LAB) will be tested for the production of fermented milks evaluating different contributes of microbial synergies to biofortification of dairy products.

2.3 References

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