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Co-culture affects protein profile and heat tolerance of *Lactobacillus delbrueckii* subsp. *lactis* and *Bifidobacterium longum*

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ABSTRACT

Some strains of the genus *Bifidobacterium* are considered probiotics and can be added as adjunct cultures to functional dairy products. *Lactobacillus delbrueckii* subsp. *lactis* is used as a starter in food fermentations. The influence of co-culturing strains *Bifidobacterium longum* NCIMB 8809 and *L. delbrueckii* subsp. *lactis* 193 on the physiology and heat tolerance of these microorganisms was studied. 2DE coupled to MS protein analysis allowed the identification of several proteins from each bacterium whose expression changed when cells were grown in compartmentalized co-cultures compared to mono-cultures. Remarkably, production of stress response chaperones was enhanced in both strains and was related with increased survival to heat shock, a technologically suitable property in the manufacture of some dairy products. This study provides the first insight in understanding communication between *B. longum* and *L. delbrueckii* subsp. *lactis*.

1. Introduction

Non-intestinal *Lactobacillus delbrueckii* strains are important microorganisms for food fermentation. In particular, they are used in the production of yogurt and fermented milks (*L. delbrueckii* subsp. *bulgaricus*) and hard cheeses (*L. delbrueckii* subsp. *lactis*) (Giraffa, De Vecchi, & Rossetti, 1998). On the other hand, microorganisms of the genus *Bifidobacterium* are common inhabitants of the human gastrointestinal tract and constitute one of the predominant bacteria in the colon of newborns (Arboleya et al., 2012). Due to the health-promoting properties attributed to some strains belonging to different species they are being used as adjunct cultures in dairy products, mainly fermented milks (Gueimonde et al., 2004; Masco, Huys, De Brandt, Temmerman, & Swings, 2005).

The combination of lactic acid bacteria and probiotics could influence the viability of each other in the product (Vinderola, Mocchiutti, & Reinheimer, 2002). In this way, an association influencing viability has been found between some probiotics, as is the case of certain *Bifidobacterium* and *Lactobacillus acidophilus* strains (Gomes, Teixeira, & Malcata, 1998). In the present work, changes in protein expression and resistance to heat shock were analyzed in co-cultures of *B. longum* and *L. delbrueckii* subsp. *lactis* strains by comparison with the corresponding single cultures. To the best of our knowledge this is the first report dealing with the analysis of changes occurring in co-cultures of bifidobacteria and lactobacilli at the proteomic and physiological level.

2. Material and methods

2.1. Microorganisms and growth conditions

L. delbruekii subsp. *lactis* 193 (INLAIN collection) and *B. longum* NCIMB 8809 (National Collection of Industrial, Food, and Marine Bacteria, Aberdeen, Scotland, UK) were routinely grown in de Man-Rogosa-Sharpe broth (MRS, BD Diagnostic Systems) supplemented with 0.05% L-cysteine (w/v) (Sigma Chemical Co) (MRSC) in an anaerobic cabinet (MG500, Don Whitley Scientific, Yorkshire, UK) with an atmosphere of 10% CO_2–10% H_2–80% N_2 at 37 °C. For the co-culture setting, we followed the instructions given by Ruiz, Sánchez, de los Reyes-Gavilán, Gueimonde, and Margolles (2009).

2.2. Two-dimensional gel electrophoresis, protein identification, and statistical analysis

Samples for two-dimensional electrophoresis (2DE) were taken at 10 h after inoculation, which corresponded in all cases with the end of the exponential phase of growth. Cell-free extracts and 2DE were obtained and performed, respectively, as previously described (Ruiz et al., 2009) with minor modifications. Briefly, 500 μg of protein

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from bacterial extracts were loaded onto a strip with a non-linear immobilized pH range of 4.0 to 7.0 (GE Healthcare Biosciences, Uppsala, Sweden) and proteins were identified by peptide mass fingerprinting. Spot detection and volume quantification were performed with ImageMaster Platinum software (version 5.00, GE Healthcare). At least three independent gels were performed for each condition. The effect of co-cultivation on the production of proteins was statistically determined by one-way ANOVA of the normalized spot volume using the type of culture as a factor with two categories: single and co-culture.

2.3. Resistance to heat shock

Cultures grown overnight of both strains, either in mono- and co-cultures, were diluted to an Abs_{600} of 0.6, and aliquots of 1 mL were incubated for 0, 5, 10 or 15 min at 50 °C and 60 °C. Bacterial viability was assessed by plating on MRSC supplemented with agar (2% w/v). Experiments were performed in triplicate and results were expressed as the mean ± standard deviation. Data on the survival to heat-shock (Log cfu/mL) were statistically analyzed by means of one-way ANOVA using the software SPSS-PC 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Effect of co-cultivation in growth kinetics

The influence of compartmentalized co-cultivation on the performance of *L. delbrueckii* subsp. *lactis* and *B. longum* was investigated. In single cultures both microorganisms reached similar levels after 10 h of incubation (Fig. 1). Co-cultivation caused a delay in the growth of microorganisms under study. At 10 h, levels attained by the bifidobacteria were not affected but *Lactobacillus* reached slightly lower counts in co-culture than in mono-culture. No variation in the final pH was detected for each strain growing either alone or in co-culture (Fig. 1). Therefore, cells from single cultures and co-cultures were collected after approximately 10 h of incubation for subsequent experiments.

3.2. Proteomic analysis of co-cultures

Fourteen spots of *L. delbrueckii* subsp. *lactis* and 19 spots of *B. longum* showed statistical differences in their production in co-culture as compared to mono-culture (Table 1; Supplementary material Fig. 1). For subsequent analyses we focused only on spots which were arbitrarily considered to be up or down regulated when their variation factor (VF) was greater than 1.8 or lower than 0.55. According to this criterion, the production of 11 proteins in *L. delbrueckii* subsp. *lactis* and 9 proteins in *B. longum*, belonging to different functional categories, was modified in co-cultures with respect to mono-cultures (Table 1; Supplementary material Fig. 1). Changes in the level of production of different categories of proteins seems to be a common feature in response to stress stimuli as well as in mixed cultures of different microorganisms (De Angelis & Gobbetti, 2004; Ruiz et al., 2009; Sánchez, Ruiz, de los Reyes-Gavilán, & Margolles, 2008).

Co-cultivation strongly affected production of enzymes related with the metabolism of carbohydrates at different levels. Regarding the glycolytic pathway, a protein identified as 6-phosphogluconate dehydrogenase (Supplementary material Fig. 1 A,B, spot 1868), involved in the formation of D-ribulose 5-phosphate in the pentose phosphate pathway with the formation of NADPH, was found over-produced in co-cultivated *B. longum* cells, as it has previously been reported to occur in *B. longum* and *B. animalis* subsp. *lactis* under bile stressing conditions (Sánchez et al., 2005, 2007). On the other hand, *L. delbrueckii* subsp. *lactis* increased the synthesis of a protein identified as beta-galactosidase (Supplementary material Fig. 1 C,D, spot 8930) whereas the enzyme annotated as galactose-1-phosphate uridylyltransferase (Supplementary material Fig. 1 A,B, spot 1929) was over-produced by *B. longum*. Galactose-1-phosphate uridylyltransferase takes part of the lacto-N-biose I/galacto-N-biose, the main metabolic pathway, for using galactose in *B. longum* (Kitaoka, Tian, & Nishimoto, 2005; Nishimoto & Kitaoka, 2007). It is a common feature that changes in environmental conditions influence expression of proteins that relates with sugar metabolism in *B. longum* (Sánchez et al., 2005) and *L. delbrueckii* subsp. *lactis* (Burns et al., 2010). In addition, co-adaptation of different bacteria to the same ecological niche leads to modification of protein expression profiles (Ruiz et al., 2009; Sonnenburg, Chen, & Gordon, 2006). It should be also considered that the MRS medium used for cultures in the present work could be the source of complex carbohydrates; *Bifidobacterium* can release substrates from these complex carbohydrates, all these contributing to some extent to the variations on gene expression in both microorganisms

3.3. Co-cultivation promotes increased survival to heat-shock

Two homologous chaperonins, Hsp60 (Supplementary material Fig. 1 A,B, spot 1767) and GroEL (Supplementary material Fig. 1, C,D, spot 8850), involved in intracellular protein folding and translocation processes (Singh & Gupta, 2009; Ventura, Canchaya, Zink, Fitzgerald, & van Sinderen, 2004) were up-regulated in *L. delbrueckii* subsp. *lactis* and *B. longum*, respectively, when grown in co-culture. Remarkably, treatment of cell suspensions at 60 °C evidenced a significantly higher survival of both microorganisms when they were grown in co-culture

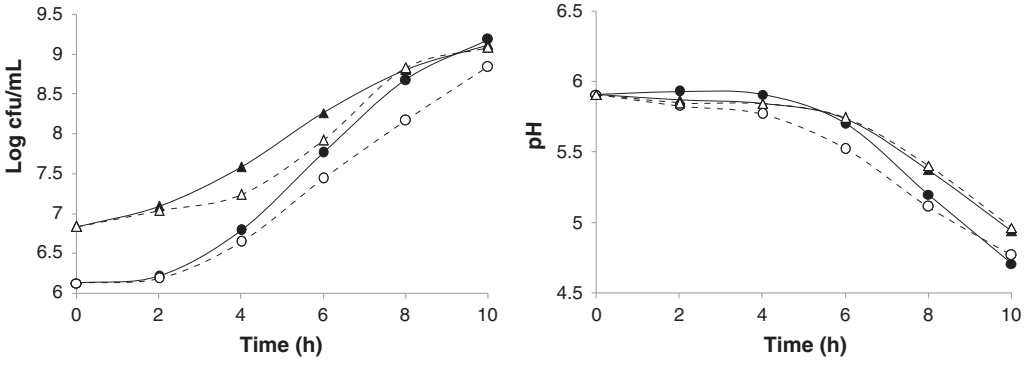

Fig. 1. Mean values of the counts (Log cfu/mL) and pH measured along the growth curves of *B. longum* NCIMB8809 (triangles) and *L. delbrueckii* subsp. *lactis* 193 (circles), grown in single culture (dark symbol-full line) and in co-culture (white symbol-dotted line). The coefficient of variation [(SD∗100)/mean] of data obtained from the three replicates was lower than 1%.

Table 1
Proteins whose production was affected by the compartmentalized co-culture of *L. delbrueckii* subsp. *lactis* 193 and *B. longum* NCIMB 8809.

COG[a] Functional category	Spot[b]	Putative function[c]	pI[d]	MW[e]	Coverage[f]	MOWSE[g]	Norm. V[h]		VF[i]
							L.d. 193	*L.d.* 193*	
Carbohydrate metabolism	10141	Phosphoglycerate kinase	5.28	42742	69	254	0.09 ± 0.19	0.26 ± 0.06	2.78
	8930	Beta-glucosidase/6-phospho-beta-glucosidase/ beta-galactosidase	5.43	55281	12	130	0.12 ± 0.14	0.45 ± 0.07	3.79
	11268	Pyruvate kinase	5.22	63139	50	244	2.28 ± 0.53	1.60 ± 0.20	0.70
	8551	Putative phosphoketolase	5.15	90929	41	282	0.07 ± 0.13	0.29 ± 0.05	4.34
Amino acid metabolism	8985	Dipeptidase	4.77	53646	36	120	0.26 ± 0.02	0.11 ± 0.13	0.44
Nucleotide metabolism	10138	Adenine phosphoribosyltransferase	5.25	19170	82	168	0.03 ± 0.06	0.15 ± 0.06	4.93
Cofactors and vitamin metabolism	10016	Xanthosine triphosphate pyrophosphatase	5.42	22337	56	129	1.01 ± 0.35	0.49 ± 0.34	0.49
	9219	Putative cysteine sulfinate desulfinase/cysteine desulfurase	5.28	42123	48	200	0.09 ± 0.07	0.19 ± 0.04	2.01
Folding, sorting and degradation	8773	Chaperone protein DnaK	4.68	66074	32	139	0.57 ± 0.12	0.35 ± 0.14	0.62
	8850	hsp60	4.68	33771	58	175	0.02 ± 0.04	0.31 ± 0.23	16.79
	9134	Putative oxidoreductase	5.07	50350	54	243	0.53 ± 0.32	0.12 ± 0.17	0.22
Translation	9695	30S ribosomal protein S2	4.98	28369	56	144	0.32 ± 0.39	0.92 ± 0.13	2.92
Transcription	9241	DNA-directed RNA polymerase subunit alpha	4.83	34906	66	233	0.09 ± 0.11	0.42 ± 0.11	4.68
Energy metabolism	9574	Putative manganese-dependent inorganic pyrophosphatase	4.76	34318	42	140	0.69 ± 0.23	0.96 ± 0.13	1.40

COG[a] Functional category	Spot[b]	Putative function[c]	pI[d]	MW[e]	Coverage[f]	MOWSE[g]	Norm. V[h]		VF[i]
							B.l. 8809	*B.l.* 8809*	
Carbohydrate metabolism	2391	Phosphoglycerate mutase 1	5.32	27645	87	250	0.27 ± 0.09	0.36 ± 0.04	1.34
	2091	Dihydrodipicolinate synthase/N-acetylneuraminate lyase	5.39	29612	47	82	0.02 ± 0.05	0.18 ± 0.06	7.87
	1868	6-Phosphogluconate dehydrogenase	5.16	52510	43	159	0.12 ± 0.11	0.28 ± 0.01	2.36
	1929	Galactose-1-phosphate uridylyltransferase	5.27	47043	63	229	0.23 ± 0.13	0.54 ± 0.14	2.37
	1853	UDP-N-acetylglucosamine pyrophosphorylase	5.12	49235	40	86	0.09 ± 0.08	0.24 ± 0.10	2.68
	1672	Phosphoketolase	5.06	92683	40	226	1.84 ± 0.87	2.69 ± 0.33	1.47
	1919	3-Phosphoglycerate kinase	4.93	41840	71	248	1.38 ± 0.12	1.17 ± 0.09	0.84
	1977	Transaldolase	4.87	39739	71	279	4.34 ± 0.72	3.23 ± 0.34	0.74
	2433	Enolase	4.73	46002	52	164	0.99 ± 0.33	0.58 ± 0.22	0.58
	2428	Succinyl-CoA synthetase, alpha subunit	5.33	30823	28	135	0.08 ± 0.09	0.17 ± 0.03	2.19
Cofactors and vitamins metabolism	2414	Protoporphyrinogen oxidase	5.58	61072	13	202	0.07 ± 0.06	–	–
Amino acid metabolism	2395	LuxS protein involved in autoinducer AI2 synthesis	5.48	18648	69	93	0.07 ± 0.07	0.16 ± 0.05	2.32
	2022	Tetrahydrodipicolinate N-succinyltransferase	5.59	35705	42	82	0.08 ± 0.06	0.13 ± 0.02	1.67
	1834	Adenylosuccinate lyase	5.32	53744	58	181	0.27 ± 0.17	0.48 ± 0.13	1.75
Nucleotide metabolism	1880	DNA polymerase sliding clamp subunit	4.54	40999	52	151	0.43 ± 0.22	0.25 ± 0.07	0.58
Folding, sorting and degradation. Stress protein	1767	Chaperonin GroEL	4.72	56860	75	294	0.34 ± 0.45	0.74 ± 0.18	2.18
	2399	Peroxiredoxin	4.53	21588	61	131	0.78 ± 0.14	0.24 ± 0.17	0.31
Translation	1846	Elongation factor Tu	4.88	44137	55	169	2.90 ± 0.21	2.69 ± 0.05	0.93
	2243	Elongation factor P	5.05	20698	45	89	0.16 ± 0.16	0.43 ± 0.10	2.79

[a] COG: cluster of orthologous genes.
[b] Spot numbers refer to the proteins excised from gels and labeled in Fig. 2.
[c] Putative functions were assigned from the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways (http://www.genome.jp/kegg).
[d] Theoretical isoelectric point expressed in pH.
[e] Theoretical molecular masses expressed in daltons.
[f] Percentage of sequence coverage.
[g] Molecular weight search score.
[h] Normalized volume for each protein derived of cells from *L. delbrueckii* subsp. *lactis* 193 grown in single culture (*L.d.* 193) and in co-culture with *B. longum* NCIMB 8809 (*L.d.* 193*) or from *B. longum* NCIMB 8809 grown in single culture (*B.l.* 8809) and in co-culture with *L. delbrueckii* subsp. *lactis* 193 (*B.l.* 8809*); Values: mean ± standard deviation (n = 3 for each growth condition); –: not detected.
[i] Variation factor: ratio of normalized volume for each protein, i.e., normalized volume value of co-culture divided by that of single culture.

than when they were incubated alone (Fig. 2). A recent paper reported the over-production of some stress-response related proteins when two different *Bifidobacterium* strains were co-cultivated (Ruiz et al., 2009). This may be a technologically suitable property in those cases where mild heating is needed at any step during the production process, as occurs during the manufacture of some rennet-coagulated cheeses (Ruas-Madiedo, Alonso, Delgado, Bada-Gancedo, & de los Reyes-Gavilán, 2002).

In short, co-cultivation induced metabolic changes in *B. longum* and *L. delbrueckii* subsp. *lactis*., mainly (i), affecting the expression of proteins related with carbohydrate metabolism and (ii), promoting accumulation of higher amount of molecular chaperones related to increased survival to heat-shock.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.01.026.

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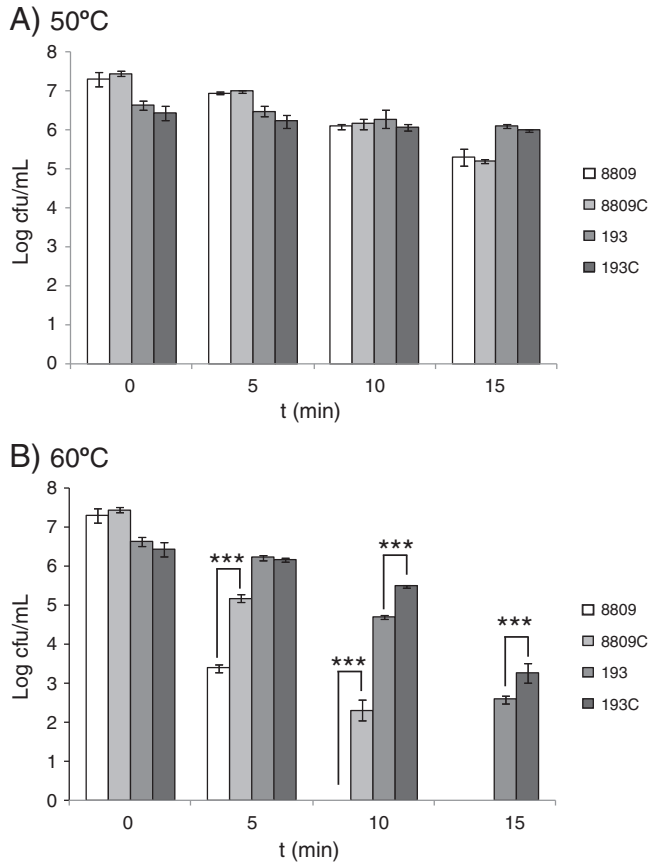

Fig. 2. Resistance to heat shock (50 and 60 °C) of *B. longum* NCIMB8809 and *L. delbrueckii* subsp. *lactis* 193, grown independently and in co-culture. For each microorganism, and in each sampling point, independent one-way ANOVAs were made to compare results obtained in single-culture with that of the co-culture. Statistical differences are denoted with asterisks (***$p<0.001$).

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