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2 **intestinal symbiont *Bacteroides thetaiotaomicron*: impressive antioxidative**
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4
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17
18 **Abbreviations**

19 Superoxide dismutase (SOD), glutathione reductase (GR), reactive oxygen species (ROS), glucose-
20 yeast medium (GYE medium), aliquots of the extracts from both yeasts obtained after culture in broth
21 (“*K. marxianus* S-02-5 liq” and “*K. marxianus* MUCL 29917 liq”, respectively), aliquots of the
22 extracts from both yeasts obtained after aerobic culture on agar medium (“*K. marxianus* S-02-5 surf”
23 and “*K. marxianus* MUCL 29917 surf”, respectively), transepithelial electrical resistance (TER),
24 acetonitrile (ACN), distilled water (DW), Trifluoroacetic acid (TFA).

25

Abstract

The effects of yeast *Kluyveromyces marxianus* S-2-05, of cheese origin, were assessed on the intestine anaerobe symbiont *Bacteroides thetaiotaomicron* ATCC 29741 to unveil any changes in its antioxidant properties. To this end, these microorganisms were grown and incubated either separately, or co-incubated, under anaerobic atmosphere. Afterwards, the microbial cells were recovered and washed, and extracts were prepared using a sterile detergent solution to mimic the intestine detergent content. The extracts prepared from *K. marxianus* S-2-05 and reference strain *K. marxianus* MUCL 29917, grown under different conditions, were assessed for their antioxidant properties against superoxide anion and hydrogen peroxide. Extracts from both yeasts showed antioxidative effects, which were particularly important for *K. marxianus* S-02-5 after anaerobic incubation. Moreover, *K. marxianus* S-02-5 displayed a high level of activity against the aforementioned reactive oxygen species, enhancing that of *B. thetaiotaomicron* ATCC 29741, after the co-incubation process. Two-dimensional polyacrylamide gel electrophoresis was used to separate the proteins extracted. Superoxide dismutase, thiol peroxidase, rubrerythrin -intensively produced by *B. thetaiotaomicron* induced by the yeast- were identified by mass spectrometry. The antioxidative potential evidenced for *K. marxianus* S-02-5 is another advantage which could justify the utilization of this strain as a probiotic for countering intestinal inflammatory processes.

Keywords: *Kluyveromyces marxianus*, *Bacteroides thetaiotaomicron*, anaerobic, antioxidant, probiotic

Chemical compounds studied in this article:

Acetonitrile (PubChem CID: 6342); Acrylamide (PubChem CID: 6579); α -cyano-4-hydroxy-cinnamic acid (PubChem CID: 5328791); Ammonium bicarbonate (PubChem CID: 14013); 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (PubChem CID: 71774074); Dithiotreitol (PubChem CID: 446094); Formic acid (PubChem CID: 284); Iodoacetamide (PubChem CID: 3727); Ringer solution (PubChem CID: 56841909); Trifluoroacetic acid (PubChem CID: 6422).

52 1. Introduction

53 *Bacteroides thetaiotaomicron* can be considered as a model of anaerobic bacterium, a Gram-negative
54 bacillus symbiont highly adaptable to its environment (Xu & Gordon, 2003), and belonging to the
55 predominant intestinal microbiota which is mainly composed of anaerobes. While this bacterium was
56 well known before the 2000s as an opportunistic pathogen with an alarming profile of antibiotic
57 resistance, a novel vision was then born after 2004 indicating that *B. thetaiotaomicron* could have
58 beneficial attributes for humans (Kelly et al., 2004). For the treatment of severe colitis or Crohn's
59 disease, fecal microbiota transplantation is the infusion of liquid filtrate feces from a healthy donor
60 (who is negative to a screening panel of laboratory exams) into the gut and *B. thetaiotaomicron* is a
61 part of stool or fecal microbiota transferred to patients (Cammara et al., 2015; Petrof & Khoruts,
62 2014). This bacterium has also received Orphan Designation for paediatric Crohn's disease by the US
63 Food and Drug Administration (2013). Previously, we established that under defined conditions, *B.*
64 *thetaitotaomicron* displayed antioxidant and anti-inflammatory properties and antioxidant
65 enzyme/proteins, chaperonin proteins produced by this bacterium were identified (Hochart-Behra et
66 al., 2008; Hochart-Behra, Drobecq, Turret, Dubreuil, & Behra-Miellet, 2014). To gain more insights,
67 we investigated the interactions between *B. thetaiotaomicron* ATCC 29741 and *Kluyveromyces*
68 *marxianus* S-02-5 recently isolated from cheese and characterized for its beneficial attributes
69 (Ceugniz, Drider, Jacques, & Coucheney, 2015). Here we established that co-incubation of *B.*
70 *thetaitotaomicron* ATCC 29741 with *Kluyveromyces marxianus* S-02-5 could either retain or enhance
71 its antioxidant potential. The antioxidative properties of yeasts were reported to induce microbial
72 production of SOD (Dellomonaco et al., 2007). *K. marxianus* species were previously found to
73 enhance superoxide dismutase (SOD) and glutathione reductase (GR) when they were exposed to
74 hydrogen peroxide (Pinheiro, Belo, & Mota, 2002), delineating their antioxidative properties. In direct
75 line, Maccaferri *et al.* (Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012) reported the capacities
76 of *K. marxianus* B0399 to diminish the levels of production of pro-inflammatory cytokines in PBMCs
77 and Caco-2 cells, caused by stimulation with lipopolysaccharide.

78 The present work aimed at studying, under anaerobic conditions, the interactions between *B.*
79 *thetaitotaomicron* ATCC 29741 and *K. marxianus* S-02-5. To this end, they were placed into a mild

80 detergent mixture containing a cholic acid derivative preserving viability, in order to detect if these
81 microorganisms are able to reject antioxidant elements from their surface at a higher level than
82 previously described for *B. thetaiotaomicron* grown alone (Hochart-Behra et al., 2008; Hochart-Behra
83 et al., 2014). The activities against superoxide anion and hydrogen peroxide were measured *in vitro*.
84 These reactive oxygen species (ROS) are the first described in the oxidative cascade of phagocytes
85 (Hochart-Behra et al., 2008; Hochart-Behra et al., 2014). Two-dimensional (2D) gels and matrix-
86 assisted laser desorption ionization time of flight – Time of flight (MALDI TOF/TOF) were used to
87 separate and identify any antioxidant enzymes/proteins in the compounds secreted both
88 microorganisms.

89

90 **2. Materials and methods**

91 *2.1. Strains and growth conditions*

92 *B. thetaiotaomicron* strain ATCC 29741 (American Type Cultured Collection) and *K. marxianus*
93 strain S-02-5 recently isolated from cheese (Ceugnietz et al., 2015), stored at -32°C, were revitalized
94 and pre-cultured in/on suitable broth or agar medium before any experiment. *B. thetaiotaomicron*
95 ATCC 29741 was grown on anaerobe broth (DIFCO, BD Dickinson, Le Pont de Claix, France), and
96 yeasts *K. marxianus* strain S-02-5 and *K. marxianus* MUCL 29917, used as reference strain, were
97 grown on GYE (glucose-yeast) agar medium (2% Glucose, 0.5% yeast extract, 1.5% Agar).

98 Preliminary growth of *B. thetaiotaomicron* ATCC 29741 and yeasts were observed after a three-day
99 incubation in anaerobic jars (AnaeroGen, Oxoid, Basingstoke, Hampshire, England), at 35-36°C and
100 aerobically at 30°C for *B. thetaiotaomicron* ATCC 29741 and *K. marxianus* S-02-5, respectively.

101

102 *2.2. Interactions inter-species: B. thetaiotaomicron ATCC 29741 vs. K. marxianus S-02-5*

103 After a preliminary growth, *B. thetaiotaomicron* ATCC 29741 and *K. marxianus* S-02-5 were cultured
104 separately or co-cultured anaerobically at 35-36°C for two days in pre-reduced Brucella broth
105 (DIFCO) supplemented with K3 vitamin (1 mg/L) (Sigma, Saint-Quentin Fallavier, France), suitable
106 for *Bacteroides*. For *K. marxianus* S-02-5, the moistened yeast equivalent to 400 mg of the dried

107 microorganism (determined by drying the strains for 2.5 hours using a SpeedVac) (LabConco
108 Corporation, Kansas City, USA), was inoculated into the growth medium. Counts before and after *B.*
109 *thetaitotaomicron* ATCC 29741 and *K. marxianus* S-02-5 together, were performed. After three-days
110 co-culture of both strains together in a growth medium, the broth containing strains was transferred
111 into pre-sterile centrifuge tubes, and tubes were centrifuged at 10,000g for 13 mins at 4°C. The
112 supernatant was decanted off and the pellet cells were washed and diluted in Ringer ¼ medium
113 (Oxoid, Hampshire, England). The spreading of the suitably diluted cells was done on Brucella agar
114 surface (enriched with K3 vitamin and haemin at the concentrations of 1 and 5 10⁹ mg/L, respectively)
115 to assess the viability and/or growth of microorganisms after co-culture. The same process was applied
116 to *B. thetaitotaomicron* ATCC 29741 and *K. marxianus* S-02-5 grown concomitantly and separately, in
117 the same conditions as for their co-culture. After centrifugation, followed by three successive washes
118 in Ringer medium ¼, the pellets of pure *B. thetaitotaomicron* ATCC 29741 and of pure *K. marxianus*
119 S-02-5 and of *B. thetaitotaomicron* ATCC 29741 co-culturing with *K. marxianus* S-02-5 were placed
120 in agitation in mild detergent solution containing 7 mM 3-[(3-cholamidopropyl) dimethylammonio]-1
121 propanesulfonate, 0.5 mM tris(hydroxymethyl) aminomethane hydrochloride at pH 6.8 (Serva,
122 Heidelberg, Germany) - a cholic acid derivative - overnight at 4°C, as previously described for *B.*
123 *thetaitotaomicron* (Hochart-Behra et al., 2008; Hochart-Behra et al., 2014) at a rate of 4 ml of
124 extraction solution per g of microorganisms. The suspensions were centrifuged and the supernatant
125 was filtered 0.22 µ (Millipore corporation, Bedford, MA, USA), and assayed for protein content
126 (Hochart-Behra et al., 2008). The extracts were supposed to contain antioxidant compounds released
127 from the surface of microorganisms. Counts were also performed before and after extraction in order
128 to assess the viability of *B. thetaitotaomicron* ATCC 29741 and *K. marxianus* S-02-5. The *in vitro*
129 pharmacological assays were then carried out as noted below.

130

131 2.3. Comparison of intra yeast species: *K. marxianus* S-02-5 vs. *K. marxianus* MUCL 29917

132 After the preliminary growth, both yeast strains *K. marxianus* S-02-5 and *K. marxianus* MUCL 29917
133 were separately incubated in the pre-reduced Brucella broth as mentioned above, under anaerobic
134 atmosphere, before proceeding with the extraction of elements secreted at the surface of the yeasts,

135 using the method described above. The same extraction procedure was performed on both yeast strains
136 collected from GYE agar after an aerobic two-day incubation.

137 The aliquots of the extracts from both yeasts were named “*K. marxianus* S-02-5 liq” and “*K.*
138 *marxianus* MUCL 29917 liq” (after culture in broth), “*K. marxianus* S-02-5 surf” and “*K. marxianus*
139 MUCL 29917 surf” (after aerobic culture on agar medium). Protein extracts were assayed using the
140 Folin and Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

141

142 2.4. *Pharmacological in vitro assays*

143 A superoxide anion produced using a cell-free model and hydrogen peroxide (Sigma) was placed into
144 a reaction mixture at a concentration of about 10 nmol/ml. Both ROS were in contact with increasing
145 concentrations of the different extracts to study their inhibitory effect on ROS. Measurements of $O_2^{\cdot -}$
146 superoxide anion and H_2O_2 were made by spectrophotometry, according to Aruoma *et al.* (Aruoma,
147 Halliwell, Hoey, & Butler, 1989), and using methods largely tested and adapted to prevent interference
148 (e.g., with peroxidases) (Leavey *et al.*, 2002).

149

150 2.5. *Statistical analysis*

151 To study the variations between the populations of *B. thetaiotaomicron* ATCC 29741 and *K.*
152 *marxianus* S-02-5 and *K. marxianus* MUCL 29917 during co-culture and extraction, the non-
153 parametric sign-test was used at the 5% level (n=5 independent experiments) and the Mann and
154 Withney test was used at the 5% level, in case of small non-paired samples (Anastat). For
155 pharmacological *in vitro* assays, the data was analysed from five independent assays using the Kruskal
156 & Wallis test at the 5% level (p=0.05) (Anastat). Results were presented as box-plots where the
157 extremities of the lowest and the highest bars represent the 10th and 90th percentiles of percentages for
158 each extract concentration. The 25th, 50th and 75th percentiles correspond to the inferior, interior and
159 superior horizontal bars of the boxes constructed for each extract concentration.

160

161 2.6. *Proteomic analysis*

162 The extracts (all at a volume equivalent to 500 µg proteins secreted by *K. marxianus* S-02-5) were
163 desalted, re-solubilized, lyophilized and the protein separation performed in gels as previously
164 described for 2D PAGE (Hochart-Behra et al., 2014). Proteins were loaded on precast immobilized pH
165 3-10 gradient ReadyStrip (17 cm, BioRad) and isoelectric focusing (IEF) was performed in the
166 BioRad Protean IEF cell system. Vertical gels with 4-20% acrylamide (BioRad) gradient (non-
167 denaturing gels) were performed for SDS PAGE. Separate proteins in the gels were then stained
168 (colloidal Coomassie blue) fixed and washed. Gel images were acquired using a GS800 densitometer
169 (BioRad). PDQuest software (BioRad) was used to analyze the protein profile of the 2D-gels of pH 3-
170 10 gradient. Protein spots of interest were excised and prepared to perform protein identification by
171 peptide mass finger printing technique using MALDI TOF/TOF mass spectrometer. They were in-gel
172 trypsin-digested according a method adapted from that of Shevchenko *et al.* (Shevchenko, Wilm,
173 Vorm, & Mann, 1996). Protein spots were first discolored [20 mM ammonium bicarbonate (Prolabo,
174 Fontenay-Sous-Bois, France), 50% (vol/vol) acetonitrile (ACN, Carlo Erba Reagents) in distilled
175 water (DW) solution], dried by rotary evaporation (miVac QUATTRO concentrator, Genevac®,
176 Ipswich, England). Then reduction for 45 min at 56 °C [20 mM ammonium bicarbonate, 10 mM 1,4-
177 dithiotreitol in DW solution (Sigma)], removal of supernatants and alkylation for 45 min at 20 °C in
178 the dark [20 mM ammonium bicarbonate, 55 mM iodoacetamide (Sigma)] were performed. After
179 removal of supernatants, washing of the gels with acetonitrile and drying by rotary evaporation, the
180 trypsin digest was then made using a 12,5 mg L⁻¹ trypsin solution (Trypsin/Lys-C Mix, Promega,
181 Madison, WI). The peptides were extracted from protein spots with 50% ACN, 50% ammonium
182 bicarbonate in DW (vol/vol) solution and finally with a solution of 0.5% formic acid in ACN. The
183 supernatants were pooled before being concentrated and desalted on a ZipTip µ-C18® column
184 (Millipore). The peptides were eluted by a 80% ACN, 0.1% trifluoroacetic acid (TFA) (vol/vol)
185 solution. Mass analysis was performed with a Bruker Autoflex Speed MALDI TOF/TOF mass
186 spectrometer (Bruker-Franzen Analytik, Bremen, Germany) according Shevchenko *et al.* (Shevchenko
187 et al., 1996). The MALDI target plate (AnchorChip™) was covered with extracted peptides mixed
188 with α-cyano-4-hydroxy-cinnamic acid matrix (2.8 mg/mL in ACN:TFA, 85:0.1 vol/vol). The
189 molecular mass measurements were performed in automatic mode using FlexControl™ 3.4 software

190 on an Autoflex Speed™ TOF/TOF instrument and in reflectron mode for MALDI-TOF peptide mass
191 fingerprinting (PMF) or LIFT mode for MALDI-TOF/TOF peptide fragmentation fingerprinting
192 (PFF). External calibration over the 1000-3500 mass range was performed using the [M+H]⁺
193 monoisotopic ions of bradykinin 1-7, angiotensin I, angiotensin II, substance P, bombesin and
194 adrenocorticotrophic hormone (clips 1-17 and clips 18-39) from a peptide calibration standard kit
195 (Bruker Daltonik). Each spectrum was produced by accumulating data from 1 200 laser shots. A
196 maximum of ten precursor ions per sample were chosen for LIFT-TOF/TOF MS/MS analysis. Peak
197 lists were generated from MS and MS/MS spectra using Flexanalysis™ 3.4 software (Bruker
198 Daltonik). Database searches using combined PMF and/or PFF datasets were performed in the UniProt
199 and NCBI nr databases via BioTools 3.2 (Bruker Daltonik) [with Mascot 2.2 (Matrix Science Ltd,
200 London, UK)] or PEAKS Studio 7.0 (Bioinformatics Solutions) or Profound (taxonomy restricted to
201 Bacteria, Yeast or not restricted). A mass tolerance of 150 ppm and 1 missing cleavage site for PMF
202 and an MS/MS tolerance of 0.5 Da and 1 missing cleavage site for MS/MS searching were allowed.
203 Variable cysteine carbamidomethylation and methionine oxidation were also considered. The
204 relevance of protein identities was judged according to their score in the research software (p value of
205 0.05 (p<0.05), False Discovery Rate < 1%). Searching was also made with Mascot in the National
206 Center for Biotechnology Information (NCBI), SwissProt and Uniprot databases.

207

208 3. Results

209 3.1. The interaction benefited the intestine bacteria and the yeast population was stable

210 As expected, when grown alone under anaerobic conditions, the cell count of *B. thetaiotaomicron*
211 ATCC 29741 increased by about 1 log (CFU/L of culture) ($p=0.0313$). On the other hand, the yeast
212 cell count remained stable during transit in the anaerobic atmosphere. Means of CFU counts varied
213 from 10.35 ± 0.07 log to 10.22 ± 0.09 log. Nevertheless, during co-incubation, the cell count of *B.*
214 *thetaiotaomicron* ATCC 29741, expressed in CFU/L of culture, significantly increased by a mean
215 value of about 0.9 log ($p=0.0313$) whilst that of *K. marxianus* S-02-5 has a drop of about 0.3 log
216 CFU/L of culture. Clearly, *B. thetaiotaomicron* ATCC 29741 took advantage of this interaction but

217 the cell counts of yeasts remained stable. During the extraction of the surface elements from these
218 microorganisms, the viabilities of *K. marxianus* S-02-5 and *B. thetaiotaomicron* ATCC 29741 were
219 also stable after single cultures. The counts of these populations, expressed in log CFU per total
220 suspension of microorganisms in solution of extraction, were 10.33 ± 0.27 log before extraction and
221 11.07 ± 0.27 log after extraction ($p=0.6875$) for *B. thetaiotaomicron* ATCC 29741; 9.93 ± 0.45 log
222 before extraction and 9.81 ± 0.56 log after extraction for *K. marxianus* S-02-5 ($p=0.1875$). The
223 bacterial population remained stable overall during the extraction after co-culture. Indeed, the counts
224 ranged from 10.71 ± 0.81 to 10.96 ± 1.17 log, with p value 0.500, and a small but not statistically
225 significant loss was observed for *K. marxianus* S-02-5, the counts of which ranged from 10.08 ± 0.08
226 to 9.88 ± 0.63 log with p value of 0.1875). Notably, similar protein quantities were found in the
227 extracts (2.94 ± 1.75 , 1.82 ± 0.94 and 1.36 ± 0.42 mg/ml in the extracts related to co-culture, to ATCC
228 29741 grown alone and to *K. marxianus* S-02-5, respectively).

229
230 As depicted in Fig. 1 and 2, the data of the pharmacological *in vitro* assays show the effects of the
231 extracts on superoxide anion and hydrogen peroxide, respectively. The antioxidative effect of the
232 extract from the co-culture appeared to be similar against superoxide anion (concentration of
233 inhibition at 50% or IC50 of about 51 μ l of extract/ml) and higher against hydrogen peroxide (IC50 of
234 about 31 μ l of extract/ml), than the effect of the extract obtained from *B. thetaiotaomicron* ATCC
235 29741 alone. The IC50 was 51 μ l of extract/ml for superoxide anion, and was not reached at the
236 highest concentration of extract tested on hydrogen peroxide. The extract obtained from *K. marxianus*
237 S-02-5 cultured alone inhibited superoxide anion at the concentration of 10 nmol/ml with an IC50 of
238 about 25 μ l of extract/ml. IC50 for hydrogen peroxide was obtained at 6 μ l of extract/ml.

239
240 The protein analysis of the *B. thetaiotaomicron* extract revealed the presence of antioxidant or
241 oxidation-reduction enzymes (2D gel and protein identifications available in Supplementary
242 material in Fig. S1 and Tables S5 and S6) such as thiol peroxidase (spots o1 and o2), ferritin
243 (o3), antibiotic biosynthesis monooxygenase (o4). However, superoxide dismutase is not

244 detected in these extracts. As previously described (Hochart-Behra et al., 2008), chaperonin
245 proteins were also found such as chaperone protein DnaK (c1), SCO1/SenC chaperone.
246 Glutamate decarboxylase (s1), is another stress protein previously widely described in our
247 extracts (Hochart-Behra et al., 2008). The other proteins concern the carbohydrate catabolic
248 pathway from *Bacteroides* (phosphoenolpyruvate carboxykinase in spots p1 and p2,
249 lactate/malate dehydrogenase in spots p5 and p6, glyceraldehyde-3-phosphate dehydrogenase
250 in spots p7, p9 to p11). Tetratricopeptide repeat protein (spot p3), magnesium chelatase,
251 subunit I/ATPase (p4) and glutaminase (in spot p8) were also found.
252 As native 2D-gels were used for protein migration, protein associations could be possible
253 such as dimers (or more associated monomers) and they could migrate at different molecular
254 weights in the gels. With this technique, protein interactions can be visualized and one spot
255 can correspond to several proteins. Conversely, although our method was less denaturing for
256 the proteins migrating in the gels, their breaking in fragments could however occur and appear
257 at a molecular weight lower than expected.
258 The proteins extracted from both co-cultivated microorganisms were mainly identified for *B.*
259 *thetaitaomicron* (Fig. 3; protein identification available in supplementary material Tables S7
260 and S8) in different categories: the group of antioxidant proteins with superoxide dismutase
261 (spots BT-o1 and BT-o2), rubrerythrin (BT-o3), probable thiol peroxidase/lipid hydroperoxide
262 peroxidase (BT-o4 to BT-o7), the group of chaperones with the Chaperone protein DnaK
263 (BT-c1), Molecular chaperone DnaJ (BT-c2), Cationic outer membrane protein OmpH (BT-
264 c3), Heat shock protein Hsp20 (BT-c4), 10 kDa chaperonin (BT-c5) and other stress proteins
265 such as Glutamate decarboxylase (BT-s1 and BT-s2). Proteins of the carbohydrate catabolic
266 pathway from *Bacteroides* were also found in these extracts (phosphoenolpyruvate
267 carboxykinase in spots BT-p1, BT-p3 and BT-p4 or lactate/malate dehydrogenase
268 /glyceraldehyde-3-phosphate dehydrogenase (in spots BT-p2 and BT-p6 to BT-p9) or 2,3-

269 bisphosphoglycerate-dependent phosphoglycerate mutase in spots BT-p12 and BT-p13.
270 Succinate-CoA ligase in spot BT-p14), NAD dependent epimerase (BT-p15) were also
271 identified. Enolase was found for *K. marxianus* S-02-5 (K-p1). Tetratricopeptide repeat protein
272 (BT-p5), Choloylglycine hydrolase (BT-p10), Glutaminase (BT-p11).

273
274 In the extract obtained from *K. marxianus* S-02-5, protein analysis (2D gel and protein identifications
275 available supplementary material Fig. S2 and Tables S9 and S10) could underpin among others the
276 presence of glucose-6-phosphate isomerase (p3), hexokinase (in spot p4), enolase (p1, p5, p7, p12, p14,
277 p19, p20), enoate reductase 1 (p10), phosphoglycerate kinase (p9, p12, p13, p15), (exo)inulinase (p2),
278 NAD(P)H-dependent D-xylose reductase or Xyl1 (o2), NADPH-dependent alpha-keto amide reductase
279 (o1), phosphoglycerate mutase 1 (p16 à p18), peptidyl-prolyl cis-trans isomerase (s1).

280
281 *3.2. Comparison of both K. marxianus strains: stability of the yeasts incubated under anaerobic*
282 *condition or during the extraction of antioxidant metabolites*

283 *K. marxianus* S-02-5 and *K. marxianus* MUCL 29917 strains were compared for their antioxidant
284 potential. Thus, their incubation under anaerobic conditions at 35-36°C confirmed the stability of the
285 cell counts of *K. marxianus* S-02-5 or *K. marxianus* MUCL 29917 under these conditions.

286 Remarkably, both yeasts survived during the process of extraction as indicated by colony forming unit
287 (CFU) counts (only 0.1 log CFU-loss during the extraction). When yeasts were collected at the GYE
288 surface after aerobic culture, cell counts before and after extraction underlined the stability of *K.*
289 *marxianus* S-02-5 and *K. marxianus* MUCL 29917 during the extraction ($p>0.05$).

290 Overall, the protein contents of the extracts appeared to depend on the method of yeast production.
291 The mean protein concentration of the extract “*K. marxianus* S-02-5 liq” (2.6 ± 0.41 mg/ml) was
292 tenfold higher than that of “*K. marxianus* S-02-5 surf” (0.25 ± 0.14 mg/ml). For “*K. marxianus* MUCL
293 29917 liq”, protein concentration was quadrupled (2.08 ± 0.35 mg/ml), relatively to that of “*K.*
294 *marxianus* MUCL 29917 surf” (0.53 ± 0.31 mg/ml).

295 After growth of *K. marxianus* S-02-5 and *K. marxianus* MUCL 29917 under anaerobic conditions in
296 broth, the mean IC_{50} of the extracts against superoxide anion were about 7 μ l/ml for both “*K.*
297 *marxianus* S-02-5 liq” and “*K. marxianus* MUCL 29917 liq” (Fig. 4). A strong action of both extracts
298 was found against hydrogen peroxide, with however a superiority to “*K. marxianus* S-02-5 liq” which
299 IC_{50} was still of about 5 μ l of extract/ml. That of “*K. marxianus* MUCL 29917 liq” reached 14 μ l of
300 extract/ml (Fig. 5).

301 After aerobic growth of yeast strains on GYE agar, an inhibitory effect was observed for the extract
302 “*K. marxianus* S-02-5 surf” against superoxide anion, with a maximal activity (39.6% inhibition of the
303 positive control of O_2^- production) at 100 μ l of extract/ml. For “*K. marxianus* MUCL 29917 surf” the
304 maximal mean inhibition was higher with 84.6% at 100 μ l of extract/ml (IC_{50} 30.75 μ l of extract/ml).
305 The maximal mean inhibitions of hydrogen peroxide of 23.5% and 43% were observed for “*K.*
306 *marxianus* S-02-5 surf” and “*K. marxianus* MUCL 29917 surf” at 100 μ l of both extracts/ml,
307 respectively.

308

309 4. Discussion

310 In the intestinal environment, the interactions between the symbiont *B. thetaiotaomicron*, known for
311 its anti-inflammatory and antioxidative properties (Hochart-Behra et al., 2008; Hochart-Behra et al.,
312 2014) and *K. marxianus*, were reported to produce antioxidant enzymes. Here, novel insights
313 regarding the anti-oxidative properties of the anaerobe in contact with yeast isolated from cheese *K.*
314 *marxianus* S-02-5 and *K. marxianus* MUCL 29917 used as control were examined and the cell counts
315 of these microorganisms were determined. Overall, the experiments performed here were aimed at
316 establishing whether *K. marxianus* S-02-5, recently isolated from cheese, could provide further
317 probiotic attributes for its utilisation in inflammatory bowel diseases.

318 The interactions between *B. thetaiotaomicron* ATCC 29741 and *K. marxianus* S-02-5 and *K.*
319 *marxianus* MUCL 29917 privileged the *B. thetaiotaomicron* ATCC 29741 population under anaerobic
320 conditions. The extracts obtained in the detergent solution, mimicking therefore the intestinal
321 detergent conditions, confirmed an inhibitory effect of *B. thetaiotaomicron* ATCC 29741 metabolites
322 on both O_2^- and H_2O_2 . The proteins identified in the intense spots in the gel, relative to the bacterial

323 extract, were similar to those previously reported (Hochart-Behra et al., 2014), such as ferritin, thiol
324 peroxidase. They are likely involved in the antioxidative effects observed for the extract. However,
325 SOD was not detected, especially in its hexameric form (Hochart-Behra et al., 2008). Stress proteins
326 and chaperones were also found as expected in this type of extract: DnaK, SCO1/SenC chaperone
327 which family has been extensively studied for its role in the incorporation of copper in the catalytic
328 site of oxidases (Fujimoto et al., 2012) and other proteins such as glutamate decarboxylase (largely
329 described in our previous extracts) which genes are known to be positively regulated by acidic shock,
330 salt stress and stationary growth phase in *Escherichia coli* (De Biase, Tramonti, Bossa, & Visca,
331 1999). Magnesium chelatase, subunit I/ATPase was identified in the *B. thetaiotaomicron* extract
332 whose ATPase activity could be regulated by thioredoxin redox. In pea plants, it has been reported to
333 modulate the redox-mediated signaling in tetrapyrrole biosynthesis and the homeostasis of reactive
334 oxygen species (Luo et al., 2012). A group of main enzymes involved in the carbohydrate catabolic
335 pathway of *Bacteroides* which lead to acetate, butyrate, propionate, succinate and lactate (Fischbach &
336 Sonnenburg, 2011) was found here yet . We identified a tetratricopeptide repeat protein (TPR). It
337 could participate in the formation of protein complexes. TPRs generally form amphipathic helix pairs
338 that mediate protein–protein interactions and multi-protein complex assemblies with other TPR-
339 containing or non-TPR-containing proteins (Bakolitsa et al., 2010). Such patterns have been described
340 for a protein complex of *B. thetaiotaomicron* involved in the starch utilization. Under our experimental
341 conditions, we had previously shown using electronic microscopy that the anaerobic bacterium
342 exported to the extraction medium an assembly of survival proteins in the form of budding vesicles
343 which were probably responsible for the antioxidant effect of the extract (Hochart-Behra et al., 2014).

344 The effect of the extract on ROS was enhanced especially on H₂O₂, when both microorganisms (*K.*
345 *marxianus* and *B. thetaiotaomicron*) were co-incubated. Moreover, the viabilities of both
346 microorganisms were stable during the extraction. The effect of the extract obtained from *K.*
347 *marxianus* S-02-5 incubated alone in broth under anaerobic conditions showed strong antioxidant
348 activity. Indeed, as little as 25 µl of extract/ml was sufficient to inhibit 50% of superoxide anion or
349 hydrogen peroxide. The superoxide dismutase from *K. marxianus* was previously reported to act

350 efficiently against O_2^- (Stefanova, Ratcheva, Nikolova, & Neychev, 2014), and could explain the
351 inhibition observed here upon addition of our extracts. Curiously, no yeast SOD was identified among
352 the major spots in the gels relative to the extracts resulting from the co-culture or from *K. marxianus*
353 S-02-5 grown alone. The eukaryotic yeast Cu/Zn SOD has been studied and would appear in gel at
354 around 34 kDa as dimeric form or at 17 kDa as monomer (Dellomonaco et al., 2007; Nedeva et al.,
355 2009; Stefanova et al., 2014). *K. marxianus* was viable during the extraction arguing that cell
356 disruption did not occur during the process. The *K. marxianus* SOD is cytosolic and was not released
357 into the extraction medium. Furthermore, we could also expect the presence of peroxidases and /or of
358 thiol-containing proteins in the extracts, countering H_2O_2 . The unique yeast protein identified in 2D-
359 gel performed with the extract from the co-culture was enolase, enzyme of the glycolytic pathway. The
360 yeast seemed overshadowed by the contact of *B. thetaiotaomicron* after anaerobic co-culture. As a
361 reminder, *B. thetaiotaomicron* is able to produce a large amount of acetate *via* the carbohydrate
362 anaerobic pathway. It has been recently reported (Martynova et al., 2016) that *K. marxianus* was
363 inhibited in its growth by acetate. It could extend lag phase, during which cells adapt to elevated
364 acetate concentrations, and growth reoccurs at a slower rate. In contrast, the anaerobe seemed
365 rapidly benefit from the presence of yeast and produced key-antioxidant/stress proteins, much more
366 abundant way than when it is cultivated at the same level out of the presence of the yeast. The full
367 range of enzymes/proteins useful in the detoxification of ROS was highlighted in 2D-gels, especially
368 the SOD in its hexameric (in spot o1 in Fig. 3) or monomeric (spot BT-o2 in Fig. 3) form. The
369 presence in gels of an intense spot of SOD hexamer seemed related to a powerful effect against
370 superoxide anion (Hochart-Behra et al., 2008). Rubrerythrin (spot BT-o3), probable thiol
371 peroxidase/lipid hydroperoxide peroxidase (spots BT-o4 to BT-o7) as shown in Fig. 3
372 constitute a full arsenal against H_2O_2 . Rubrerythrins are non-heme di-iron proteins involved in
373 oxidative stress defense as peroxide scavengers in a wide range of organisms. These proteins
374 belonging to the ferritin-like superfamily contain a C-terminal rubredoxin-like domain
375 involved in electron transport used during catalysis in anaerobic conditions (Cardenas,
376 Quatrini, & Holmes, 2016). *B. thetaiotaomicron*, was reported by Mishra and Imlay (Mishra

377 & Imlay, 2013) to use a consortium of enzymes to scavenge hydrogen peroxide, and
378 rubrerythrin devoid of any secretory leader sequence. It appears, for these authors, to be
379 devoted to scavenging H₂O₂ in the cytoplasmic compartment. However, in our study, the
380 bacterial cell viability was preserved and we observed an intense protein spot for rubrerythrin
381 in 2D-gel. Our previous studies have also shown a particular protein export mode by budding
382 of vesicles on the bacterial wall which could be used for rubrerythrin and peroxidases export.
383 Chaperones are useful for refolding aggregated proteins after stress such as (in Fig. 3) DnaK
384 (in spot BT-c1), DnaJ (BT-c2), cationic outer membrane protein OmpH (BT-c3), Heat shock
385 protein Hsp20 (BT-c4), 10 kDa chaperonin (BT-c4) and other stress proteins such as
386 glutamate decarboxylase (BT-s1 and BT-s2). So the *B. thetaiotaomicron* glutaminase found in our
387 extract may be essential to provide from glutamine the glutamate substrate to glutamate
388 decarboxylase. TPR was once again identified in the extract, performed this time with both
389 microorganisms. The extract from the co-culture contained also the full enzyme necessary for
390 the carbohydrate catabolism et to provide energy (Miller & Wolin, 1979). As for
391 choloylglycine hydrolase, this enzyme catalyses the hydrolysis of amide bonds of conjugated
392 bile salts and could act under our experimental conditions on the 3-[(3-cholamidopropyl)
393 dimethylammonio]-1 propanesulfonate detergent mimicking bile salts (Ridlon, Kang, & Hylemon,
394 2006).

395 When the extract was made with *K. marxianus* S-02-5 only, the identification of the protein in the gel
396 has especially shown glycolysis enzymes, (exo)inulinase, Xyl1 which is , NADPH-dependent alpha-
397 keto amide reductase. (Exo)inulinase from *K. marxianus* is a powerful extracellular enzyme of
398 pharmaceutical interest, which rapidly hydrolyzes inulin to produce fructo-oligosaccharides and
399 fructose whose metabolism bypasses the metabolic pathway of glucose and therefore does not
400 require insulin (Singh & Lotey, 2010). Inulinase from *K. marxianus* exists in two forms. The
401 inulinase present in the culture fluid is a dimer, and the enzyme retained in the cell wall is a
402 tetramer. In our case, the spot p2 found at a molecular weight of around 125 kDa in the non-

403 denaturing gel (available in supplementary content 7) could be compatible with the dimer
404 (Rouwenhorst, Hensing, Verbakel, Scheffers, & van Duken, 1990). *K. marxianus* is capable
405 of growing under anaerobic fermentation conditions that lead to ethanol production even if
406 alternating aerobic and anaerobic stages has been shown to promote the growth of the yeast.
407 *K. marxianus* is also capable to use various hexoses or pentoses for its fermentation to ethanol
408 (Banat, Singh, & Marchant, 1996). Inulinase is produced by *K. marxianus* under aerobic
409 conditions while its fermentation occurs under anaerobic conditions or with limited oxygen
410 supply (Yuan, Zhao, Ge, & Bai, 2008). So we put the hypothesis that ethanol was released
411 into our yeast extracts. Moreover, the NADPH-dependent alpha-keto amide reductase found in gel
412 is in favor of the conversion of various carbonyl compounds into their corresponding alcohol products
413 and this enzyme was studied in *Saccharomyces cerevisiae* (Chang, Griest, Harter, & Petrash, 2007).
414 The xylose fermentation to ethanol under anaerobic conditions (Zhang et al., 2015) can also be
415 evoked in our study, since NAD(P)H-dependent D-xylose reductase or Xyl1 was found in gel for our
416 strain *K. marxianus* S-02-5. This enzyme catalyzes the first step of the xylose fermentation.
417 Thus, an ethanol release into the yeast extract could be expected in this study. To summarize,
418 under anaerobic conditions, *K. marxianus* S-02-5 was likely limited in its growth and
419 developed a fermentative metabolism at least partially using pentose pathway. Was this yeast
420 subject to stress? Peptidyl-prolyl cis-trans isomerase was identified in the extract, which is known as
421 a chaperone refolding aggregated proteins (Göthel & Marahiel, 1999).
422
423 Both *K. marxianus* S-02-5 and *K. marxianus* MUCL 29917 demonstrated remarkable stability during
424 their anaerobic incubation and in the presence of the mild detergent derived from cholic acid supposed
425 to mimic intestinal conditions during the extraction process. Extracts prepared from these strains
426 demonstrated strong action against O_2^- as well as against H_2O_2 . Moreover, these microorganisms were
427 able to secrete antioxidant compounds at a higher level than did *B. thalioaomicron* ATCC 29741,
428 previously studied for its antioxidant properties (Hochart-Behra et al., 2008; Hochart-Behra et al.,

2014). These compounds showed remarkable activity *in vitro* against superoxide anion and hydrogen peroxide, which are the first ROS described in the oxidative cascade of phagocytes (Hochart-Behra et al., 2008; Hochart-Behra et al., 2014). Remarkably, these *species* were shown to be the first ROS produced in case of inflammatory burst by phagocytes, especially by neutrophils (Van Eeden, Klut,, & Hogg, 1999).

K. marxianus species could be a good yeast candidate either to modulate or stop the oxidative-inflammatory cascade. Furthermore, *K. marxianus* was reported to exhibit stronger properties relative to the enhancement of transepithelial electrical resistance (TER) across a monolayer of differentiated human colonocytes (Caco-2 cells) (50% TER increase) than probiotic *S. boulardii* (30% TER increase) (Smith, Baker, Arneborg, & Jespersen, 2015). *K. marxianus*, as well as *Metschnikowia gruessii*, were reported as the non-*Saccharomyces* yeasts to protect human epithelial cells from pathogen invasion (Smith et al., 2015), and the antioxidative effects evidenced here may explain, partially, the protective action of the *K. marxianus species*.

However, when *K. marxianus* S-02-5 and *K. marxianus* MUCL 29917 were cultured under aerobic atmosphere, their extracts did not show the same antioxidative potency. The protein contents of the extracts were also different depending on the mode of yeast incubation. Under anaerobic conditions, *K. marxianus* S-02-5 was more efficient. One hypothesis could be advanced that the alcoholic fermentation process could be at the origin of this difference. Indeed ethanol which is often used as solvent in the extraction of active natural products has been shown to have strong scavenger properties against ROS and probably to interfere with the effects that the authors seek to demonstrate. One should be vigilant before to attribute antioxidant effects to ingredients that are extracted with alcoholic solutions (Li, 2013). In our study, antioxidant proteins were not identified in the intense spots in gels for *K. marxianus*. Antioxidant compounds could be of lower molecular weight than 10 kDa, because the extracts were desalted on filters with cut-off level of 10 kDa. Some thiols of low molecular weight could be powerful. So, assays were finally performed on the corresponding filtrates that were stored in freezing to search their possible antioxidant power. Antioxidant effects were found with notable variations from one filtrate to the other which could be explained by the lability of ethanol or thiols that can be rapidly oxidized in disulphide bonds over time.

457 The intestinal content is characterized by anaerobic conditions, and detergent medium and a strong
458 representation of *Bacteroides* genus in intestinal flora. Basically we can expect the inhibition of the
459 oxidative-inflammatory cascade in the presence of *K. marxianus* S-02-5, especially in the case of
460 inflammatory bowel diseases.

461

462 **5. Conclusion**

463 *K. marxianus* S-02-5 appears to bring a benefit for antioxidant activity when it is co-incubated with *B.*
464 *thetaitotaomicron* under conditions mimicking the intestinal environment. Indeed, the antioxidant
465 power of the extract performed from both microorganisms grown together was much more powerful
466 than that obtained with the extract from the single bacterium. Moreover, *K. marxianus* S-02-5
467 exhibited strong inhibition of superoxide anion and hydrogen peroxide as well as the reference strain
468 *K. marxianus* MUCL 29917. However, that could be due to ethanol fermentation. Animal models of
469 inflammation will be of great interest in the study of the antioxidant and anti-inflammatory status of
470 the tissues in contact with *K. marxianus* S-02-5. The data obtained in this study underpinned the
471 antioxidative properties of *K. marxianus* species, mainly those of *K. marxianus* S-02-5, recently
472 isolated from cheese, when it is incubated under anaerobic conditions in the presence of the *B.*
473 *thetaitotaomicron* intestine symbiont. This attribute could then support the health claim of this yeast as
474 a potential probiotic to counter intestinal inflammatory processes.

475

476 **Conflict of interest**

477 The authors do not declare any conflict of interest.

478

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489

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625

626 **Figure captions**

627

628 **Fig. 1. Inhibition of superoxide anion by three types of extracts obtained from single cultures of**
629 ***Bacteroides thetaiotaomicron* ATCC 29741 and *Kluyveromyces marxianus* S-02-5 or after their**

630 **co-culture.** Superoxide anion was *in vitro* produced in a cell-free model using the xanthine-xanthine
631 oxidase system. The effects of the extracts obtained from *B. thetaiotaomicron* ATCC 29741, *K.*
632 *marxianus* S-02-5 (isolated from cheese) and both microorganisms co-cultured, were measured on this
633 reactive oxygen *species* (* Superoxide anion concentration statistically different from that obtained
634 with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent
635 experiments). Means \pm standard deviations are available in supplementary material Table S1.

636

637 **Fig. 2. Inhibition of hydrogen peroxide by 3 types of extracts obtained from single cultures of**
638 ***Bacteroides thetaiotaomicron* and *Kluyveromyces marxianus* S-02-5 or after their co-culture.**

639 Hydrogen peroxide was *in vitro* incubated in cell-free model with the extracts obtained from *B.*
640 *thetaitotaomicron* ATCC29741, *K. marxianus* S-02-5 from cheese origin and both microorganisms co-
641 cultured. The H_2O_2 inhibition was significant for the extracts obtained from *K. marxianus* after single
642 culture or from both microorganisms co-cultured (* Hydrogen peroxide concentration statistically
643 different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat,
644 $n=5$ five independent experiments). Means \pm standard deviations are available supplementary material
645 Table S2.

646

647 **Fig. 3. Two-dimensional gel obtained with the proteins extracted from *Bacteroides***
648 ***thetaitotaomicron* ATCC 29741 and *Kluyveromyces marxianus* S-02-5 grown together. (MW)**
649 **Molecular weight standards. (pI) Isoelectrics points. Stress proteins were identified by peptide mass**
650 **finger printing through two different search algorithms (“PEAKS” and “MASCOT”) in the NCBI**
651 **(National Center for Biotechnology Information), UniProt, SwissProt: oxidative stress proteins (BT-o1**
652 **to BT-o7); chaporenin proteins (BT-c1 to BT-c5); other stress proteins (BT-s1, BT-s2); other proteins**
653 **(BT-p1 to BT-p15; K-p1); control (serum albumin).**

654

655 **Fig. 4. Inhibition of superoxide anion by both *Kluyveromyces marxianus* extracts.** They were
656 obtained from single cultures of the strains S-02-5 and MUCL 29917 grown in broth medium (liq).
657 Superoxide anion was *in vitro* produced in a cell-free model using the xanthine-xanthine oxidase

658 system (* Superoxide anion concentration statistically different from that obtained with the control
659 without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent experiments). Means \pm
660 standard deviations are available supplementary material Table S3.

661

662 **Fig. 5. Inhibition of hydrogen peroxide by both *Kluyveromyces marxianus* extracts.** They were
663 obtained from single cultures of the strains S-02-5 and MUC 29917 (liq). Hydrogen peroxide was *in*
664 *vitro* incubated in cell-free model with the extracts. (* Hydrogen peroxide concentration statistically
665 different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat,
666 $n=5$ five independent experiments). Means \pm standard deviations are available supplementary material
667 Table S4.

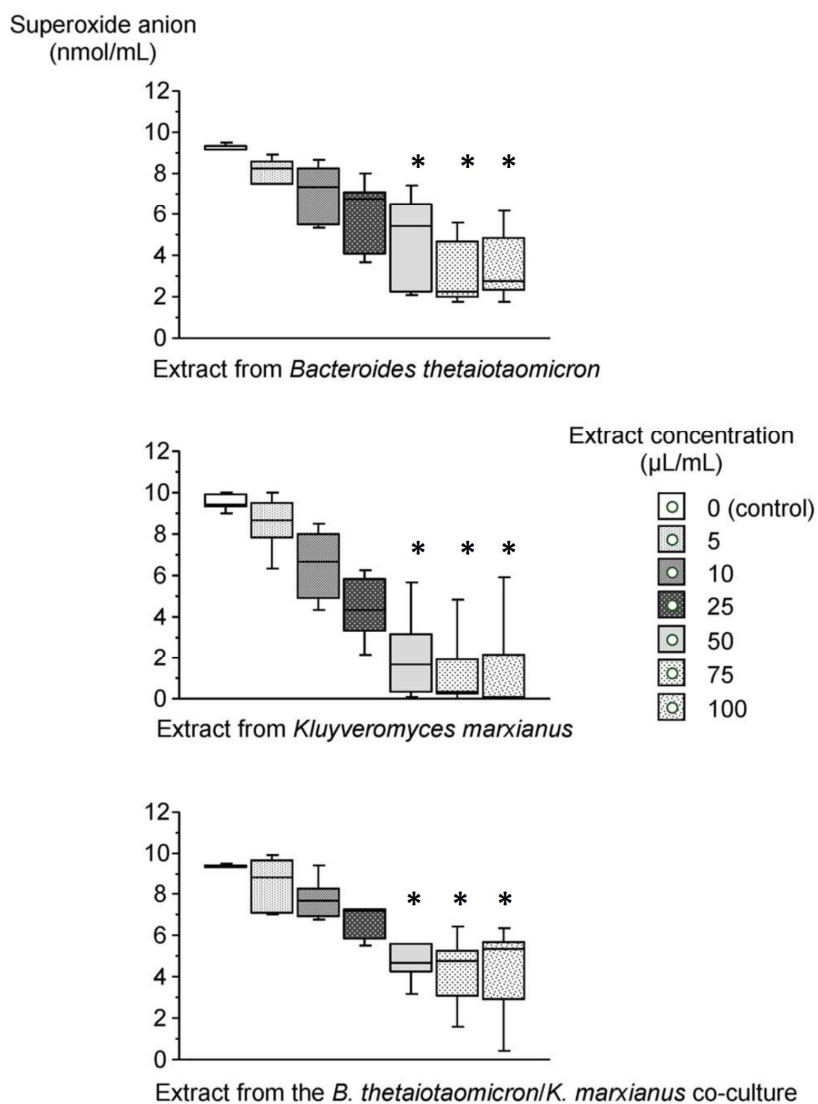


Fig. 1. Inhibition of superoxide anion by three types of extracts obtained from single cultures of *Bacteroides thetaiotaomicron* ATCC 29741 and *Kluyveromyces marxianus* S-02-5 or after their co-culture. Superoxide anion was *in vitro* produced in a cell-free model using the xanthine-xanthine oxidase system. The effects of the extracts obtained from *B. thetaiotaomicron* ATCC 29741, *K. marxianus* S-02-5 (isolated from cheese) and both microorganisms co-cultured, were measured on this reactive oxygen species (* Superoxide anion concentration statistically different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent experiments). Means \pm standard deviations are available in supplementary material Table S1.

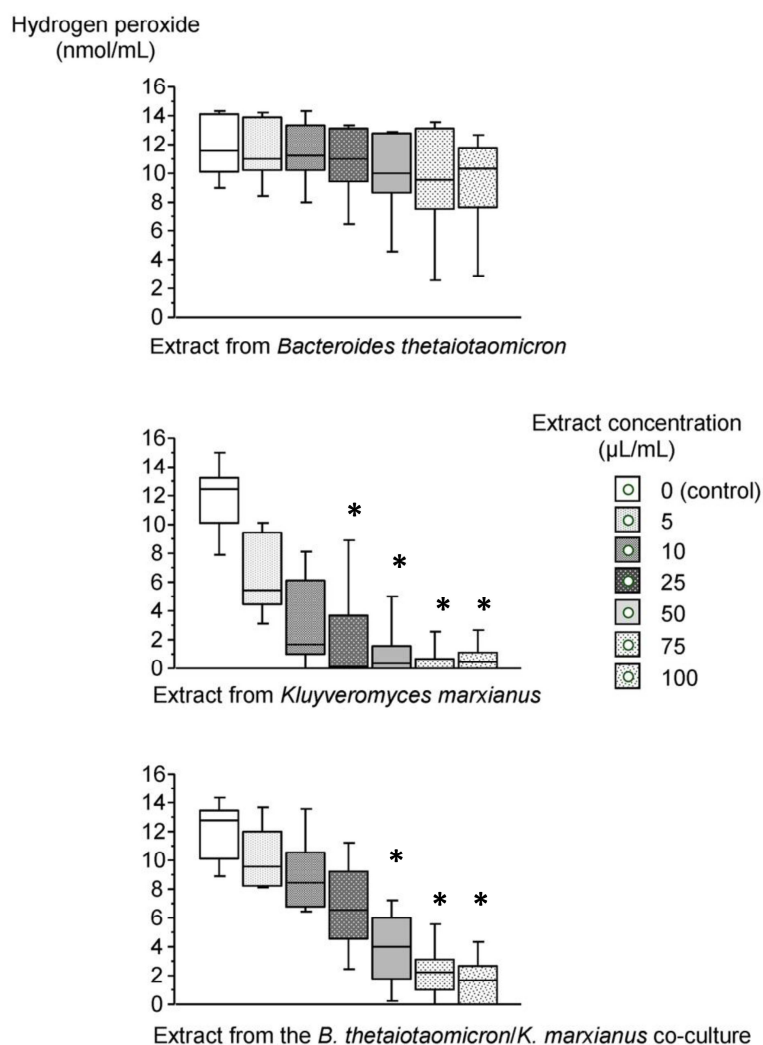


Fig. 2. Inhibition of hydrogen peroxide by 3 types of extracts obtained from single cultures of *Bacteroides thetaiotaomicron* and *Kluveromyces marxianus* S-02-5 or after their co-culture. Hydrogen peroxide was *in vitro* incubated in cell-free model with the extracts obtained from *B. thetaiotaomicron* ATCC29741, *K. marxianus* S-02-5 from cheese origin and both microorganisms co-cultured. The H_2O_2 inhibition was significant for the extracts obtained from *K. marxianus* after single culture or from both microorganisms co-cultured (* Hydrogen peroxide concentration statistically different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent experiments). Means \pm standard deviations are available in supplementary material Table S2.

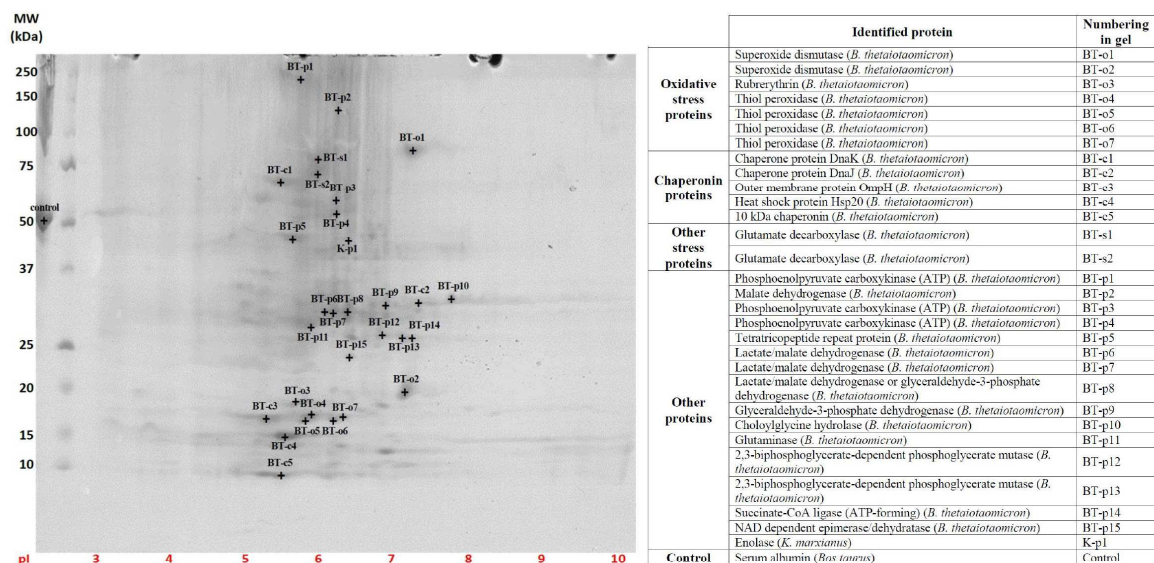


Fig. 3. Two-dimensional gel obtained with the proteins extracted from *Bacteroides thetaiotaomicron* ATCC 29741 and *Kluyveromyces marxianus* S-02-5 grown together. (MW) Molecular weight standards. (pI) Isoelectric points. Stress proteins were identified by peptide mass fingerprinting through two different search algorithms ("PEAKS" and "MASCOT") in the NCBI (National Center for Biotechnology Information), UniProt, SwissProt databases: oxidative stress proteins (BT-o1 to BT-o7); chaperonin proteins (BT-c1 to BT-c5); other stress proteins (BT-s1, BT-s2); other proteins (BT-p1 to BT-p15; K-p1); control (serum albumin).

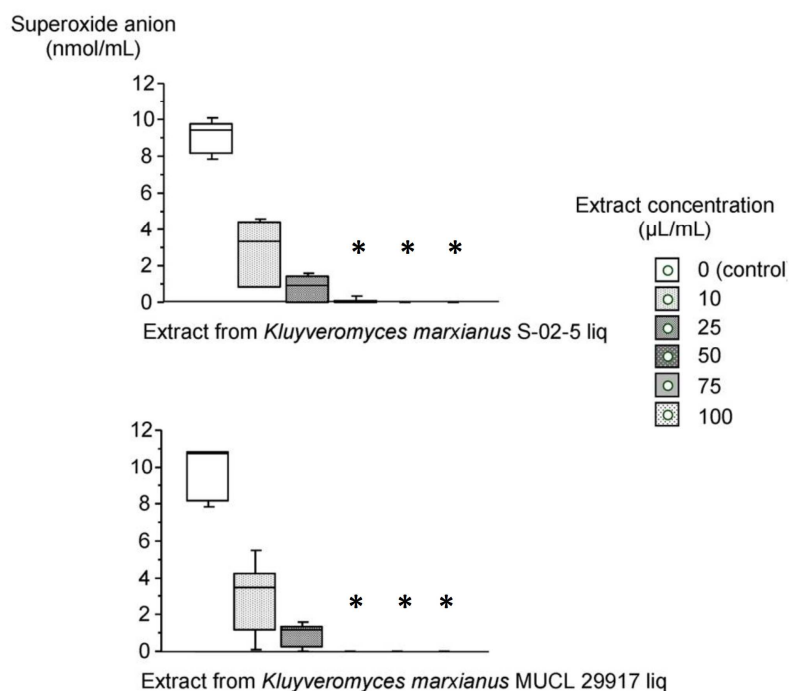


Fig. 4. Inhibition of superoxide anion by both *Kluveromyces marxianus* extracts. They were obtained from single cultures of the strains S-02-5 and MUCL 29917 grown in broth medium (liq). Superoxide anion was *in vitro* produced in a cell-free model using the xanthine-xanthine oxidase system (* Superoxide anion concentration statistically different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent experiments). Means \pm standard deviations are available in supplementary material Table S3.

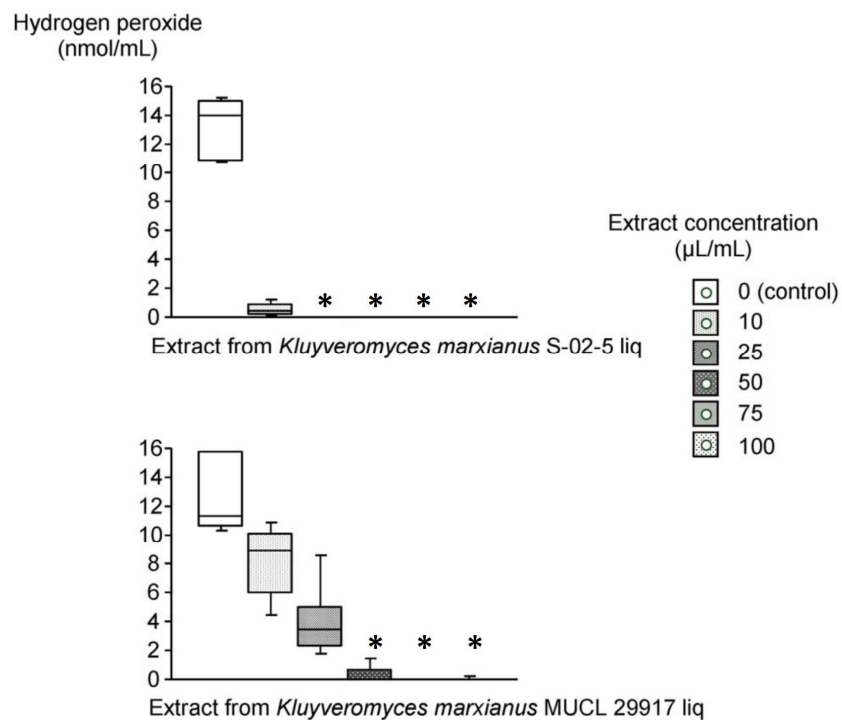


Fig. 5. Inhibition of hydrogen peroxide by both *Kluveromyces marxianus* extracts. They were obtained from single cultures of the strains S-02-5 and MUC 29917 (liq). Hydrogen peroxide was *in vitro* incubated in cell-free model with the extracts (* Hydrogen peroxide concentration statistically different from that obtained with the control without extract, Kruskal & Wallis test, $p=0.05$, AnaStat, $n=5$ five independent experiments). Means \pm standard deviations are available in supplementary material Table S4.

Highlights

- An impressive antioxidant power of the yeast *Kluyveromyce marxianus* was found.
- *Bacteroides thetaiotaomicron* was used as a model of intestine bacterial anaerobe.
- The release of antioxidant compounds by this symbiont depends on its environment.
- Co-incubating both microorganisms benefited the bacterial population.
- Together, they produced more antioxidant extracts than did the single bacteria.