

1 **Lysozyme affects the microbial catabolism of free arginine in raw-milk hard cheeses**

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10 **Abstract**

11 Lysozyme (LZ) is used in several cheese varieties to prevent late blowing which results from
12 fermentation of lactate by *Clostridium tyrobutyricum*. Side effects of LZ on lactic acid bacteria
13 population and free amino acid pattern were studied in 16 raw-milk hard cheeses produced in eight
14 parallel cheese makings conducted at four different dairies using the same milk with (LZ+) or
15 without (LZ-) addition of LZ. The LZ- cheeses were characterized by higher numbers of cultivable
16 microbial population and lower amount of DNA arising from lysed bacterial cells with respect to
17 LZ+ cheeses. At both 9 and 16 months of ripening, *L. delbrueckii* and *L. fermentum* proved to be
18 the species mostly affected by LZ. The total content of free amino acids indicated the proteolysis
19 extent to be characteristic of the dairy, regardless to the presence of LZ. In contrast, the relative
20 patterns showed the microbial degradation of arginine to be promoted in LZ+ cheeses. The data
21 demonstrated that the arginine-deiminase pathway was only partially adopted since citrulline
22 represented the main product and only trace levels of ornithine were found. Differences in arginine
23 degradation were considered for starter and non-starter lactic acid bacteria, at different cheese
24 ripening stages.

25 **Keywords:** Raw milk cheese, Lysozyme, Arginine deiminase, Non-starter LAB, Free amino acids.

26

27 **1. Introduction**

28 Nowadays hen's egg white lysozyme (EC 3.2.1.17) (LZ) is used in Grana Padano as well as in other
29 hard cheeses to prevent the "late blowing" defect (Brasca et al, 2013; Jiménez-Saiz et al., 2013). In
30 fact, LZ is efficient in lysing the vegetative cells of Clostridia, specifically of *Clostridium*
31 *tyrobutyricum*, by splitting β (1–4) linkages between N-acetylmuramic acid and N-
32 acetylglucosamine of the peptidoglycan of the bacterial cell wall (Hughey and Johnson, 1987).
33 These bacteria are capable of producing spores that survive the thermal treatment applied in making
34 hard cheeses and can later germinate and produce gas causing the defect. The origin of the
35 contamination by this bacterium has been identified in the wide use of silage in livestock feeding
36 (Jonsson, 1991; Vissers et al., 2006).

37 The different sensitivity to LZ of various bacteria, both Gram positive and negative, is due to the
38 different cell wall composition and structure and, thus, to the binding of the enzyme to its specific
39 substrate (Bester and Lombard, 1990; Carini et al., 1985; Hughey and Johnson, 1987). Due to its
40 wide spectrum, LZ activity can also occur against lactic acid bacteria (LAB) involved in curd
41 acidification and cheese ripening. The interference of the enzyme with the acidification process
42 occurring during hard cheese production has been indirectly studied by considering the sensitivity
43 of LAB responsible of curd acidification. For example, LZ inhibitory activity has been extensively
44 evaluated for the main species present in the natural whey starter used to produce Grana Padano, i.e.
45 *Lactobacillus helveticus*. It was found that sensitivity of *L. helveticus* was strain-dependent, and
46 acquisition of resistance can be due to strain adaptation rather than selection of spontaneous mutants
47 (Fortina et al., 1998; Neviani et al., 1991). Resistance to LZ was also reported for *Lactobacillus*
48 *delbrueckii* (Vinderola et al., 2007). Moreover, a correlation was observed between LZ resistance

49 and bacteriophage sensitivity in *L. helveticus* (Neviani et al., 1992) and the authors suggested the
50 possibility of using LZ as a selective agent to isolate phage-resistant starter strains.

51 To author's knowledge, few literature data are available on LZ resistance of non-starter LAB
52 (NSLAB). These are part of the raw milk cheese microbiota and are not involved in curd
53 acidification but play a relevant role in cheese ripening (Gatti et al 2014). Carini et al. (1985)
54 reported LZ resistance of *L. casei* species. Ugarte et al. (2006) studied NSLAB isolated from soft
55 and semi hard Argentinean cheeses and found most of the species to tolerate 2.5 mg 100g⁻¹ of LZ.
56 More recently, LZ sensitivity of NSLAB was studied as one of the criteria suitable to evaluate their
57 probiotic aptitude (Solieri et al., 2014). A strain-dependent resistance to LZ at the concentration of
58 10 mg 100g⁻¹, close to that adopted in Grana Padano, was found for *L. rhamnosus*, *L. paracasei*, *L.*
59 *casei*, *L. harbinensis*, and *L. fermentum*.

60 The aim of this work was to investigate the effects of LZ in cheese with respect to both the
61 microbial populations and proteolysis pathways responsible of cheese ripening. In particular, the
62 focus was on Grana Padano PDO cheese, usually made with LZ and extensively studied for its
63 microbial and chemical features (Pellegrino et al., 1997; Masotti et al., 2010; Santarelli et al., 2013;
64 Pogacic et al., 2013). Eight cheese makings were therefore conducted at four different dairies, using
65 in parallel, the same milk either added or not with LZ. The 16 derived cheeses were analysed after 9
66 months of ripening, i.e. the minimum ripening period for Grana Padano PDO cheese, and after 16
67 months. The microbial populations were characterized by Length Heterogeneity-PCR (LH-PCR)
68 considering both the intact and lysed cells. Furthermore, the free amino acids (FAA) patterns of the
69 cheeses were evaluated. Since FAA mostly result from the action of intracellular proteinases and
70 peptidases released after the bacterial cell lysis, different patterns could be expected between
71 cheeses produced with and without addition of LZ.

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73

74 **2. Materials and methods**

75 *2.1 Cheese manufacture*

76 Grana Padano cheeses were manufactured at four dairies belonging to the Consorzio Tutela Grana
77 Padano, following the traditional manufacturing process (European Parliament and Council, 2012).
78 At each dairy, the cheese makings were carried out on two different days for a total of eight trials.
79 For each cheese making trial, raw bulk milk was partially skimmed (fat content: 2.2-2.3%) by
80 natural creaming, divided into two vats (1000 L each), one of which was added with 20 g LZ
81 (Sacco, Cadorago, Italy) carefully dispersed in 200 mL water, and the two vats were worked in
82 parallel. The natural whey starter (titratable acidity: 30-32°SH/50 mL), obtained from the residual
83 whey of the previous days' cheese-making, and the calf rennet were added to coagulate the vat milk
84 at 32°C in 8-10 min. The curd was gently cut into small granules while progressively heated up to
85 52-54°C, then it was allowed to compact at the bottom of the vat for 60 min before extraction. The
86 cheeses (two wheels per vat) were molded for 48 h to allow lactic acid fermentation (pH was
87 measured in the core of the wheels) and then salted in brine for 18-20 days. During ripening, all the
88 cheeses were regularly inspected by X-ray tomography (Philips CT Brilliance 16P, Zürich,
89 Switzerland) to evidence possible development of defects. The twin cheeses obtained from each vat
90 were cut after 9 and 16 months of ripening respectively. A portion representative of the whole
91 wheel was taken from each, grated and deep-frozen until analysis. Samples for microbiological
92 analyses were kept at 4°C until arrival at the laboratory and immediately analyzed. Cheese samples
93 either containing or not LZ were coded as LZ+ and LZ- respectively, whereas numbering from 1 to
94 8 identifies the cheese making trial they come from.

95 *2.2 Bacterial counts*

96

97 Bacterial counts were determined on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke,
98 United Kingdom). Representative samples (10 g) of the grated cheese were suspended in 90 mL of

99 20 g L⁻¹ tri-sodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenised for 2 min in
100 a blender (Seward, London, United Kingdom). For enumerating mesophilic lactobacilli, as main
101 microbiota of cheese during ripening, decimal dilutions of cheese homogenates were made in
102 quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) and spread plated in
103 triplicate on MRS. The plates were incubated at 30 °C for 72 h under anaerobic conditions.

104

105 *2.3 DNA extraction*

106

107 Bacterial genomic DNA was extracted directly from samples by using a General Rapid Easy
108 Extraction System (GREES) DNA kit (InCura S.r.l., Cremona, Italy) according to the
109 manufacturer's instructions. Cheese samples were pre-treated in order to discriminate the DNA
110 from whole and lysed cells as described by Gatti et al. (2008). Briefly, cheese samples resulted in
111 two fractions, the free-cell fraction was obtained by filtration and the whole-cell fraction was
112 obtained by treating samples with DNase to digest free DNA arising from lysed cells. DNA was
113 extracted from 1 mL of the filtered untreated fraction (lysed cells) and from 1 mL of the treated
114 fraction (whole cells).

115

116 *2.4 Length heterogeneity (LH)-PCR*

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118 LH-PCR was used in order to determine the microbial community composition. V1 and V2 16S
119 rDNA gene regions were amplified with primers 63F and 355R (Lazzi et al., 2004). The forward
120 primer was 5'-end labelled with a 6-carboxyfluorescein (6-FAM) dye. Amplicons were then
121 separated by capillary electrophoresis in an automated sequencer (Applied Biosystems, Foster City,
122 USA). PCR and capillary electrophoresis conditions were as described by Bottari et al. (2010). The
123 fragment sizes (base pairs) were determined with GeneMapper software version 4.0 (Applied
124 Biosystems), local Southern method to generate a sizing curve from the fragment migration of the

125 internal size standard (GS500 LIZ®; Applied Biosystems) and a threshold of 150 fluorescence
126 units. The fragment analysis software converted fluorescence data into electropherograms. The
127 peaks represent fragments of different sizes and the areas under the peaks are the amount of the
128 fragments. Total area were considered to directly correlate to the total amount of the DNA arising
129 from whole or lysed cells depending on the two fractions previously described. Each peak,
130 corresponding to amplicon of specific length on the electropherogram profile, was attributed to
131 bacterial species according to published databases (Lazzi et al., 2004; Gatti et al., 2008) and the
132 areas under the recognized peaks were used to estimate the amount of the assigned species in the
133 samples. Total area under all the peaks (sum of attributed and unattributed peaks) of the LH-PCR
134 electropherograms was used for measuring total amount of DNA arising from both intact and lysed
135 cells.

136

137 *2.5 Determination of free amino acids by ion-exchange chromatography*

138 *2.5.1 Free amino acid extraction*

139 The grated cheese was weighted (1.5 g) in a 100 mL beaker, added with 40 mL 0.2 N tri-sodium
140 citrate buffer at pH 2.2 (SCB), kept under magnetic stirring for 15 min then carefully homogenized
141 with Ultra-Turrax (5 min at low speed). The extract was filtered (Whatman 42 paper filter, GE
142 Healthcare, Milan, Italy) and 10 mL of the filtrate were transferred into a 25 mL volumetric flask,
143 dropwise added with 10 mL 7.5% (w/v) 5-sulphosalicylic acid (pH 1.7-1.8) under stirring, diluted
144 to the mark with SCB and filtered. Finally, 10 mL of this filtrate were transferred into a 100 mL
145 volumetric flask, added with 2 mL norleucine solution (60 mg norleucine in 100 mL SCB) as an
146 internal standard, made up to the mark with 0.2N Lithium citrate pH 2.2 (dilution buffer), and
147 filtered on 0.2 µm disposable filter (Minisart® RC 25, Sartorius, Goettingen, Germany) prior to
148 injection. All cheese samples were analysed in duplicate.

149 *2.5.2 Amino acid standard solutions*

150 A stock solution was prepared containing: 15 mg of arginine, asparagine, citrulline, glycine,
151 glutamine, γ -aminobutyric acid, methionine, ornithine, threonine, tyrosine; 30 mg of alanine,
152 aspartic acid, phenylalanine, isoleucine, histidine, serine; 40 mg of leucine, proline, valine, glutamic
153 acid and lysine (Sigma-Aldrich) per 100 mL of SCB. Aliquots of 0.5, 1.0, 2.0 and 5.0 mL of this
154 solution were transferred into 100 mL volumetric flasks, added with 2 mL of the norleucine
155 solution, and made up to the mark with dilution buffer to prepare working solutions with four
156 different concentrations.

157 *2.5.3 Chromatographic conditions*

158 A Biochrom 30+ chromatograph (Biochrom Ltd., Cambridge, UK) equipped with an Accelerated
159 Lithium Column (Biochrom Ltd.) was used and the elution conditions recommended by the
160 manufacturer were followed. Reagents of analytical grade and MilliQ water (Millipore, Vimodrone,
161 Italy) were used. Ready-to-use Ninhydrin reagent was purchased from Erreci s.r.l. (Pieve Emanuele,
162 Italy).

163 *2.6 Statistical analysis*

164 Statistical treatment of data was performed by means of SPSS Win 12.0 program (SPSS Inc.,
165 Chicago, IL, USA). Data were analysed by Principal Component Analysis (PCA) by means of
166 Statistica (StatSoft Inc., Tulsa, OK, USA) and comparison of means was carried out by Student's t-
167 test. A $P < 0.05$ was assumed as significance limit.

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171 3. Results and discussion

172

173 3.1 Microbial characterization of 9-month ripened cheeses

174

175 Microbial counts in MRS at 30°C (i.e. mesophilic lactobacilli) and community composition were
176 different in all couples of samples and not clearly correlated with presence or absence of LZ.
177 However, some peculiarity emerged. Overall, cultivable bacterial population varied of almost two
178 log units among samples (Table 1). The plate count in LZ+ cheeses showed a great variability
179 (ranging from 4.44 log cfu/g in sample 6 to 6.64 log in sample 7). Differently, the counts in LZ-
180 cheeses were higher and less variable. In particular, the cultivable population in LZ- cheeses were
181 higher than in the corresponding LZ+ for 6 out of the 8 couples (Table 1).

182 The LH-PCR was performed with DNA extracted from intact cells to estimate which bacterial
183 species were still present in the cheeses after 9 months of ripening. Only peaks attributed to the
184 database set (Lazzi et al., 2004; Gatti et al., 2008) were considered. The same species evidenced in
185 Grana Padano by Santarelli et al. (2013) and by Pogacic et al. (2013), i.e. *Lactobacillus delbrueckii*,
186 *L. helveticus*, *L. rhamnosus*, *L. fermentum* and *Pediococcus acidilactici*, were found in cheeses but
187 never simultaneously in the same sample. *L. helveticus*, *L. delbruecki* and *L. rhamnosus* were the
188 most frequently detected species in LZ+ cheeses, while in LZ- cheeses *L. helveticus*, *L. rhamnosus*
189 and *L. fermentum* prevailed, with *L. delbruecki* only found in two LZ- cheeses (Table 1).
190 *Pediococcus acidilactici* was seldom found, preferentially in LZ- cheeses.

191 The evaluation of the free DNA fraction allowed estimating which LAB underwent lysis during 9
192 months of ripening leaving their DNA still amplifiable. This method, developed by Gatti et al.
193 (2008) to highlight LAB lysis in Parmigiano-Reggiano, was also adopted by Santarelli et al. (2013)
194 for Grana Padano. Lysed species found in the presently studied cheeses were *L. delbrueckii*, *L.*
195 *helveticus*, *L. rhamnosus* and, in one sample, *L. fermentum*. Interestingly, lysed *L. delbrueckii* was
196 detected in the same samples where the intact cells were still present, seven out of eight of these

197 samples being LZ+ (Table 1). Differently, lysed *L. helveticus* in LZ+ cheeses was always below the
198 detection limit. Recently, Sgarbi and colleagues demonstrated the ability of NSLAB to grow using
199 cell lysates of SLAB as the exclusive source of nutrients (Sgarbi et al., 2014). Total area under all
200 of the peaks (attributed and unattributed) in the LH-PCR electropherograms either from intact or
201 lysed cells was used for measuring the respective total amounts of DNA (Table 1). The amount of
202 DNA of intact cells was higher than the amount of DNA of lysed cells for 14 out of 16 samples,
203 irrespective of LZ presence. Importantly, higher amount of DNA arising from lysed cells was
204 observed in 6 out of 8 LZ+ cheeses (Table 1).

205 Considering individual species, in the LZ- cheeses *L. delbrueckii* was below the detection limit in
206 six out of eight samples, whereas presence of *L. fermentum* was often higher than in the
207 corresponding LZ+ cheese. Taking together microbial counts and LH-PCR results, lower numbers
208 of cultivable population and higher amount of DNA arising from lysed cells found in LZ+ cheeses
209 are reasonably due to the hydrolytic activity of the additive (Hughey et al., 1987). On the other
210 hand, its efficacy on different LAB species depends on their different sensitivity which was found
211 to be strain specific in *L. helveticus* (Neviani et al., 1991; Fortina et al., 1998), *L. casei* group
212 (Solieri et al., 2014), and potentially variable in *L. delbrueckii* (Vinderola et al., 2007).

213 Despite this variability, PCA showed that LZ+ samples spread along the first component, mostly
214 due to the high amounts of *L. delbrueckii* (both intact and lysed), intact *L. helveticus*, DNA from
215 lysed cells on one side, and to high MRS plate counts and high amount of *L. rhamnosus* on the
216 other side (Figure 1a and 1b). Moreover, LZ- cheeses appeared to distribute along the second
217 component, where the amount of *L. fermentum* and *P. acidilactici* showed greater weight. The
218 positioning of the samples (cheeses) was likely determined by LZ, whose presence affected both
219 SLAB (*L. helveticus* and *L. delbrueckii*) and NSLAB (*L. rhamnosus*, *L. fermentum* and *P.*
220 *acidilactici*) species with a direct effect on LZ-sensitive species and a consequent effect of selection
221 on the others.

222

223 3.2 Free amino acid pattern in 9-month ripened cheeses

224

225 During cheese ripening, casein is progressively degraded into peptides and free amino acids (FAA)
226 by a pool of proteolytic enzymes coming from both starter and non-starter microflora and acting in
227 combination. Since the pattern of peptides is changing over time (Ferranti et al., 1997), we have
228 disregarded this intermediate fraction and focused the attention on the pattern of free amino acids
229 (FAA) that proved to represent an accurate descriptor of proteolysis behavior in Grana Padano
230 cheese (Cattaneo et al., 2008).

231 The total content of FAA on cheese protein basis was in the range 17-22% (data not shown), in
232 agreement with previously published data for Grana Padano cheese of the same age (Masotti et al.,
233 2010). The values were not significantly different ($P=0.48$) between cheeses produced with and
234 without LZ, indicating that proteolysis has proceeded at the same rate. The whole data set was thus
235 analyzed by PCA to highlight possible differences among cheese samples. The plot showed a
236 significant dispersion of the observations, and the cheese samples did not cluster together depending
237 on the presence or absence of LZ (Figure 2a). Nevertheless, within each couple of twin cheeses, the
238 LZ+ cheeses always fell on the upper side with respect to the corresponding LZ- cheese.
239 Interestingly, this positioning appeared to be due to four FAA, namely arginine, citrulline, ornithine
240 and γ -aminobutyric acid (g-ABA) (Figure 2b), although their content only accounts for 5-6% of the
241 total FAA. In particular, g-ABA is a non-protein amino acid generated through decarboxylation of
242 glutamic acid as a defense mechanism for resistance to an acidic environment (van de Gukte et al.,
243 2002). The ability to produce g-ABA was reported for several SLAB species typically present in
244 Grana Padano natural whey starter, including *L. helveticus*, *L. delbruecki*, as well as for *L.*
245 *plantarum*, *L. brevis*, all producing a glutamate decarboxylase (Li and Cao, 2010). Although the
246 values were very low, the average content of g-ABA was approximately double in LZ+ cheeses
247 (Table 2) and confirmed the presence of g-ABA-producing strains. The other three FAA are all

248 involved in a common metabolic pathway, being citrulline and ornithine non-protein amino acids
249 deriving from arginine catabolism. Thus we have focused our attention on this pathway.

250

251 3.2 *Arginine metabolism*

252

253 The total amount of arginine liberated from casein in cheese was calculated as the sum of free
254 arginine plus citrulline plus ornithine as molar concentration. Values were in the range from 48 to
255 63 mmol/kg (Figure 3a), roughly corresponding to 22-28% of the arginine in casein (Farrel et al.,
256 2004). By comparing the twin cheeses obtained from different cheese makings (Figure 3a), the total
257 amount of arginine liberated from casein was found to be characteristic of the dairy. In contrast, the
258 amount of arginine converted into both citrulline and ornithine was always lower in the LZ-
259 cheeses, regardless of the dairy of origin, being the amount of ornithine marginal. Not much
260 literature is available to clarify the progress of microbial degradation of arginine in real cheeses
261 (Brandsma et al., 2012; Diana et al., 2014; Laht et al., 2002), since studies are mostly based on
262 single-strain fermentation trials. Our data show that in cheeses it is significantly affected by the
263 presence of LZ, as it is further discussed.

264 The ability to catabolize arginine through the arginine deiminase (ADI) pathway is rather common
265 in LAB species, typically in heterofermentative LAB (Fröhlich-Wyder et al., 2015; Nicoloff et al.,
266 2001; Price et al., 2012). The accepted model for this pathway (Figure 3b) implies the uptake of free
267 arginine into the bacterial cell by an antiporter system and its degradation into citrulline by ADI.
268 Intracellular citrulline can either be excreted or converted into ornithine by the cytoplasmic
269 enzymes ornithine transcarbamoylase and carbamate kinase. Ornithine is then excreted in the
270 medium. Overall, the ADI pathway brings to the production of one mol of ATP and two mol of
271 ammonia per mol of degraded arginine. Therefore, arginine catabolism is considered to represent
272 both a way to counteract the acid stress and an alternative source of energy. Our data indicated that
273 availability of free arginine did not represent a limiting factor in Grana Padano cheese since it was

274 continuously liberated from peptides. This fact, along with the low levels of citrulline released and
275 extremely low levels of citrulline converted into ornithine, suggested a limited adoption of the ADI
276 pathway during cheese ripening, possibly because of environmental conditions only slightly
277 stressing LAB. Actually, in hard cheeses like Grana Padano, cell stressing conditions, such as high
278 temperature or highly acidic pH, principally occur during the cheese molding, when growth of
279 SLAB largely prevails (Gatti et al., 2014). As already mentioned, SLAB species typical to Grana
280 Padano are mostly represented by homofermentative species, i.e. *L. helveticus* and *L. delbrueckii*
281 subsp. *lactis* (Gatti et al., 2014). *L. helveticus* has been reported to harbor an incomplete ADI
282 operon (Christiansen et al., 2008), whereas the complete pathway has been observed in strains of *L.*
283 *delbrueckii subsp lactis* (El Kafsi et al., 2014; Nicoloff et al., 2001). The limited adoption of the
284 ADI pathway by SLAB of Grana Padano is supported by our recent data (Pellegrino et al., 2015)
285 showing that, during lactic acid fermentation occurring in the natural whey starter, SLAB mostly
286 coped the strong acidic conditions by converting glutamic acid into g-ABA. Indeed, free arginine
287 was used during cell growth, since most of SLAB species are auxotrophic for this amino acid
288 (Christiansen et al., 2008), but only trace levels of both citrulline and ornithine were detected in
289 whey starter. Considering this, metabolites deriving from ADI pathway could be produced by
290 NSLAB throughout the ripening period more actively in LZ+ than in LZ- cheeses.

291 To better clarify these aspects, we have analyzed the remaining cheeses after a total ripening period
292 of 16 months. To compare cheeses at different ages, the average relative contents of arginine,
293 citrulline and ornithine in LZ+ and LZ- cheeses were considered (Figure 4). Remarkably, the ADI
294 pathway was in use even in late ripening, being the contents of both arginine and citrulline
295 significantly different between 9- and 16-month ripened cheeses (P= 0.001 and P= 0.000,
296 respectively). Moreover, the presence of LZ still showed a promoting effect on this mechanism, as
297 the residual content of arginine was different (P= 0.000) between LZ+ and LZ- cheeses at 16
298 months of ripening. Overall, arginine proved to be freely available throughout the whole ripening
299 period and to be progressively converted into citrulline with a minimum further degradation into

300 ornithine. Thus our data indicated that only the first step of the ADI pathway is commonly adopted
301 in Grana Padano and, according to the accepted scheme (Figure 3), this step would be likely
302 adopted by living cells in response to acid stress, since it only brings to production of ammonia.
303 This fact is difficult to explain because the pH values in the ripened cheeses were all in the range
304 5.68-5.84, thus far from being stressing to LAB cells, and no systematic differences were found
305 between LZ+ and LZ- cheeses.

306 Interestingly, also the microbial profile of the 16-month ripened cheeses confirmed the main
307 features observed in cheeses after 9 months of ripening: the most relevant differences between LZ+
308 and LZ- cheeses regarded *L. delbrueckii*, largely dominating in LZ+ cheeses, and *L. fermentum*
309 dominating in LZ- cheeses (data not shown). To author's knowledge, the only subspecies,
310 belonging to *Lactobacillus* genus and *delbrueckii* species, isolated from whey starter and unripened
311 Grana Padano, is *lactis*, whereas this species was never isolated from ripened Grana Padano cheese,
312 even if presence of intact cells have been revealed by culture independent methods (Pogacic et al.,
313 2013). Recently, the evolutionary adaptation of *L. delbrueckii* subsp. *lactis* to the milk environment
314 through the acquisition of functions, including genes encoding for ADI pathway, that allow an
315 optimized utilization of milk resources, has been demonstrated (El Kafsi et al., 2014). Accordingly,
316 ADI pathway could be used as stress response for *L. delbrueckii* subsp. *lactis* to stay viable,
317 although not cultivable, and thus isolable, in the ripened cheese.

318 With respect to *L. fermentum*, its presence was smaller in LZ+ cheeses at both the ripening stages,
319 in spite of the claimed LZ-resistance (Solieri et al., 2014). Since in these cheeses the degradation of
320 arginine was more intense, this species was unlikely involved in it, although some strains were
321 reported to harbor the genes for ADI pathway (Vrancken et al., 2009a). Through accurate kinetic
322 studies in controlled-condition batch fermentations, Vrancken et al. (2009a, 2009b) evaluated the
323 response of the ADI pathway to different stress conditions in *L. fermentum* IMDO 130101. These
324 authors demonstrated that both the arginine conversion rate and final citrulline-to-ornithine ratio
325 were strongly pH- and salt-dependent, whereas the temperature was not influent in the range 20-45

326 °C. In particular, when pH was set either below 4.0 or above 7.0, arginine was completely
327 converted into ornithine. Differently, at pH in the range 5-6, such as in Grana Padano, citrulline was
328 the main end-product. This strong dependence of the ADI pathway on the environmental pH might
329 explain the prevalence of arginine conversion into citrulline in the studied cheeses regardless of the
330 responsible species.

331 The different amounts of arginine metabolites in LZ+ and LZ- cheeses may be related also to the
332 known biodiversity of *L. rhamnosus* (Bove et al., 2011). This species was present in both types of
333 cheese and thus was not discriminant in our study. However, regarding to LZ resistance, Solieri et
334 al. (2014) found a great variability among strains of *L. rhamnosus*, isolated from Parmigiano-
335 Reggiano cheese, which turned out also to be the most LZ-resistant NSLAB (Solieri et al., 2014). It
336 has been demonstrated that the genetic polymorphisms of *L. rhamnosus* is a response to cheese
337 environmental adaptation also for arginine repressor (*ArgRI*) (Bove et al., 2012) and for formation
338 of ammonia through the ADI pathway (Liu et al., 2003). Thus, we can hypothesize that different
339 strains of *L. rhamnosus*, having different ability to adopt the ADI pathway, were able to develop in
340 the two types of cheese. This hypothesis needs to be confirmed by further characterization of *L.*
341 *rhamnosus* strains that have been isolated from the two types of cheese.

342

343 **4. Conclusions**

344 The presented results indicate that the functionality of the most relevant LAB species in GP
345 production is not hindered by LZ, but a significant effect on a specific metabolic process, i.e. the
346 degradation of arginine, has been evidenced for the first time. Although it was not possible to
347 identify the LAB species and strains actually responsible for the arginine degradation, a role of the
348 dominant *L. helveticus* species could be largely excluded. *L. delbrueckii subsp. lactis* could be one
349 of the responsible species. However, NSLAB species, such as *L. fermentum* and *L. rhamnosus*, may
350 also contribute depending on strain ability to degrade arginine.

351

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353

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358

359 **References**

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530 **Captions to figures**

531

532 **Fig. 1.** PCA of different LAB species in cheeses produced with (LZ+) or without (LZ-) addition of
533 lysozyme. Component plot (Panel a), and loadings of individual species (Panel b). Abbreviations
534 are as in Table 1.

535

536 **Fig. 2.** PCA of free amino acids composition of cheeses produced with (LZ+) or without (LZ-)
537 addition of lysozyme. Component plot (Panel a), and loadings of individual FAA (Panel b).

538

539 **Fig. 3.** Content of free arginine, citrulline and ornithine in four couples of 9-month ripened cheeses
540 produced with (LZ+) or without (LZ-) lysozyme (Panel a), and scheme of the arginine deiminase
541 pathway (Panel b).

542

543 **Fig. 4.** Relative content of arginine (Arg), citrulline (Cit) and ornithine (Orn) in Grana Padano
544 cheeses produced with (LZ+) and without (LZ-) lysozyme and ripened for different periods.

545

546

547

548 **Table 1.**

549 Microbial characterization of 9-month ripened cheeses produced with (LZ+) or without (LZ-) lysozyme.

Sample	Log cfu/g	peaks area of recognized species (whole cells DNA)					peaks area of recognized species (lysed cells DNA)					Total DNA area	
		<i>Lactobacillus delbrueckii</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus fermentum</i>	<i>Pediococcus acidilactici</i>	<i>Lactobacillus delbrueckii</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus fermentum</i>	Whole cells	Lysed cells	
MRS plate count	MRS*	Ldw*	Lhw*	Lrw*	Lfw*	Paw*	Ldl*	Lhl*	Lrl*	Lfl*	DNAw*	DNA1*	
1LZ+	6,07	840	16156	11394	1928	<LOD	2587	<LOD	<LOD	<LOD	31153	2587	
1LZ-	6,26	<LOD**	3910	1260	15929	<LOD	<LOD	<LOD	<LOD	<LOD	21099	576	
2LZ+	6,28	1365	215	13141	<LOD	<LOD	995	<LOD	784	<LOD	15223	1779	
2LZ-	6,25	<LOD	8992	<LOD	37974	<LOD	<LOD	<LOD	<LOD	1443	48232	3739	
3LZ+	6,12	<LOD	3434	64202	7011	<LOD	<LOD	<LOD	2402	<LOD	79422	3339	
3LZ-	6,25	<LOD	223	12306	4896	<LOD	<LOD	<LOD	1374	<LOD	18224	1374	
4LZ+	4,58	4188	1586	<LOD	14287	<LOD	1465	<LOD	<LOD	<LOD	20061	24360	
4LZ-	5,99	<LOD	8799	2360	<LOD	6162	<LOD	1024	518	<LOD	17321	16294	
5LZ+	5,74	3282	6928	5422	<LOD	3446	1747	<LOD	<LOD	<LOD	19078	30697	

5LZ-	5,83	<LOD	5853	16483	56959	<LOD	<LOD	778	<LOD	<LOD	80651	7632
6LZ+	4,44	10652	28041	<LOD	2393	<LOD	2089	<LOD	<LOD	<LOD	41086	6834
6LZ-	5,50	<LOD	6658	5082	16437	1916	<LOD	<LOD	<LOD	<LOD	30093	815
7LZ+	6,64	4464	5578	7826	<LOD	4403	1295	<LOD	877	<LOD	22271	16750
7LZ-	6,64	1957	13958	23302	<LOD	8643	482	1948	1094	<LOD	47860	8432
8LZ+	5,87	2535	2669	4717	<LOD	<LOD	610	<LOD	<LOD	<LOD	10538	1988
8LZ-	6,23	503	1507	20791	<LOD	4738	<LOD	1519	<LOD	<LOD	27539	5133

550

551 * code used in PCA analysis

552 **below limit of detection

553