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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1 Cheese manufacture

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

2.2 *Bacterial counts*

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

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

2.3 *DNA extraction*

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

2.4 *Length heterogeneity (LH)-PCR*

LH-PCR was used in order to determine the microbial community composition. V1 and V2 16S rDNA gene regions were amplified with primers 63F and 355R (Lazzi et al., 2004). The forward primer was 5′-end labelled with a 6-carboxyfluorescein (6-FAM) dye. Amplicons were then separated by capillary electrophoresis in an automated sequencer (Applied Biosystems, Foster City, USA). PCR and capillary electrophoresis conditions were as described by Bottari et al. (2010). The fragment sizes (base pairs) were determined with GeneMapper software version 4.0 (Applied 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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3. Results and discussion

3.1 Microbial characterization of 9-month ripened cheeses

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

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

The evaluation of the free DNA fraction allowed estimating which LAB underwent lysis during 9 months of ripening leaving their DNA still amplifiable. This method, developed by Gatti et al. (2008) to highlight LAB lysis in Parmigiano-Reggiano, was also adopted by Santarelli et al. (2013) for Grana Padano. Lysed species found in the presently studied cheeses were *L. delbrueckii*, *L. helveticus*, *L. rhamnosus* and, in one sample, *L. fermentum*. Interestingly, lysed *L. delbrueckii* was 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 ϒ-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

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

3.2 Arginine metabolism

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

The ability to catabolize arginine through the arginine deiminase (ADI) pathway is rather common in LAB species, typically in heterofermentative LAB (Fröhlich-Wyder et al., 2015; Nicoloff et al., 2001; Price et al., 2012). The accepted model for this pathway (Figure 3b) implies the uptake of free arginine into the bacterial cell by an antiporter system and its degradation into citrulline by ADI. Intracellular citrulline can either be excreted or converted into ornithine by the cytoplasmic enzymes ornithine transcarbamoylase and carbamate kinase. Ornithine is then excreted in the medium. Overall, the ADI pathway brings to the production of one mol of ATP and two mol of ammonia per mol of degraded arginine. Therefore, arginine catabolism is considered to represent both a way to counteract the acid stress and an alternative source of energy. Our data indicated that 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

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

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

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

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

The different amounts of arginine metabolites in LZ+ and LZ- cheeses may be related also to the known biodiversity of *L. rhamnosus* (Bove et al., 2011). This species was present in both types of cheese and thus was not discriminant in our study. However, regarding to LZ resistance, Solieri et al. (2014) found a great variability among strains of *L. rhamnosus*, isolated from Parmigiano-Reggiano cheese, which turned out also to be the most LZ-resistant NSLAB (Solieri et al., 2014). It has been demonstrated that the genetic polymorphisms of *L. rhamnosus* is a response to cheese environmental adaptation also for arginine repressor (*ArgR1*) (Bove et al., 2012) and for formation of ammonia trough the ADI pathway (Liu et al., 2003). Thus, we can hypothesize that different strains of *L. rhamnosus*, having different ability to adopt the ADI pathway, were able to develop in the two types of cheese. This hypothesis needs to be confirmed by further characterization of *L. rhamnosus* strains that have been isolated from the two types of cheese.

4. Conclusions

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

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Captions to figures

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

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

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

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

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	
	MRS plate count	*Lactobacillus delbrueckii*	*Lactobacillus helveticus*	*Lactobacillus rhamnosus*	*Lactobacillus fermentum*	*Pediococcus acidilactici*	*Lactobacillus delbrueckii*	*Lactobacillus helveticus*	*Lactobacillus rhamnosus*	*Lactobacillus fermentum*	Whole cells	Lysed cells
	MRS*	Ldw*	Lhw*	Lrw*	Lfw*	Paw*	Ldl*	Lhl*	Lrl*	Lfl*	DNAw*	DNAl*
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