# Application of a commercial digestive supplement formulated with enzymes and probiotics in lactase non-persistence management

Alvaro Ferreira-Lazarte, F. Javier Moreno, Mar Villamiel*

Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM).

C/Nicolás Cabrera, 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

*Author to whom correspondence should be addressed:

Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM),

C/ Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid,

E-28049 Madrid (Spain).

Tel: +34 910017951

E-mail: m.villamiel@csic.es

**Abstract**

Strategies to avoid lactose malabsorption, which affects 70% of the world's population, are focused on the restriction of milk and dairy products or the use of non-human β-galactosidases or probiotics endowed with β-galactosidase activity added at mealtime. The evaluation of a commercial blend of probiotics and enzymes (protease, lactase, lipase and amylase) and its potential application in lactase non-persistence management are described in this work. Recommended amounts (460-1000 mg) of commercial probiotics/enzymes blend showed to be adequate for in vitro lactose hydrolysis in standard solutions (0.25-5 %) and commercial dairy products, milk (5% lactose) and yogurts (3% lactose) reaching hydrolysis values between 44-96%. According to these percentages, the use of the enzymatic preparation would guarantee the intake of less than 12 g, recommendations of the EFSA for lactose intolerant. Furthermore, formation of prebiotic galactooligosaccharides was also detected increasing the potential benefits of the enzymatic preparation in the gastrointestinal system.

**Keywords:** lactose malabsorption; probiotics; enzymatic preparation; carbohydrase activity; *in vitro* digestion; milk products

## 1. Introduction

Over the past years, the interest toward human well-being and disease prevention has increased the consumption of healthy diets and a prudent addition of dietetic supplements,[1] being the gastrointestinal function one of the main targets. Particularly, the digestion and absorption of carbohydrates has been one of the main topics widely studied during the last years. Digestible di-, oligo - and polysaccharides are hydrolysed to their corresponding monomers before being absorbed in the small intestine; however, in some specific physiological or pathological situations, as in the case of lactose intolerance, these carbohydrates are hardly hydrolysed and absorbed, reaching the gut lumen where they are fermented by the intestinal microbiota.[2]

Lactose intolerance is a common problem resulting from β-galactosidase (i.e., lactase) deficiency at the level of the small intestine. With the rare exception of congenital hypolactasia, this enzyme is always present in the new-born, but its activity naturally diminishes after weaning. In Caucasians, a specific mutation favoured by the high intake of milk, permits the presence of lactase also in adults. However, in other geographical area these changes are not common and, approximately, a 70% of the world's population have non-persistence of lactase. Particularly, in some Asian countries this rate increases up to 100%.[2,3]

Although lactose intolerance is not considered as a true ailment, its symptoms (abdominal spasms, swelling, flatulence and diarrhoea, with a considerable intraindividual and interindividual variability in the severity) may disturb the quality of life. The strategies to avoid this problem are mainly focused on the restriction of milk and dairy products and the intake of lactose-depleted and lactose-free products, non-exempt of nutritional and/or technical problems.[4] In this sense, some published studies have shown that thermal processing could involve a strong advance of the Maillard

63 reaction (MR), with loss of available lysine and modifications in the sensorial properties
64 when lactase is added before the heat treatment. As after hydrolysis the increase in
65 galactose and glucose can greatly favour the evolution of this reaction, the addition of
66 the enzyme after thermal processing is recommended; however, this involves the use of
67 aseptic conditions with the consequent increase in the price of the products.[5,6]
68 Moreover, in pack addition of lactase after milk sterilisation can have adverse
69 organoleptic and nutritional concerns related to the enzyme side proteolytic activity
70 especially for extended storage time.[7]

71 The intake of commercially lactase enzyme preparations in solid from fungal or
72 yeast origin before lactose consumption has been also suggested as a possibility for
73 people with these problems. Although there are interesting studies that underline their
74 potential applications, its usefulness is not fully established due to technical and dose
75 discordances. In spite of this, the EFSA Panel on Dietetic Products, Nutrition and
76 Allergies concluded that there is a cause-effect relationship between their consumption
77 and breaking down lactose in individuals with symptomatic lactose malabsorption.[8] The
78 administration of probiotics endowed with a lactase activity has been also showed to be
79 very useful to treat patients with this problem.[3]

80 Recently, a new commercial product formulated with enzymes (protease,
81 lactase, lipase and amylase) and non-dairy, heat-stable and stomach acid resistant
82 probiotics (*Lactobacillus gasseri, Bifidobacterium bifidum* and *Bifidobacterium*
83 *longum*) is offered as a supplement to support healthy digestive function and help
84 alleviate occasional gas and bloating. The same supplement without enzymes has been
85 proved to have anti-inflamatory effect due to the changes in the gut microbiota
86 communities. An intervention study reported a higher percentage of participants who
87 had an increase in bifidobacteria and lactobacillus in their faecal samples during the

probiotic intervention versus the placebo.[9] However, to the best of our knowledge, no studies on the potential application of this preparation on malabsorption of carbohydrates have been carried out. Thus, the objectives of this work have been: i) to characterise the carbohydrase activity of the commercial preparation of probiotics with enzymes and ii) to evaluate its *in vitro* effectiveness during the hydrolysis of lactose in lactose solutions and commercial dairy products such as whole milk, skimmed milk and two different yogurts.

## 2. Materials and methods

### *2.1. Chemicals and reagents*

Fructose (Fru) standard was purchased from Fluka analytical™. D-galactose (Gal), D-glucose (Glc), lactose, sucrose, phenyl-β-glucoside, o-nitrophenyl (o-NP), p-nitrophenyl (p-NP), o-nitrophenyl-β-D-glucopyranoside (o-NPG) and p-nitrophenyl-α-glucopyranoside (p-NPG) standards were obtained from Sigma-Aldrich (St Louis, MO). Commercial whole and skimmed milk and yogurt samples were purchased from local markets in Madrid, Spain. Commercial enzymatic preparation (Kyo-Dophilus® plus enzymes) (human strains of *Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1 and *Bifidobacterium longum* MM2 together with protease (*Aspergillus melleus*), lactase (*A. oryzae*), lipase (*A. niger*) and amylase (*A. oryzae*)) was kindly supplied by Vitae® Natural Nutrition S. L. (Barcelona, Spain).

### *2.2. Characterisation of commercial preparation*

Commercial enzymatic preparation was used to prepare an enzymatic solution according to the method of Olaokun, *et al*,[10] with minor modifications. Probiotics plus enzymes (10 mg/mL) was homogenized in ice-cold 0.05 M sodium phosphate buffer

112 solution (pH 7.0, 6.5 and 6.5). Then, the clear solution was used for determining protein
113 content, enzymatic activity and subsequent analysis by HPLC-ELSD and GC-FID.
114 Furthermore, pH, water activity and dry matter analysis were carried out directly on the
115 enzymatic preparation.

116 2.2.1. Physico-chemical characterisation

117 The dry matter content was gravimetrically determined in an oven at 110 °C during
118 48 hours until constant weight according to the Association of Official Analytical
119 Chemists (AOAC).[11] Water activity ($a_w$) measurement was carried out in an AW Sprint
120 TH-500 instrument (Novasina, Lachen, Switzerland). The pH of enzymatic preparation
121 (1%, w/v) was obtained using a pHmeter (Mettler Toledo GmBH, Schwerzenbach,
122 Switzerland). Protein content in enzymatic solution was determined by the Kjeldahl
123 method as described by AOAC.[12]

124 2.2.2. Carbohydrase characterisation

125 The determination of β-galactosidase or lactase activity was adapted from
126 Warmerdam *et al.*[13] A solution of o-NPG (o-nitrophenyl-β-D-glucopyranoside) in
127 phosphate buffer 0.05 M, (pH 7.0, 6.5 and 6.0) with a concentration of 0.5 mg/mL
128 (0.05% w/v) was prepared. Enzymatic activity was determined by incubating 1,900 µL
129 of the o-NPG solution and 100 µL of enzyme solution from this commercial product (10
130 mg/mL in phosphate buffer 0.05 M, pH 7.0, 6.5 and 6.0) for 2 h at 37 ºC. The method is
131 based on the measuring of the continuous release of o-NP from o-NPG. Absorbance of
132 released o-NP was measured at 420 nm every 20 s using a spectrophotometer
133 (Specord® Plus, Analytik Jena) together with a temperature controller (Jumo dTRON
134 308, Jumo Instrument Co.). Considering the lactase content on the enzymatic
135 preparation, specific enzymatic activity (U) was expressed in µmol $min^{-1}$ $g^{-1}$, where one

136 unit was defined as the amount of enzyme that produced 1 μmol of o-NP in one min of
137 reaction ($n$ = 6).

138 Similar procedure was used to determine the maltase activity but using a solution of
139 p-NPG (p-nitrophenyl-α-glucopyranoside) in phosphate buffer 0.05 M, pH 7.0, 6.5 and
140 6.0 with (0.05% w/w) and monitoring the release of p-NP at 410 nm every 20 s ($n$ = 4).

141 Invertase activity was determined following the method described by Ghazi *et al*,[14]
142 with slight modifications. An individual solution of sucrose (1 mg/mL) in sodium
143 phosphate buffer 0.05 M, pH 7.0 was used. 50 mL of this solution were incubated
144 together with 50 mg of enzymatic preparation with enzymes at 37 ºC during 2 h.
145 Aliquots were taken at different times (15, 30, 60, 90 and 120 min) and reaction was
146 stopped on boiling water during 5 min. Sucrase activity was determine by monitoring
147 sucrose hydrolysis and increase of fructose by GC-FID. The specific enzymatic activity
148 (U) was expressed in μmol $min^{-1}$ $g^{-1}$, where one unit was defined as the amount of
149 enzyme that produced 1 μmol of reducing sugars in one min of reaction ($n$ = 4).

150 2.3. *In vitro* digestion of buffered standard solutions of lactose

151 To determine the effectiveness of the commercial preparation in the hydrolysis
152 of lactose, an *in vitro* digestion study was carried out under simulated physiological
153 conditions. This is a first common approach to understand the digestion of functional
154 ingredients.[15]

155 First of all, to evaluate the resistance of lactose to the enzymatic preparation,
156 several assays of lactose digestion were carried out using different carbohydrate-
157 enzymatic preparation ratios (Table 1). Lactose concentrations were chosen to cover
158 most of the commercial lactose-content products, such as milk, yogurt, cheese, and free-
159 lactose products.[16,17] In regard of the enzymatic preparation, doses were chosen taking

160 into account recommended prescription (2 capsules/920 mg per day). Thus, 460 mg (1
161 capsule), 1000 mg and 155 mg of preparation were tested with each lactose
162 concentration. Reactions that presented similarity on the ratio preparation/lactose with
163 other reaction were discarded.

164 Hence, assays aimed to determine the capability of enzymatic preparation to
165 hydrolyse lactose at different concentrations. Thus, different solutions of lactose (5.0,
166 1.0 and 0.25 %, w/v) in phosphate buffer 0.05 M pH 7.0 and 6.5 were tested with the
167 following doses of enzymatic preparation: 155, 460 and 1000 mg. Finally, 250 mL of
168 solution of lactose (5.0, 1.0 and 0.25 %, w/v) were mixed with each dose of enzymatic
169 preparation (155, 460 and 1000 mg). The mixture was incubated at 37 ºC (pH 7.0 and
170 6.5) under continuous agitation (400 rpm) for 2 h. Aliquots were taken at 15, 30, 60, 90
171 and 120 min of digestion and heated in boiling water for 5 min to stop the reaction. The
172 digestion of lactose was monitored by analysis of the trimethyl silylated oximes
173 (TMSO) of carbohydrates by GC-FID as described below.

174 In addition, a series of control samples, based on the incubation of enzymatic
175 preparation without lactose during the same reaction times, were also analysed. Results
176 showed a minor increase of monosaccharides, galactose and glucose as the digestion
177 proceeded. These values were conveniently subtracted in order to avoid any
178 overestimation of the monosaccharide fraction.

179 2.4. *In vitro* digestion of lactose commercial products

180 Since buffered standard solutions of lactose would be more prone to changes as
181 they are not protected in a food medium, the effectiveness of enzymatic preparation on
182 lactose commercial products (two commercial milk and two yogurts) was tested. First,
183 commercial products were characterised (Table 2) by measuring its pH, protein content,

184 total carbohydrates and lactose content by GC-FID. Later, 250 mL of commercial milk
185 and yogurt were mixed with 1000 mg of the commercial preparation of probiotics with
186 enzymes and the mixture was incubated at 37 ºC (pH of milk and yogurt), 400 rpm for 2
187 h. Aliquots were taken at 15, 30, 60, 90 and 120 min of digestion and the reaction was
188 stopped by heating samples in boiling water for 5 min.

189 Before chromatographic analysis, samples were subjected to a clarification
190 procedure using Carrez reagents in order to remove interfering compounds 6.
191 Carbohydrates analysis was performed by GC-FID as described below.

192 *2.5. Carbohydrate analysis by GC-FID*

193 Trimethyl silylated oximes (TMSO) of carbohydrates (mono-, di- and
194 trisaccharides) present in samples were determined following the method of Cardelle-
195 Cobas.[18] Chromatographic analysis was carried out on an Agilent Technologies gas
196 chromatograph (Mod7890A) equipped with a flame ionization detector (FID). The
197 TMSO were separated using a 15 m x 0.32 mm x 0.10 µm film, fused silica capillary
198 column (DB-5HT, J&W Scientific, Folson, California, USA). Nitrogen was used as
199 carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and
200 385 ˚C, respectively. The oven temperature was programmed from 150 to 380 ˚C at a
201 heating ratio of 3 ˚C/min. Injections were made in the split mode (1:20 or 1:5)
202 depending on lactose content of the solution.

203 The TMSO derivatives were formed following the method of Ruiz-Matute *et al.*[6]
204 First, a volume of 100 or 200 µL of the digested sample was added to 400 µL of internal
205 standard solution, containing 0.5 mg/mL of phenyl-β-glucoside. Afterwards, the mixture
206 was dried at 40 ˚C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).
207 Sugar oximes were formed by adding 250 µL hydroxylamine chloride (2.5%) in

208 pyridine and heating the mixture at 70 ˚C for 30 min. Subsequently, the oximes
209 obtained in this step were silylated with hexamethyldisylazane (250 µL) and
210 trifluoroacetic acid (25 µL) at 50 ˚C for 30 min.[19] Derivatization mixtures were
211 centrifuged at 6,700 x *g* for 2 min and supernatants were injected in the GC.

212 Data acquisition and integration were done using Agilent ChemStations software
213 (Wilmington, DE, USA). Response factors were calculated after the duplicate analysis
214 of standard solutions (fructose, galactose, glucose, lactose, sucrose, raffinose and
215 stachyose), at different concentrations ranging from 0.005 to 4 mg/mL.

216 *2.6. Statistics*

217 All digestions were carried out in duplicate and two GC-FID analysis were
218 carried out for each digestion treatment ($n$ = 4). The comparisons of means using
219 analysis of variance (ANOVA) were made using the statistical package (SPSS Inc.,
220 Chicago, Il). The differences were considered significant when $P < 0.05$.

221

222 **3. Results and discussion**

223 *3.1. Characterisation of the commercial preparation of probiotics with enzymes*

224 The overall characterisation of the enzymatic preparation showed that the pH
225 was 6.46, similar to that of small intestine in adults and children, where the hydrolysis
226 of carbohydrates takes places.[15,20] The $a_w$ (0.186) and dry matter (94.6%) values
227 guarantee its microbiological stability. Regarding protein, data obtained by the Kjeldhal
228 method were slightly higher (17.4%) than the sum of the amounts of all enzymes
229 reported in the product (for 1 capsule of 460 mg: 35 mg protease, 17.5 mg lactase, 12.5
230 mg lipase y 12.5 mg amylase; total 77.5 mg, 16.8%). This small difference could be due

231 to the different methods used and the presence of proteins coming from the probiotic
232 bacteria.

233 The next step was the evaluation of the main carbohydrase activities in the
234 enzymatic preparation, being lactase, maltase and invertase the tested activities as
235 indicated in Material and Methods. Although, according to the data sheet, the
236 commercial preparation presented other enzymatic activities different from those related
237 to carbohydrates, they were not evaluated. This was out from the aim of the work and,
238 as indicated earlier, nowadays a lot of attention is paid to the carbohydrate
239 malabsorption. Figure 1 shows the evolution of the corresponding activities carried out
240 at pH 6, 6.5 and 7. Maltase (B) and invertase (C) followed a similar behaviour with a
241 constant increase through the time; however, lactase (A) had a different pattern with a
242 first lineal phase to reach a plateau after 10 min at pH 7 and 5 min at pH 6.5 and 6. In
243 lactase and maltase the highest activity was detected at pH 7. Taking into account the
244 data of Figure 1, the specific enzymatic activities were calculated (Table 3), the highest
245 activity being lactase, followed by maltase and invertase. Cardelle-Cobas, (2009)[18]
246 tested an enzymatic preparation from the same source (*Aspergillus oryzae*) and found a
247 β-galactosidase activity of ~7000 U/g. Taking into account these results, the main
248 objective of this work was focused on the usefulness of the commercial enzymatic
249 preparation on lactose hydrolysis, in order to broaden its applicability.

250 *3.2. Hydrolysis of lactose in buffered standard solutions*

251 Figure 2 depicts the chromatograms obtained by GC-FID of the hydrolysis of
252 lactose (A, 5%; B, 1%; C, 0.25%) after 2 h of digestion with 1000 mg of commercial
253 preparation. Galactose and glucose were formed together with different di- and
254 trisaccharides derived from the transgalactosylation of lactose. It has been previously

255 described that β-galactosidase can hydrolyse or transgalactosylate lactose forming
256 molecules of higher molecular mass depending on the reaction conditions.[21,22] As
257 lactases from *A. oryzae* synthesise galactooligosaccharides (GOS) prebiotic with β(1-6)
258 linkages it is plausible that the structures formed during the *in vitro* digestion of lactose
259 with the enzymatic preparation are prebiotics. In addition to the action of lactase derived
260 from *A. oryzae*, it is also presumable that the probiotic bacteria (bifidobacteria and
261 lactobacilli) also present in the supplement contribute to these reactions. In this sense, β-
262 galactosidases derived from lactic acid bacteria and bifidobacteria are also of valuable
263 interest for production of GOS with better selectivity for the growth and metabolic
264 activity of these two bacteria genera in the gut, which may lead to an improved prebiotic
265 effect.[23]

266 Tables 4 and 5 show the results obtained after all the reactions carried out with
267 lactose solutions at pH 7 and 6.5, taking into account the ratios commercial
268 preparation/lactose (w/w) indicated in Materials and Methods (Table 1). As expected,
269 lactose hydrolysis increased with the increase of preparation and with the decrease of
270 lactose concentration. In general, the highest hydrolysis was found in the reactions
271 performed at the lowest pH. At pH 6.5, three reactions led to percentage values of
272 hydrolysis higher than 90%, and in one of them was almost 99%, whereas at pH 7 only
273 in one reaction the hydrolysis value exceeded 90% (maximum amount of enzyme, 1000
274 mg, and minimum of lactose, 0.25%). However, the β-galactosidase activity carried out
275 with o-NPG above mentioned, was higher at pH 7 than at pH 6.5. These dissimilarities
276 could be ascribed to different selectivity of enzymes (from *A. oryzae* and probiotics
277 bacteria) toward substrates, o-NPG and lactose.

278 Figure 3 shows the evolution at pH 7 (A) and 6.5 (B) of GOS (di- and
279 trisaccharides) formed during the corresponding reactions of lactose hydrolysis. At both

280 pH values, the highest GOS content was detected at the maximum lactose concentration
281 (5%) since at lower concentration of substrate the transgalactosylation is not favoured
282 and the GOS formed are hydrolysed rapidly by the enzymes.[22] Values of GOS
283 concentrations in the range 30-120 mg/g lactose were found.

284 *3.3. Hydrolysis of lactose in commercial products*

285 Once we confirmed the high level of lactose hydrolysis with this commercial
286 preparation in buffered standard solutions of lactose, we studied the matrix effect in the
287 digestion of lactose present in commercial milks and yogurts with the composition
288 indicated in the Table 2. The evolution of the hydrolysis of lactose in these products is
289 revealed in Figure 4. It is clear that both types of products had a different behaviour
290 against the enzymatic preparation with a higher hydrolysis in yogurts (>91%) than in
291 milk (>55%), probably due to the lower amount of initial lactose in yogurts and/or the
292 lower pH, since the maximum activity of lactase from *A. oryzae* takes places at pH
293 values of 2.5-5.5. In addition, the presence of lactase coming from the live starter
294 cultures could also contribute to the lactose hydrolysis.[24]

295 On the other hand, lactose presented in a solid food may be less likely to induce
296 symptoms than an identical load of lactose presented in solution[8]. This fact might be
297 attributed to the rate of gastric emptying which could be a relevant factor, so the fat
298 content of the foodstuff consumed may slow the entrance of lactose into the small
299 intestine, and, hence, increase the fraction of lactose digested and slow the rate of
300 presentation of unabsorbed lactose to the colon[25].

301 When whole and skimmed milks were compared, the hydrolysis occurred in
302 significant less extent in the former (54.8%) than in the latter (60.4%). These values are
303 slightly lower than those obtained during the hydrolysis of lactose in buffered standard

304 solutions under the same conditions which gave rise to a value of 69.3%. Therefore, a
305 protection effect of milk composition against the hydrolysis of lactose with lactase from
306 *A. oryzae* was observed. The upper thickness and, therefore, reduced distribution rates
307 of both the enzyme and the substrate, in addition to possible hydrophobic binding by fat
308 globules in whole milk, are likely reasons of these data.[26]

309 Similarly to the results with lactose solutions, there was higher formation of GOS
310 (Table 6) in the commercial products with lower hydrolysis (milks, around 5,500 mg/L)
311 and this amount was almost kept as a plateau during all the digestion process and were
312 much higher than the values of GOS found by Ruiz-Matute *et al*.[6] in commercial UHT
313 milks (average, 2,134 mg/L). Later, Larsen et al (2015)[27] reported a patented method for
314 preparing lactose-depleted and rich GOS products having a stable content of GOS using
315 lactase from *B. bifidum*.

316 **3. Conclusions**

317 The data found in this research allow us to conclude that the studied commercial
318 supplement of enzymes and probiotics, in the quantities (≤1000 mg, two capsules) and
319 conditions here assayed, is adequate for the hydrolysis of lactose in buffered solutions
320 (0.25 - 5%) and in commercial dairy products, milk (5% lactose) and yogurts (3%
321 lactose). Hydrolysis of lactose values ranged from 27 to 99%, depending on the
322 relationship of enzyme preparation / lactose and the type of the product. The highest
323 hydrolysis was found in lactose solutions followed by yogurts, and especially a
324 protective effect of the matrix was also observed in whole milk. In this commercial
325 product, considering the intake of 1000 mg, a hydrolysis higher than 55% was observed,
326 which would guarantee the intake of less than 12 g, recommendations of the EFSA
327 Panel on Dietetic Products, Nutrition and Allergies for lactose intolerant 8. In addition,

due to the transgalactosylation potential of lactases present in this commercial preparation under the conditions tested, prebiotic GOS are also formed, expanding the applications of probiotics plus enzymes. Although more research is needed, this preparation could be taken with meals to assist in the digestion of lactose or be also used to easily prepare lactose-depleted and enriched in GOS products before consumption.

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*Conflict of interest*

The authors have no competing personal or financial interests to declare.

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