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1 ***Polyol-producing lactic acid bacteria isolated from sourdough and their***
2 ***application to reduce sugar in a quinoa-based milk substitute***

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8 ***Abstract***

9 The interest for plant-based dairy substitutes is expanding rapidly and consumers are opting for
10 nutritious and healthy dairy alternatives. The reduction of sugar using different exogenous enzymes in
11 combination with lactic acid fermentation in a quinoa-based milk substitute was explored in this study.
12 Different amylolytic enzymes were used to release sugar from the raw material, which were further
13 metabolised to mannitol, due to fermentation with two heterofermentative lactic acid bacteria. Using
14 these two biotechnological techniques enables the reduction of sugar, while also preserving some of
15 the sweetness. *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 were isolated from
16 sourdough. Both strains showed high viable cell counts with *L.eueconostoe citreum* TR116 > 8.4 and
17 *L.actobacillus brevis* TR055 > 9.3 log cfu/ml, and a reduction in pH to 3.7 and 3.5 respectively. When
18 fructose was available, mannitol was produced in conjunction with acetic acid in addition to lactic
19 acid. Due to these processes, the original glucose value was reduced from 50 mmol/100g to
20 approximately 30 mmol/100g, which equates to a glucose reduction of 40%. In respect to mannitol
21 production, both strains performed well: *L.eueconostoe citreum* TR116 showed a conversion factor of
22 1:1 from fructose to mannitol, while *L.actobacillus brevis* TR055 showed a lower yield, with a
23 conversion factor of 1:0.8. Glycaemic load was reduced by more than a third, bringing it down to the
24 low range with a value of about 10. Overall, enzymatic modification in conjunction with mannitol-
25 producing lactic acid bacteria shows great potential for further possible application in the development
26 of nutritious and sugar reduced plant-based milk substitutes.

27 **Keywords**

28 Milk substitute, Lactic acid bacteria, Quinoa, Sugar reduction, Mannitol, Glycaemic index

29 **1. Introduction**

30 Plant-based milk substitutes (PBMSs) are gaining popularity and the interest is expanding rapidly.
31 Consumers are choosing dairy substitutes over dairy products for various reasons; obviously so in the
32 case of individuals suffering from milk allergies and intolerance, but an increasing consumption is
33 based on preference. In this regard, PBMSs can serve as a sustainable, ethical and nutritious option to
34 meet the needs of consumers. Owing to this increasing interest, the market is expected to grow at a
35 significant rate: MarketsandMarkets (2017) estimated the value of the dairy alternative market to be
36 7.37 Billion USD for 2016 and predicted a growth rate of 11.7% from 2017 on, reaching a forecasted
37 market value of 14.36 Billion USD in 2022. Nevertheless, many studies reported several concerns
38 about the nutritional value of some products (Jeske et al., 2017; Katz et al., 2005; Sousa et al., 2017).
39 In particular, the low protein content was found to be a major risk. Furthermore, PBMSs based on
40 starchy raw materials, such as rice or quinoa contain high amounts of sugar due to hydrolysis of starch
41 and release of maltose and/or glucose thereof. Sugar contents and *in-vitro* glycaemic indices of
42 commercial PBMSs were analysed, and rice-, and coconut-based products especially showed high
43 values for the glycaemic indices with 97.74, and 96.82, respectively, with sugar content of 7.02 and
44 1.86 g/100g, respectively (Jeske et al., 2017). High sugar consumption affects human health, being a
45 major inducer for obesity and chronic diseases (Lustig et al., 2012). The public awareness of this
46 problem is increasing and consumer behaviour is changing: 64% of consumers on the Island of Ireland
47 are concerned about their sugar intake (James Wilson, 2018), and similarly German consumers have
48 reduced their sugar consumption by 48% (Mintel Press Team, 2017). For this reason, research and
49 industry are investigating methods for sugar-reduction or use of sweeteners as alternatives to sugar.
50 Mannitol, as one promising alternative, is a natural sugar alcohol, prevalent in several plants, fungi,
51 yeast and bacteria (Wisselink et al., 2002). It has a sweet taste, being perceived about 40% less sweet
52 than sucrose, and its incorporation in food has several beneficial effects; these include health claims

1

Abbreviation:

PBMS: Plant-based milk substitutes

QBMS: Quinoa-based milk substitute

53 relating to protection against tooth-decay and reduction of the glycaemic response, both due to
54 mannitol not being absorbed in the human intestine, and thus exhibiting a low calorific value. Both
55 claims are approved by the European Food Safety Authority (2011). Although an increasing amount of
56 consumers (17% in Germany) believe that plant-based yoghurt alternatives are healthier, the biggest
57 challenge lies in the taste for these products (Mintel Press Team, 2017). In this regard, mannitol could
58 improve the properties of these products, increasing both health benefits and flavour at the same time.
59 Industrially, mannitol is produced by catalytic hydrogenation of a glucose/fructose syrup, producing a
60 mixture of sorbitol and mannitol. However, the yield is low and costs are high for this chemical
61 process (Grembecka, 2015). As an alternative, lactic acid bacteria (LAB) can be used to produce
62 mannitol in a more sustainable and efficient way. Heterofermentative LAB can reduce fructose
63 directly to mannitol. It is catalyzed by the enzyme mannitol dehydrogenase and, metabolically, serves
64 to regenerate NAD^+ (Wisselink et al., 2002). Fermented foods are attracting increased interest and
65 recently much emphasis has been granted to their unique functional properties and contribution to the
66 health of consumers. Their application has evolved from preserving food to understanding and
67 exploiting metabolites, other than organic acids and antifungal compounds. Studies focus on
68 compounds associated with health benefits and additional functional properties, such as mannitol or
69 exopolysaccharides (Chilton et al., 2015; Lynch et al., 2018; Selhub et al., 2014; Tamang et al., 2016).
70 Further, new raw materials are explored as substrate for LAB fermentation and for the development of
71 novel products. For instance, the ancient pseudocereal quinoa has received renewed interest,
72 particularly in Western countries due to its high nutritional value (Arendt and Zannini, 2013). It is
73 especially rich in protein and essential amino acids, contains adequate levels of important
74 micronutrients such as minerals and vitamins, and significant amounts of other bioactive compounds,
75 such as polyphenols (Alvarez-Jubete et al., 2010; Arendt and Zannini, 2013). As a versatile substrate,
76 quinoa has been used for different fermented products; Axel et al. (2015) improved the nutritional
77 value and bread quality using quinoa flour as a base for sourdough with exceptionally high amounts of
78 antifungal compounds; Zannini et al. (2018) developed a quinoa-based yoghurt, having a higher water
79 holding capacity and viscosity than a chemically-acidified control, due to dextran exopolysaccharide
80 excretion by an LAB strain. In addition, fermentation has been shown for many cereals and legumes to

81 improve sensorial and textural properties (Peyer et al., 2016) and could be used as a tool to ameliorate
82 grassy and bitter off-flavours, characteristic for quinoa.

83 In this study, a quinoa based milk substitute was used as a substrate to study the production of
84 mannitol by means of a two-step process including enzyme treatment, and fermentation with two
85 heterofermentative LABs. The samples were treated with amylases and a glucose-isomerase, in order
86 to generate fructose as a substrate, which was further metabolized to mannitol by the action of LAB.
87 The aim was to study the potential for sugar-reduction using this two-step process.

88 **2. Materials and methods**

89 **2.1 Materials, strains and culture conditions**

90 Organic quinoa was obtained from Ziegler & Co. GmbH Naturprodukte (Wunsiedel, Germany).
91 Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated.
92 The microorganisms *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 were isolated from
93 yellow pea sourdough and teff sourdough, respectively, and belong to the culture collection of the
94 Department of Biological Sciences, Cork Institute of Technology, Ireland. These cultures were
95 selectively chosen for their ability to produce mannitol. The LAB isolates were maintained as frozen
96 stocks in 40% (w/w) glycerol at -80°C. The strains were routinely sub-cultured on de Man Rogosa and
97 Sharp (MRS) agar under anaerobic conditions for 24 h at 30 °C.

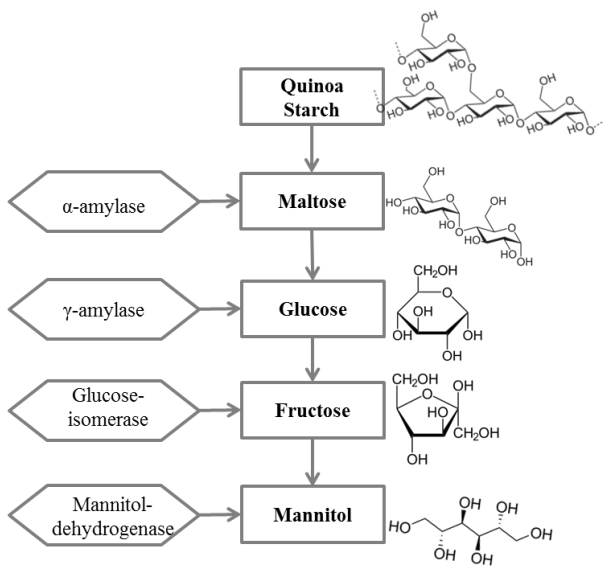
98 **2.2 Preparation of quinoa-based milk substitute**

99 50 g organic quinoa flour and 350 g water were mixed in a semi-industrial blender (Kenwood Major
100 Titanium, New Lane, Havant, UK) at maximum speed for 3 minutes. To each sample 250 mg α -
101 amylase (Hitempase 2XP, Kerry, Carrigaline, Ireland), 300 μ L amyloglucosidase (Attenzuzyme,
102 Novozymes), and 36.6 μ L protease (Flavourzyme, Novozymes) were added. The samples were mixed
103 again for 30 s at lower speed. Additionally, 0.8 g glucose-isomerase were added to some of the
104 samples (labelled as “iso”). All samples were kept in a stirring water bath at 60 °C for 24 h for enzyme
105 action and cooled to 25 °C within 20 min (Lochner mashing device LP electronic, Berching,
106 Germany). Samples were cooled on ice straight after, filtered with cheese clothes and homogenized

107 (APV Homogenizer, SPX FLOW, Inc., Charlotte, USA) at 150 bar for the 1st stage, 30 bar for the 2nd
108 stage. Finally, the samples were pasteurized in a water bath at 65 °C for 30 min.

109 2.3 Fermentation

110 Single colonies of each LAB strain were propagated twice in 10 mL MRS broth in anaerobic and static
111 conditions for 24 h at 30 °C. Cultures were cultivated until the late exponential phase (ca. 14 h) and
112 enumerated by performing a viable plate count in duplicate. After cell count determination,
113 suspensions were prepared in the same manner for inoculation and harvested by centrifugation at 9000
114 g for 10 min at 4 °C and washed twice with Ringer's solution. The inoculation was performed at 7 log
115 cfu/mL directly into tempered quinoa-based milk substitute (QBMS) samples. Fermentation was
116 performed anaerobically, under static conditions at 30 °C for 24 h. Figure 1 depicts the enzymatic
117 processing of quinoa starch, using the exogenous enzymes (α -amylase, γ -amylase, and glucose-
118 isomerase) added at the before outlined part of the preparation of QBMS samples, and the endogenous
119 enzyme mannitol-dehydrogenase, which is secreted by *Leuconostoc citreum* TR116 and *Lactobacillus*
120 *brevis* TR055 during the fermentation test.



122 **Figure 1** Enzymatic processing of quinoa starch with exogenous enzymes (α -amylase, γ -amylase, and
123 glucose-isomerase), and endogenous enzymes, secreted by LAB (mannitol-dehydrogenase)

124 **2.4 Compositional analysis**

125 Compositional analyses were performed on the quinoa flour and unfermented samples. Total nitrogen
126 content was determined according to the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen content was
127 converted into protein using the factor 5.75 according to Fujihara et al. (2008). Fat content was
128 measured following the Soxhlet method. Ash content was determined in a muffle furnace by
129 incineration (4 h, 600 °C), pre-heated in crucibles (1 h, 100 °C). The moisture content was determined
130 by drying in an oven at 103 °C until constant mass was reached. Total starch was analysed using the
131 enzyme kit K-TSTA supplied by Megazyme, Ireland.

132 **2.5 Viable cell counts**

133 Total cell counts of LAB were performed on MRS agar plates after incubation for 48 h under
134 anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) at 30 °C.

135 **2.6 Measurement of titratable acidity, and pH**

136 The total titratable acidity (TTA) was determined by suspending 5 g of sample in 45 mL distilled
137 water and titrating against 0.1 N NaOH to pH 8.5 (Katina et al., 2006). After 3 min, the pH was
138 readjusted to 8.5. The TTA was expressed as the number of millilitres of NaOH used for titration. The
139 pH was monitored using a commercial digital pH meter.

140 **2.7 Determination of sugar and organic acids profiles**

141 ~~For sugar analysis, s~~ Samples were diluted with water, ~~and~~ filtered (0.2 µm). ~~Sugar profiles were and~~
142 analysed by high performance liquid chromatography using an Agilent Infinity 1260 HPLC System.
143 ~~For sugar analysis the system was~~ equipped with a Waters Sugar-Pak, 300 x 6.5 mm HPLC column at
144 0.5 mL/min flow rate of 0.0001 mmol/L CaEDTA at 80 °C, ~~and detected by using~~ a refractive index
145 detector (Agilent Technologies, Palo Alto, CA) ~~for detection~~. Glucose, maltose, fructose, and mannitol
146 were used as external standards. Results were reported in mmol/100g QBMS. ~~Organic acids were~~
147 ~~determined using an Agilent Hi-Plex H, 7.7 x 300 mm, pack size 8 µm HPLC column with a 178 PL~~
148 ~~Hi-Plex Guard column mounted upstream at a flow rate of 0.5 mL/min of 0.005 mmol/L H₂SO₄, and a~~
149 ~~column temperature of 60 °C. Lactic acid, and acetic acid were used as external standards. Results~~
150 ~~were reported in mmol/100g QBMS.~~

151

152 ***2.8 Determination of organic acids***

153 ~~Organic acids were determined by high performance liquid chromatography using an Agilent Infinity~~
154 ~~1260 HPLC System equipped with a diode array detector (Agilent Technologies, Palo Alto, CA). All~~
155 ~~measurements were performed using an Agilent Hi-Plex H, 7.7 x 300 mm, pack size 8 µm HPLC~~
156 ~~column with a 178 PL Hi-Plex Guard column mounted upstream. Samples were previously sterile~~
157 ~~filtered through (0.2 µm) and analysed at a flow rate of 0.5 mL/min of 0.005 mmol/L H₂SO₄ at a~~
158 ~~column temperature of 60 °C. Lactic acid, and acetic acid were used as external standards. Results~~
159 ~~were reported in mmol/100g QBMS.~~

160 ***2.9 Glycaemic index***

161 In vitro determination of the glycaemic index (GI) was evaluated according to Magaletta & DiCataldo
162 (2009) using a calculation designed by an artificial neural network. A certain amount of sample
163 (equivalent to 0.5 g of available carbohydrates, based on the results of sugar and starch analysis) was
164 digested by a multi-enzyme preparation. The digestate was analysed for glucose, fructose, lactose,
165 galactose, and maltitol with HPLC, described as above. These results, together with the results from
166 the protein and fat determination, were used to feed the calculation:

$$GI = 26.264529 - 1.048186 \cdot \textit{Protein} [\%] - 0.248138 \cdot \textit{Fat} [\%]$$

$$+ 621.7824 \cdot \textit{Glucose} [\%] - 52.7993 \cdot \textit{Fructose} [\%]$$

$$- 233.67679 \cdot \textit{Lactose} [\%] - 61.21071 \cdot \textit{Galactose} [\%] - 84.689245 \textit{Maltitol} [\%]$$

167 Glycaemic load (GL) was calculated according to Atkinson et al. (Atkinson et al., 2008):

$$GL = (GI \cdot \textit{available carbohydrate (g) per portion}) / 100$$

168 The portion size was set to 250 g.

169 ***2.10 Physicochemical Properties***

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170 Rheological behaviour of the products was characterised using a controlled stress rheometer
171 (MCR301, Anton Paar GmbH, Austria) equipped with a sensor system of coaxial cylinders (C-CC27-
172 T200/SS, Anton Paar GmbH, Austria). The shear stress (σ) was measured as a function of shear rate
173 ($\dot{\gamma}$) ranging from 0.5 to 100 s⁻¹ within 500 s. The measurements were carried out at 10 °C. The
174 apparent viscosity measured at 10 s⁻¹ is referred to as viscosity. Stability was determined through
175 phase separation analysis using an analytical centrifuge (LUMiSizer; LUM GmbH, Berlin, Germany).
176 The instrumental parameters used were as follows: 1000 rpm for 30 min followed by 3000 rpm for 60
177 min at 24 °C. Separation rate in %/h was determined by plotting the % of transmission over the time.
178 Syneresis of quinoa milk was analysed using a slight modification of the centrifugation method
179 previously reported by Keogh and O’Kennedy (1998). 40 g of sample were centrifuged at 220 g for 10
180 min at 4 °C. The supernatant was poured off and weighed again. Syneresis was expressed as a %.
181 Colour values were measured using the CIE L*a*b* colour system and obtained using illuminant D65.
182 The instrument used was a colorimeter (CR-400, Konica Minolta, Osaka, Japan). Colour of samples
183 was characterised according to whiteness index (WI), defined as:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

184 **2.11 Statistics**

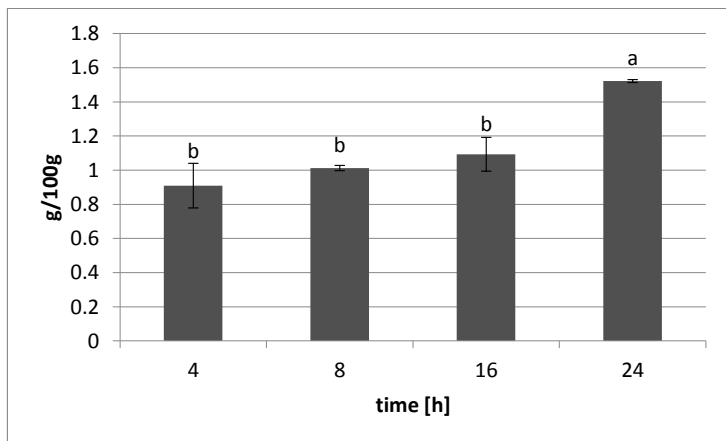
185 All analyses were carried out at least in triplicate. Means were compared with one-way analysis of
186 variance (ANOVA) and Tukey pairwise. The significance level was set to $\alpha = 0.05$.

187 **3. Results and discussion**

188 **3.1 Compositional analysis**

189 The composition of the quinoa flour used for the preparation of the samples given in % (w/w) was as
190 follows: moisture: 9.6% \pm 0.4, protein: 14.64% \pm 0.14, ash: 2.59% \pm 0.03, fat: 7.24% \pm 0.00, and total
191 starch (dry basis): 67.4% \pm 3.55. The prepared QBMS samples contained 0.84% \pm 0.01 protein, 0.29%
192 \pm 0.01 ash, 0.33% \pm 0.04 fat and <1.8 mg/L starch. Due to hydrolysis of starch, the samples contained
193 glucose with 9.09% \pm 0.45. The level of fructose during glucose-isomerase treatment at different time

194 points is displayed in figure 1. Fructose contents continued to increase, while finally the amount of
195 glucose was reduced to $7.21\% \pm 0.09$ and fructose levels occurred at $1.55\% \pm 0.07$ after 24 hours.



196

197 **Figure 12** Concentration of fructose in quinoa-based milk substitutes over time during glucose-
198 isomerase treatment. Values that share a label are not significantly different from one another ($p <$
199 0.05).

200 3.2 Cell Growth and acidification properties

201 The values of viable cell counts, pH, total titratable acidity, and acid profile are presented in Table 1.
202 The results showed that QBMS facilitated the growth of *L. citreum* TR116, as well as *L. brevis*
203 TR055. The latter showed a more vigorous growth, reaching values of 9.35 log cfu/mL, while *L.*
204 *citreum* TR116 reached cell counts of 8.48 log cfu/mL after 24h incubation. Ruiz Rodríguez et. al
205 (2016) found both strains to be autochthonous in spontaneously fermented sourdough produced from
206 quinoa. Both strains showed similar cell counts in QBMS, regardless of treatment with isomerase, i.e.
207 the presence of fructose in the media had no impact on cell growth. However, the presence of fructose
208 influenced acid production; TTA values increased for both strains in samples due to the treatment with
209 glucose-isomerase, from 4.38 to 6.79 mL for *L. citreum* TR116, and from 6.04 to 8.68 mL for *L.*
210 *brevis* TR055. The pH, however, dropped to 3.75 and 3.52, for *L. citreum* TR116 and *L. brevis* TR055,
211 respectively and was not decreased considerably in QBMS treated with glucose-isomerase. A closer
212 look at the acid profile revealed that in the absence of fructose, only lactic acid was produced (5.49
213 and 8.82 mmol/100g from *L. citreum* TR116, and *L. brevis* TR055, respectively), while in the

214 presence of fructose, acetic acid was additionally produced from both strains (4.79 and 4.12
 215 mmol/100g from *L. citreum* TR116 and *L. brevis* TR055, respectively).

216 **Table 1** Cell counts, pH, TTA, and organic acid values of unfermented (Unf.), isomerase treated
 217 samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis*
 218 TR055 fermented of quinoa-based milk substitutes.

	cfu [log cfu/mL]	pH	TTA [mL]	Lactic acid [mmol/100g]	Acetic acid [mmol/100g]
Unf.	n.d.	5.37±0.08 ^b	1.70±0.01 ^c	n.d.	n.d.
Unf. Iso	n.d.	5.67±0.05 ^a	1.63±0.06 ^c	n.d.	n.d.
TR116	8.48±0.03 ^b	3.75±0.02 ^c	4.38±0.35 ^d	5.49±0.42 ^b	n.d.
TR116 Iso	8.42±0.26 ^b	3.64±0.03 ^{cd}	6.79±0.47 ^b	5.59±0.37 ^b	4.79±0.41 ^a
TR055	9.35±0.10 ^a	3.52±0.05 ^{de}	6.04±0.12 ^c	8.82±0.13 ^a	n.d.
TR055 Iso	9.24±0.09 ^a	3.45±0.02 ^{de}	8.68±0.27 ^a	8.95±0.08 ^a	4.12±0.23 ^a

219 Values within a column that share a superscript are not significantly different from one another (p <
 220 0.05); n.d. detectable, limit for cfu < 3 log cfu/mL, for lactic and acetic acid < 1mM/100g

221 Heterofermentative LAB can generate additional ATP with the production of acetic acid from acetyl
 222 phosphate. However, this is only possible in the presence of fructose, which acts as an alternative
 223 electron acceptor, NAD⁺ is regenerated via the reduction of fructose to mannitol, which would
 224 otherwise happen through the production of ethanol from acetyl phosphate (Wisselink et al., 2002).

225 Table 2 shows the sugar composition with some stoichiometric parameters related to the sugar
 226 metabolism. Due to the glucose-isomerase treatment, 8.58 mmol/100g fructose were produced from
 227 glucose. Furthermore, glucose was metabolised by both strains and approximately 9 mmol/100g
 228 glucose were consumed in all fermented samples; neither the glucose-isomerase treatment and
 229 changing carbohydrate composition, nor the bacteria itself had a considerable impact on this value.
 230 When fructose was present, both LAB produced mannitol additionally. *L. citreum* TR116 produced
 231 8.58 mM/100g, while *L. brevis* TR055 produced less mannitol, at 7.18 mM/100g. *L. citreum* TR116
 232 metabolized fructose completely to mannitol, with a yield of 100%, while *L. brevis* TR055 achieved a
 233 yield of 84%. It was demonstrated previously that heterofermentative LAB can reduce fructose to
 234 mannitol with yields of up to 100%, when glucose and fructose were available (1:2) (Wisselink et al.,

235 2002). *L. citreum* is known to produce mannitol, however other studies have found lower yield values
 236 of 89.3% or 70% from fructose (Carvalho et al., 2011; Otgonbayar et al., 2011). In these studies, the
 237 ratio of fructose to glucose was higher, and the strains used were grown in a different medium. On the
 238 other hand, *L. brevis* TR055 showed a lower ratio of fructose to mannitol with about 84%. Therefore,
 239 some fructose must have entered the phosphoketolase pathway instead of being metabolized to
 240 mannitol. Due to the combined reactions of glucose-isomerase and fermentation, a total amount of
 241 about 20 mmol/L glucose were removed and transformed into metabolites like organic acids, mannitol
 242 and other compounds. This bioprocess can be used to generate sour, fermented products, while at the
 243 same time not losing too much sweetness, since mannitol is produced. Glucose was reduced by
 244 approximately 40% (equivalent to 20 mmol/100g) through the action of the glucose-isomerase
 245 treatment and being used as a carbon source for bacterial growth. However, the sweetness of the
 246 product was only reduced by about 24% and 28% for samples treated with glucose-isomerase and
 247 fermented with *L. citreum* TR116 or *L. brevis* TR055, respectively, when considering [literature values](#)
 248 [of the relative sweetness of glucose and mannitol \(0.7 and 0.6, respectively, compared to sucrose\)](#)
 249 [\(Nutrients Review, 2016\).](#)~~the reduced glucose content and the different sweetness levels of glucose~~
 250 ~~and mannitol (0.7 and 0.6, respectively when compared to sucrose) (Nutrients Review, 2016).~~

251 **Table 2** Sugar composition and stoichiometric parameters of [unfermented \(Unf.\), isomerase treated](#)
 252 [samples \(Iso\) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis*](#)
 253 [TR055 of quinoa-based milk substitutes.](#)~~*Leuconostoc citreum* TR116 and *Lactobacillus brevis*~~
 254 ~~TR055 fermented quinoa-based milk substitutes.~~

	Glucose [mmol/100g]	Fructose [mmol/100g]	Maltose [mmol/100g]	Mannitol [mmol/100g]	Reduction of glucose [mmol/100g]	Mannitol yield on fructose
Unf.	50.44±2.49 ^a	n.d.	1.09±0.13 ^a	n.d.	-	-
Unf. Iso	39.99±0.48 ^b	8.58±0.40 ^a	0.86±0.07 ^{bc}	n.d.	10.44±2.27 ^b	-
TR116	39.92±2.00 ^b	n.d.	0.99±0.04 ^{ab}	n.d.	10.52±2.24 ^b	-
TR116 Iso	31.01±0.32 ^c	n.d.	0.67±0.04 ^c	8.58±0.10 ^a	19.43±2.29 ^a	100±5 ^a
TR055	42.38±2.58 ^b	n.d.	0.94±0.08 ^{ab}	n.d.	8.05±0.22 ^b	-
TR055 Iso	30.14±1.95 ^c	0.17±0.08 ^b	0.72±0.07 ^c	7.18±0.46 ^b	20.3±2.44 ^a	84±2 ^b

255 Values within a column that share a superscript are not significantly different from one another (p <
256 0.05); n.d., not detectable, < 0.5mM/100g

257 Furthermore, the glycaemic effect of the samples was determined with an *in-vitro* method. The
258 digestion of carbohydrate-containing food products affects blood glucose levels, also known as the
259 postprandial glycaemic effect. The GI is related to the type of carbohydrates and dependent on the rate
260 of digestion (Wolever et al., 2008). In fact, only glucose can be absorbed directly by the small
261 intestine and used for energy generation; other sugars, such as fructose and galactose must be
262 metabolised by the liver to glucose, or, in the case of sucrose, and most polysaccharides, must be
263 hydrolysed into their constituent monosaccharides before being metabolised further. Therefore, the
264 postprandial rise in blood glucose levels is lower for those carbohydrates. In the case of mannitol, or
265 other non-glycaemic carbohydrates such as dietary fibre and resistant starch, no effect on the blood
266 glucose level can be observed, since these are not digested in the small intestine (Östman et al., 2002) .
267 The GI of all samples was high, ranging from 64 to 76. Only the unfermented, glucose-isomerase-
268 treated sample had a slightly lower GI, due to the conversion from glucose to fructose, which has a
269 lower impact on the blood sugar level (Foster-Powell et al., 2002). Considering the GL on the other
270 hand, considerable differences were observed. The GL relates the GI to a portion size, representing
271 both quality and quantity of carbohydrates being consumed (Barclay et al., 2008). Hence, results
272 represent the impact on the blood sugar level after consuming 250 mL of sample. For the untreated
273 sample, a GL of 16.22 was determined. With fermentation, only a slight, insignificant, reduction was
274 obtained, to 14.40 and 14.09 for *L. citreum* TR116 and *L. brevis* TR055, respectively. A remarkable
275 reduction of more than a third was obtained for both the glucose-isomerase-treated, fermented
276 samples, bringing the GL down to almost the low range (<10) (Venn and Green, 2007). *L. citreum*
277 TR116 showed a value of 10.80 and *L. brevis* TR055 one of 10.43.

278 **Table 3** *In-vitro* glycaemic index, load, and reduction of glycaemic load of unfermented (Unf.),
279 isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and
280 *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.~~for *Leuconostoc citreum* TR116, and~~
281 ~~*Lactobacillus brevis* TR055 fermented quinoa based milk substitutes.~~

	Glycaemic index [-]	Glycaemic load [-]	Reduction of Glycaemic load [%]
Unf.	69.47±1.37 ^{bc}	16.22±0.70 ^a	-
Unf. Iso	64.57±1.11 ^c	14.12±0.51 ^a	9.89±1.90 ^b
TR116	76.51±0.22 ^a	14.40±0.72 ^a	14.68±4.08 ^b
TR116 Iso	73.04±1.61 ^a	10.80±0.22 ^a	34.45±2.91 ^a
TR055	70.82±0.60 ^{abc}	14.09±0.87 ^b	13.15±2.38 ^b
TR055 Iso	72.08±3.95 ^{ab}	10.43±0.58 ^b	36.71±0.64 ^a

282 Values within a column that share a superscript are not significantly different from one another (p <
283 0.05)

284 The depletion of about 20 mmol/100g glucose and ultimate bioconversion into the non-glycaemic
285 carbohydrate, mannitol, resulted in this substantial reduction of GL. Research studies strongly indicate
286 a correlation between high GI and GL and increased risk of type 2 diabetes, breast cancer, gallbladder
287 disease and heart disease, while low GI and GL diets show many health benefits i.e. weight control,
288 protection against colon and breast cancer, obesity, cardiovascular disease, and diabetes (Brand-Miller,
289 McMillan-Price, et al. 2009).

290 **3.3 Physicochemical properties**

291 Samples were analysed for physicochemical properties to assess their characteristics as a beverage. The
292 results are shown in table 4. Due to the drop in pH during fermentation, the samples were destabilized,
293 which is evident in the results for syneresis and separation rate; both values increased from 10.20 to
294 15.21% and from 13.34 to 21.86 %/h, respectively, due to fermentation of samples with *L. brevis*
295 TR055. No considerable differences between the fermented samples were found. The pH after
296 fermentation is close to the isoelectric point of quinoa proteins, being around 4 (Elsouhaimy et al.,
297 2015), resulting in a low solubility and destabilisation. As seen in the measurements of rheology, the
298 decrease of pH did not affect the viscosity and no significant differences were found between samples.
299 Unlike other proteins, such as casein in bovine milk, quinoa proteins do not facilitate a network-
300 forming matrix and gel strength is weak, as shown also by Mäkinen et al. (2014). However, in order to
301 compensate for the lack of network forming properties of the quinoa proteins, Zannini et al. (2018)
302 used an EPS-producing culture (*Weissella cibaria* MG1) to produce a quinoa-based yoghurt substitute.

303 The resulting yoghurt showed increased viscosity (> 0.5 Pa s), and improved water holding capacity,
 304 both due to the amounts of EPS produced. A combined fermentation with an EPS-producing strain
 305 could therefore overcome the rheological challenges, generating a product with multiple new
 306 functional properties. The samples showed very slight differences for the chromaticity and similar
 307 whiteness indices, ranging from 49.49 to 54.32. These values indicate a lower whiteness of the
 308 samples compared to bovine milk (81.89), but the values are similar to other commercial PBMS (Jeske
 309 et al., 2017).

310 **Table 4** Physicochemical properties of products of unfermented (Unf.), isomerase treated samples
 311 (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of
 312 quinoa-based milk substitutes, for ~~*Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055~~
 313 fermented quinoa-based milk substitutes.

	Separation rate [%/h]	Viscosity [mPa·s]	Syneresis [%]	Whiteness Index [-]
Unf.	13.34±1.08 ^c	5.92±0.89 ^b	10.20±1.86 ^b	49.49±1.57 ^d
Unf. Iso	15.04±0.82 ^{bc}	5.83±0.48 ^b	11.68±1.96 ^b	51.18±1.23 ^{cd}
TR116	18.25±2.53 ^{ab}	5.99±1.04 ^b	15.13±1.60 ^a	52.06±1.43 ^{bc}
TR116 Iso	18.19±1.41 ^{ab}	8.14±1.41 ^a	16.20±1.05 ^a	53.25±1.94 ^{ab}
TR055	21.86±2.14 ^a	6.36±1.20 ^a	15.21±1.56 ^a	53.53±0.77 ^{ab}
TR055 Iso	15.04±1.02 ^c	7.06±0.81 ^a	16.50±0.63 ^a	54.32±1.50 ^a

314 Values within a column that share a superscript are not significantly different from one another (p <
 315 0.05)

316 4. Conclusion

317 This study demonstrates a novel biotechnological processing approach to improve nutritional
 318 properties and meet consumer demands of PBMSs. The production of mannitol was examined in a
 319 quinoa-based milk substitute, using two LAB as starter cultures. It was shown that quinoa serves as a
 320 good substrate, facilitating the growth of *L. citreum* TR116 and *L. brevis* TR055 with high viability.
 321 The hydrolysis of starch, further conversion of glucose to fructose through the enzyme glucose-
 322 isomerase, and subsequent reduction of fructose to mannitol via fermentation, reduced the glucose
 323 content by 40% and GL by 35%. *L. citreum* TR116 and *L. brevis* TR055 could be used as novel

324 functional starter cultures and this approach can be transferred to any kind of fermented food product,
325 such as sourdough or beverages. This laboratory prototype could represent an example of novel
326 PBMSs, characterised by improved nutritional and functional properties and also by a lower carbon
327 and water footprint when compared to their dairy counterparts.

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438

Highlights

- Quinoa served as a good substrate, facilitating the growth of *L. citreum* TR116 and *L. brevis* TR055
- Exogenous amylolytic enzymes in combination with lactic acid fermentation enabled the reduction of glucose by 40%
- Mannitol was produced with high conversion factors from fructose
- Glycaemic load was reduced by more than a third