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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Optimization of lactic ferment with quinoa flour as bio-preservative alternative for packed bread

Andrea Micaela Dallagnol · Micaela Pescuma · Graciela Rollán · María Inés Torino · Graciela Font de Valdez

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Abstract The consumers' demand for food with high nutritional quality and free of chemical additives increases the need to look for new products and preservation strategies. Quinoa (*Chenopodium quinoa*) is an Andean pseudocereal highly appreciated because of its nutritional properties. Moreover, it is an optimal substrate for growing and production of improved amounts of antifungal compounds by *Lactobacillus plantarum* CRL 778. The aim of this work was to optimize a lactic ferment for packaged breads with improved nutritional value and prolonged shelf life by applying a statistical experimental design model. The addition of 30 % quinoa to the wheat semiliquid ferment (QWF) could highly improve the amino acids release (4.3 g/L) during fermentation. Moreover, this quinoa proportion was sufficient to obtain the same concentration of the antifungal compounds, phenyllactic and hydroxiphenyllactic acids (PLA and OH-PLA) as with 100 % quinoa (ca. 36 and 51 mg/L, respectively). Statistical model analysis showed that citrate and skimmed milk enhanced significantly all evaluated parameters specially PLA (ca. 71 mg/L), HO-PLA (ca. 75 mg/L), and lactate (27 g/L) with a p value <0.005. The synergic effects of higher antifungal compounds production, acid release, and pH decrease allowed lowering the amount (about 50 %) of the chemical preservative calcium propionate commonly added to bread. Moreover, these breads show increased shelf life.

Keywords Lactic antifungals · RSM methodology · Packed bread

A. M. Dallagnol (✉)
Laboratorio de Microbiología de Alimentos y Biotecnología, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Argentina
e-mail: andymicd@gmail.com

M. Pescuma · G. Rollán · M. I. Torino · G. F. de Valdez
Centro de Referencia para Lactobacilos (CERELA)-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina

G. F. de Valdez
Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

Introduction

Sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (LAB) and yeasts (De Vuyst and Vancanneyt 2007); their properties depend on the production of organic acids (lactic and acetic acids) and the proteolytic activity of LAB during the fermentation process. The use of sourdough offers many advantages over baker's yeast, such as enhanced flavor, prolonged preservation, and improved dough structure (De Vuyst and Neysens 2005). In fact, sourdough bread has an extended mold-free storage-life compared to conventionally leavened products (Salovaara 2004; Smith et al. 2004). The presence of certain metabolites produced by specific LAB strains is responsible of the prolonged storage-life of sourdough bread (Coda et al. 2008; 2011; Ryan et al. 2011). For this reason, LAB are promising alternatives to chemical preservatives in bakery, as antimicrobial compounds produced by these microorganisms are able to suppress food-borne yeasts and molds (De Muynck et al. 2004).

The antimold activity of LAB in sourdough has been generally attributed to the presence of organic acids, particularly lactic and acetic acids (Rocken 1996; Cabo et al. 2002). In recent years, other low-molecular-weight compounds such as cyclic dipeptides (L-Phe–L-Pro and L-Phe–trans-4-OH-L-Pro),

phenyllactic acid (PLA), hydroxyphenyllactic acid (OH-PLA), propionic acid, coumaric acid, phenylpropanic acid, 2-methylcinnamic acid, salicylic acid, and sodium decanoate have been identified as major antifungal metabolites of sourdough lactobacilli (Lavermicocca et al. 2000; Ström et al. 2002; Schnürer and Magnusson 2005; Zhang et al. 2010; Ryan et al. 2011). However, these metabolites are either not produced in effective quantities in sourdough fermentations or adversely affect the quality of the product when produced in active concentrations. Nevertheless, the antifungal activity of metabolites from LAB in bread, to date, has not been attributed to a single compound, but rather to their synergistic activity (Black et al. 2013).

The sourdough strain *Lactobacillus plantarum* CRL 778 has antifungal properties against the major bread contaminant fungi *Aspergillus*, *Fusarium*, and *Penicillium*, mainly because of the production of PLA and acetic acid (Gerez et al. 2009). It has been reported that the synthesis of PLA by *L. plantarum* CRL 778 in a chemically defined medium (CDM) could be improved with high amounts of phenylalanine (Phe) while the production of both, PLA and OH-PLA increased by co-metabolism of glucose with citrate (Dallagnol et al. 2011). Citrate would act as an external electron acceptor stimulating the glutamate dehydrogenase (GDH) activity. The GDH enzyme is necessary for α-ketoglutarate production and the amino acids transamination, which is the bottleneck for the production of PLA and OH-PLA, in LAB (Vermeulen et al. 2006). Citrate metabolism also increases the formation of acetic acid by *L. plantarum* CRL 778.

L. plantarum CRL 778 has a good proteolytic activity on wheat protein (Gerez et al. 2006; 2012). Its ability to grow in quinoa's (*Chenopodium quinoa*) flour and to synthesize antifungal compounds was previously studied (Dallagnol et al. 2013). The production of these antifungal was related to the proteolytic activity of the strain and the consequent release of Phe and tyrosine (Tyr), which are sources for the synthesis of PLA and OH-PLA, respectively (Valerio et al. 2004). Quinoa is relatively new to the American market and has recently received much attention. Actually, it is referred to as an "ancient grain" and the "mother grain" of the Incas. The use of quinoa's flour in the bakery industry, i.e., sourdough technology, is interesting because of the nutritional quality of this grain. However, the application of the crop in this area is limited because of its high cost of market (FAO). These drawbacks can be solved using the least amount of quinoa producing the desired effects; about 10–20 % according to different authors (Lorenz and Coulter 1991; Enriquez et al. 2003). In the light of our findings, this study was focused to optimize a bio-preservative lactic starter based on quinoa/wheat flour by applying an experimental design statistical model. Classical optimization methodology involves the optimization of a single factor while keeping others. This method is not very suitable for optimization of multiple factors, not only the time required but also difficult to detect interaction effects between variables under study (Kammoun et al. 2008). This limitation is avoided by using statistical experimental design, a set of experimental strategies, mathematical methods, and statistical inferences that explains the interactions, reducing variability, time, and costs (Liu and Tzeng 1998; Ren et al. 2008; Yu et al. 2008; Pan et al. 2008).

Material and methods

Lactic acid bacteria strain

Lactobacillus plantarum CRL 778 was isolated from home-made wheat dough and belongs to the Culture Collection (CRL) of Centro de Referencia para Lactobacilos (CERELA), Tucumán-Argentina. This LAB was grown in MRS broth at 37 °C for 16 h and transferred twice before each test.

Fermentation conditions

Assessment of quinoa and wheat flour ratio—Cells of *L. plantarum* grown in MRS were harvested by centrifugation at 8000*g* for 10 min, washed twice, and suspended in sterile distilled water. This cell suspension (ca. 3×10^9 cfu/mL) was inoculated at 2 % (*v/v*) in various semiliquid dough (slurries) prepared by mixing tap water (200 g); food-grade anhydrous dextrose (2 g, Adama SA, Argentina) and flour (100 g) consisting of different ratios of quinoa/wheat flours as follows (%): 0/100, 10/90, 20/80, 30/70, 40/60, 50/50, and 100/0 and incubated at 30 °C for 24 h. Wheat flour (000 type, Molinos Florencia, S.A., Argentina) was purchased in the market whereas quinoa flour was prepared in the laboratory using commercial quinoa seeds (*Chenopodium quinoa* Wild) from Bolivia country. The seeds were washed several times with cold water to remove saponins until there was no foam in the wash water; then, they were dried at 48 °C for 24–48 h until reaching a water activity of 0.30–0.35, which was measured with a water activity meter (AquaLab LITE, Decagon, USA). Finally, the quinoa seeds were ground and sifted to obtain the flour.

Obtained slurries were adjusted to pH=6.0 with NaOH 2 N and fermented (30 °C, 24 h) under soft stirring condition (Shaker Vicking model Dubnoff, Argentina). At the beginning and the end of fermentation (24 h), samples were withdrawn for analytical methods (see below). Assays were determined in two independent experiments, and mean values±standard deviation (SD) are given. Data were compared by analysis of variance (ANOVA) and Fisher test using Minitab 12 software.

Experimental design for optimization of cultures (slurries)—The effect of different parameters (independent variables) on growth and antifungal production by

L. plantarum CRL 778 was assessed in slurries prepared as was described above. In this case, however, only the preselected quinoa/wheat ratio was used as flour.

Four factors including initial pH (IP), addition of potassium phosphate buffer K_2HPO_4/KH_2PO_4 (PB), sodium citrate (C), and skim milk (SM) were evaluated at three levels each one (%, *w/w* of flour). The IP at 5.5, 6.0, and 6.5 % (adjusted with NaOH 2 N); PB (Cicarelli, Argentina) and C (Sigma, USA) at 0.0, 0.2, and 0.4 %; and SM (La Serenísima, Argentina) at 0.0, 2.5, and 5.0 %.

Buffer concentrations were chosen taking into account the ones present in the LAB growth medium such as MRS and CDM (Hébert et al. 2004). Citrate was added because of its capacity to improve the synthesis of PLA and OH-PLA (Dallagnol et al. 2011), the maximum amount tested corresponding to that allowed by the Argentinean Food Code. Finally, milk was considered to stimulate *L. plantarum* CRL 778 growth (Gerez et al. 2010).

A complete 2^4 factorial design with center point was used to arrange experiments and to determine the interaction between variables (see statistical analysis). Assays were made in duplicate, which means that 40 ferments were evaluated in eight blocks of trials with eight replicates of the center point.

The factorial design with center point was carried out by means of Design-Expert software (version 7.0, Stat-Ease, USA). To determine the relationship between independent variables (factors) and responses, a *multiple-regression* analysis of the data was carried out by STATISTICA software (version 7.0, StatSoft, Tulsa, OK, USA), generating the corresponding response surface graphs. For each response (viability, final pH, lactic acid, acetic acid, PLA, and OH-PLA production), it was applied a model adjusted to the following linear equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum\sum \beta_{ij} x_i x_j \quad \text{with } i \neq j$$

where Y_i represents the value of expected response (experimental data); β_0, β_i, and β_{ij} are parameters of the model; x_i and x_j are cods of the studied factors; and k is the number of analyzed factors. This model considers the main effect of each factor and the interaction effects between the factors.

For a better understanding of the obtained results with the experimental design, the following concepts are introduced: (i) the main effect of a factor indicates the average variation of a measured response when the level of the factor changes, the others held constant; (ii) the interaction between factors indicates that the effect of one factor varies according to the value taken by another factor. The interaction can be positive (increasing) or negative (decreasing). In the addition, the effect is an apparent interaction, but this is not significant ($p>0.05$); and (iii) the desirability function involves the transformation of data values for each variable in desirability, which vary in a range from 0.0 (undesirable value) to 1.0 (very desirable value). An advantage of this feature is that it allows representation of expected values for multiple dependent variables in a single response surface.

Analytical methods

Responses measured (dependent variables) included growth, final pH, and production of lactic acid, acetic acid, and OH-PLA PLA.

Growth and pH—Ferment (1 mL) was suspended in 9 mL of sterile physiological solution and homogenized in vortex. For LAB cell counts, aliquots (0.1 mL) in serial 10-fold dilutions from each homogenate were spread on MRS agar with 0.01 g/L cycloheximide to inhibit yeast growth. To support the fact that the counted LAB cells corresponded to *L. plantarum* CRL 778, a naturally antibiotic [streptomycin (Sp), spectinomycin (St)]-resistant strain of *L. plantarum* CRL 778^R previously obtained (Dallagnol et al. 2013) was grown in a slurry containing quinoa and wheat in a similar way than the wild-type strain. The resistant strain was used only for comparison and was corroborated that the obtained cell count after the fermentation was that of the obtained with the wild type.

The pH value was determined using a Sartorius portable meter PT-10 model (Sartorius AG, Goettingen, Germany) equipped with a puncture electrode (Hanna Instruments, Woonsocket, RI).

Peptide assessing—Peptide profiles were evaluated by reverse-phase high-performance liquid chromatography (RP-HPLC) as previously reported (Dallagnol et al. 2013). Peptide picks were expressed as arbitraries united (AU).

Total and individual free amino acids—Total free amino acids were assessed by the cadmium-ninhydrin (Cd-Ninhydrin) method while individual free amino acids by RP-HPLC as previously reported (Dallagnol et al. 2013). Results were expressed in grams per liter.

Organic acids and antifungal compounds—Lactate, acetate, PLA, and OH-PLA were determined by HPLC in ferments as previously reported (Dallagnol et al. 2013) and expressed in grams per liter (lactate and acetate) and milligrams per liter (PLA and OH-PLA).

Bread making and bio-preservation assay

For this, control bread dough was made by mixing 600 g of commercial wheat flour 000 type (Molinos Florencia, SA), 12 g of dried yeast (Levex, Chile), 12 g of salt (Dos Anclas, Chile), 18 g of baking margarine (Dánica Dorada, Argentina), 18 g of dried skim milk (Verónica, Argentina), and 360 mL of tap water for 10 min with a spiral mixer (Santini Dal 1918, Argentina). Then, bread dough was divided into 10 loaves (100 g each) and molded manually. The loaves were leavened

at 30 °C (2 h) and baked at 180 °C (38 min). To evaluate the effect of ferments in bread dough, different percentages (10, 20, and 40 % *v/v*) of baking tap water were replaced by equal amounts of ferment and processed in a similar way to the control bread dough. The bio-preservative effect of ferment was also tested in bread prepared with suboptimal concentrations (0.2 and 0.3 % *wt/wt*) of calcium propionate (CP), a chemical preservative usually employed in packaging bread.

The pH of dough before and after leavening period was determined using a Sartorius portable meter PT-10 model (Sartorius) equipped with a puncture electrode (Hanna Instruments). Cooked breads were cooled (1 h) in an open room to allow ambient fungal contamination. After that, the loaves were stored in plastic bags at 32±2 °C for 15 days for shelf-life evaluation. The bread shelf-life was defined as the time (in days) for molds to become visible on the surface of the packaged loaves. Observations were performed daily, and results were expressed as percentage of contamination.

Results

Quinoa/wheat ratio

Seven semiliquid ferments using *L. plantarum* CRL 778 and different quinoa/wheat ratio were developed (Fig. 1). Results at 24 h incubation showed that *L. plantarum* CRL 778 growth increased from 8.6 to 9.8 log cfu/mL as quinoa concentration in the ferments increased from 0 to 100 %. The pH values were similar in all ferments (3.35–3.73) although the lactic acid concentration increased significantly from 12.9 to 22.5 g/L in ferments 1 to 7, respectively (Fig. 1). The effect of different quinoa/wheat ratio on the acetic acid production was less pronounced (from 0.62 to 0.97 g/L) compared to lactic acid production. The biosynthesis of PLA and OH-PLA increased significantly (about 2.5 folds) by the addition of 30–40 % of quinoa flour (ferments 4 and 5); however, a higher quinoa concentration (ferments 6 and 7) produced no additional changes on the antifungal compounds production.

On the bases of these results, we selected the ferment 4 which consists of 30/70 % of quinoa/wheat ratio and from now on named as quinoa/wheat ferment (QWF). This ferment had the minimal quinoa concentration able to stimulate significantly the PLA production by CRL 778 strain, the most important antifungal compound analyzed in this work.

Proteolytic activity during fermentation of QWF by *L. plantarum* CRL 778

In order to asses if the synthesis of PLA and OH-PLA was associated with proteins hydrolysis and consequent release of peptides and antifungal amino acids precursors, Phe and Tyr, 0.1 % trifluoroacetic acid-soluble peptides (≤3 kDa) in QWF were determined by RP-HPLC, before and after culturing CRL 778 strain (Fig. 2). The analysis of this peptide profiles showed that about 30 peaks were present at the beginning of fermentation (T0) while more than 42 peaks were present after fermentation period (T24). However, the areas of some of them were too small to be accurately determined. Figure 2a shows the obtained peptide profiles where several peaks whose heights (mm) were compared are listed. After 24 h, absent peptides at the beginning of fermentation increased widely and originated peaks 1 (24.0 mm), 3 (14.5 mm), 6 (4.4 mm), 7 (28.6 mm), and 12 (4.0 mm). Additionally, peptides present at T0 that corresponded to peaks 5, 10, 11, 13, and 14 showed an increase in size between 45 and 88 % at T24. On the other hand, peaks 2, 4, and 8 showed, respectively, a decrease of 65, 99, and 100 % in height, suggesting a significant degradation during fermentation.

Amino acid content also varied significantly after fermentation. The amount of total free amino acids increased four times with respect to T0, reaching 4.3 g/L after 24 h

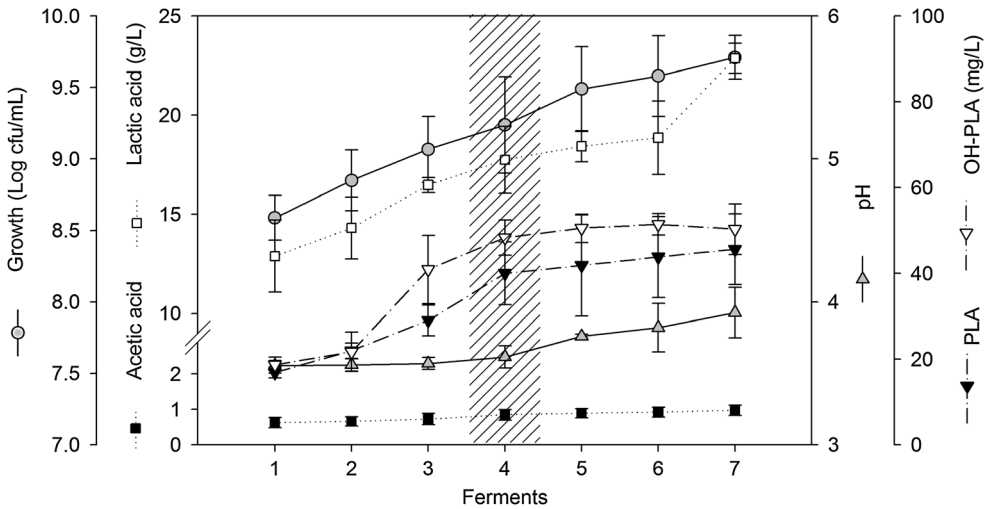

Fig. 1 Effect of quinoa/wheat ratio on growth and metabolite production by *L. plantarum* CRL 778 in semiliquid ferment (30 °C, 24 h). Ferments contained the following quinoa/wheat ratio (%, *w/w*): 1, 0/100; 2, 10/90; 3, 20/80; 4, 30/70; 5, 40/60; 6, 50/50; and 7, 100/0

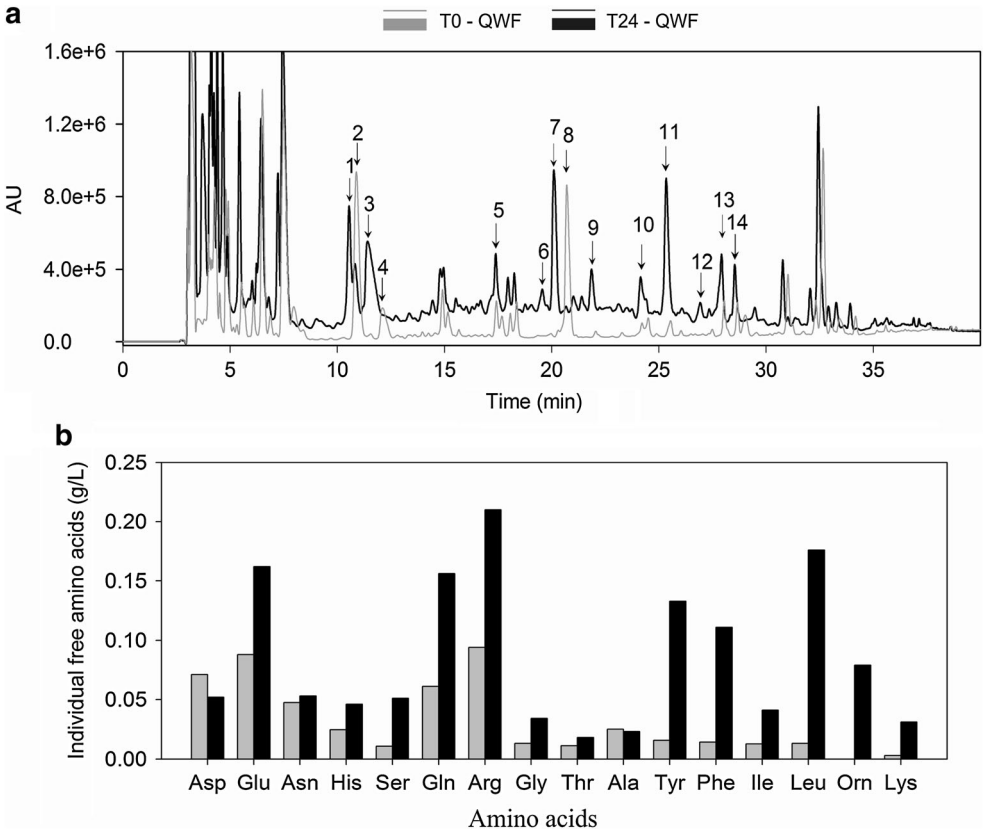

Fig. 2 Peptide profiles (**a**) and individual free amino acids (**b**) released in quinoa/wheat ferment (QWF) at the beginning (T0) and end (T24) of the fermentation period

incubation. Phe and Tyr increased approximately 10 times, being observed values of 0.11 and 0.13 g/L at the end of fermentation, respectively (Fig. 2b). Interestingly, some essential amino acids were also increased, observing at T24 a higher amount of leucine (Leu, 0.17 g/L), isoleucine (Ile, 0.041 g/L), and lysine (Lys, 0.031 g/L) than those determined at T0. Moreover, some nonessential amino acids as arginine (Arg, 0.21 g/L), glutamate and glutamine (Glu and Gln, 0.16 g/L), as well as ornitine (Orn, 0.079) were released.

Optimization of QWF

To optimize multiple factors, a 2^k factorial central point statistical experimental design was used, which would allow simultaneously seeing the effects of k factors on a response and find possible interactions between them. Factors or independent variables were citrate (C), skim milk (SM), initial pH (IP), and phosphate buffer (PB); each of them employed at three levels, including zero (see Material and methods). Responses measured (dependent variables) were growth, final pH, and production of lactic acid, acetic acid, and specific antifungals PLA and OH-PLA.

Table 1 shows the coefficients obtained from factor analysis of variance (ANOVA). p values, which indicate the significance ($p<0.05$) for each independent variable, were also used as a tool to select the response surface plots displaying the results of the analysis.

The results showed that, overall, the main effect of all factors evaluated was significant ($p<0.05$) for at least two or more dependent variables (Table 1). Conversely, the interaction effects were not significant although an additional effect ($p>0.05$) was observed in most cases. Only the C and PB combination showed a statistically significant negative interaction (effect=−0.16) ($p=0.001$) on growth. This interaction is shown in Fig. 3, where it can be observed that citrate, by itself, had the maximum effect, being unnecessary, the addition of buffer.

The statistical analysis of the results obtained for growth, final pH, and lactic acid production in QWF indicates that these variables were similarly affected by the factors tested. This allowed the desirability function to represent the three variables (growth, final pH, and lactic acid) in the same surface response. Figure 4 shows desirability surfaces obtained for the following combinations: C and SM, C and PB, C and IP, SM and PB, SM and IP, and PB and IP. Figure 4a shows that in increasing the level of C (0.0 to 0.4 %), a desirability value of 0.62 is reached, and that increasing the level of SM (0.0 to 5.0 %) leads to a desirability value of 0.46. The presence of C and SM at peak levels shows an additive effect ($p>0.05$) with a desirability value of 0.75. A similar analysis of the six graphs obtained allowed to conclude that C is the

Table 1 Effects of evaluated factors on each response in quinoa/wheat ferment according to the surface of the extracted response model

Factor	Cell viability				pH				Lactic acid				Acetic acid				PLA				OH-PLA			
	Eff.	SE	*t* value	*p* value	Eff.	SE	*t* value	*p* value	Eff.	SE	*t* value	*p* value	Eff.	SE	*t* value	*p* value	Eff.	SE	*t* value	*p* value	Eff.	SE	*t* value	*p* value
Int.	2.18	0.04	57.13	0.000	3.59	0.02	212.91	0.000	257.97	5.22	49.44	0.000	16.11	0.49	32.80	0.000	0.27	0.01	23.14	0.000	0.27	0.01	22.71	0.000
C	0.23	0.04	5.88	0.000	0.13	0.02	7.41	0.000	36.72	5.35	6.87	0.000	3.37	0.50	6.70	0.000	0.08	0.01	6.37	0.000	0.05	0.01	4.22	0.000
SM	0.13	0.04	3.30	0.003	0.06	0.02	3.55	0.002	16.94	5.38	3.15	0.004	2.19	0.51	4.32	0.000	0.06	0.01	4.76	0.000	0.05	0.01	3.77	0.001
PB	0.13	0.04	3.47	0.002	0.04	0.02	2.19	0.039	11.27	5.22	2.16	0.041	0.18	0.49	0.36	0.722	0.00	0.01	0.17	0.869	0.00	0.01	0.32	0.755
IP	0.03	0.04	0.70	0.488	0.09	0.02	5.08	0.000	19.51	5.22	3.74	0.001	−2.05	0.49	−4.18	0.000	0.01	0.01	0.61	0.549	0.02	0.01	1.65	0.113

Eff. effect, *SE* standard error, *Int.* intercept, *C* citrate, *SM* skim milk, *PB* phosphate buffer, *IP* initial pH

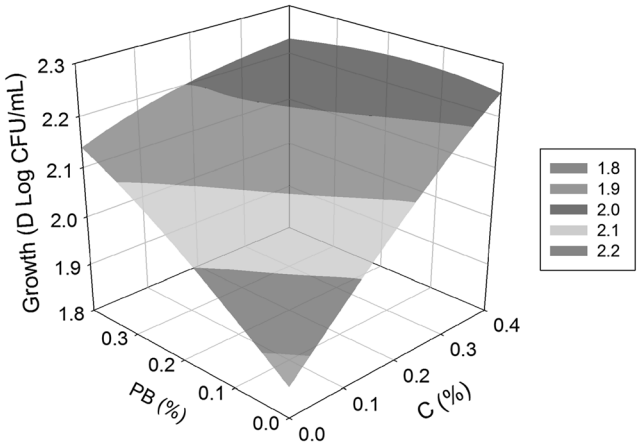

Fig. 3 Response surfaces showing the growth of CRL 778 strain in quinoa/wheat ferment (30 °C, 24 h) under the influence of citrate (C) and phosphate buffer (PB)

most important factor to improve growth and synthesis of lactate in the QWF, and that the combination of C and IP at maximum levels (0.4 and 6.5, respectively) leads to the highest value of desirability.

Acetic acid synthesis was significantly affected by three factors: C, SM, and IP (Fig. 5). The latter variable had an inverse effect compared to the other variables assessed, i.e., with increasing IP from 5.5 to 6.5, decreased acetate production (Fig. 5b). Thus, the highest concentration of acetate was obtained with the highest levels of C and SM to an IP of 5.5.

The production of both, antifungal PLA and OH-PLA, was significantly ($p<0.001$) affected by C and SM (Fig. 6). The main effect of these factors on PLA was higher (0.06–0.08) than that on OH-PLA (0.05); however, the shape of the Fig. 6b panel suggests a positive interaction ($p=0.051$) despite being in statistical limits for addition and synergy, with intermediate values fitting into the first one.

Based on these results, optimization of QWF (from henceforth, QWF_{Op}) was achieved with the maximum concentrations of C (0.4 %) and SM (5 %), whereas both compounds were the main factors that allowed to optimize all parameters evaluated, especially the synthesis of PLA, PLA-O, and acetate. The IP remained at 6.0 without adjustment, and the addition of buffer is rejected, producing no significant effect on the synthesis of an antifungal and showing a negative interaction on growth when combined with C (Fig. 3).

Results obtained in QWF and QWF_{Op} are summarized in Table 2. It can be shown that PLA was the compound highly increased although lactate, OH-PLA and acetate were also improved.

Bio-preservative effect on packaged breads

Different concentrations of QWF or QWF_{Op} (10, 20, and 40 % *v/v*) and CP (0.2 and 0.3 % *w/w*) were analyzed in baked breads made at a laboratory scale (Fig. 7). The breads prepared

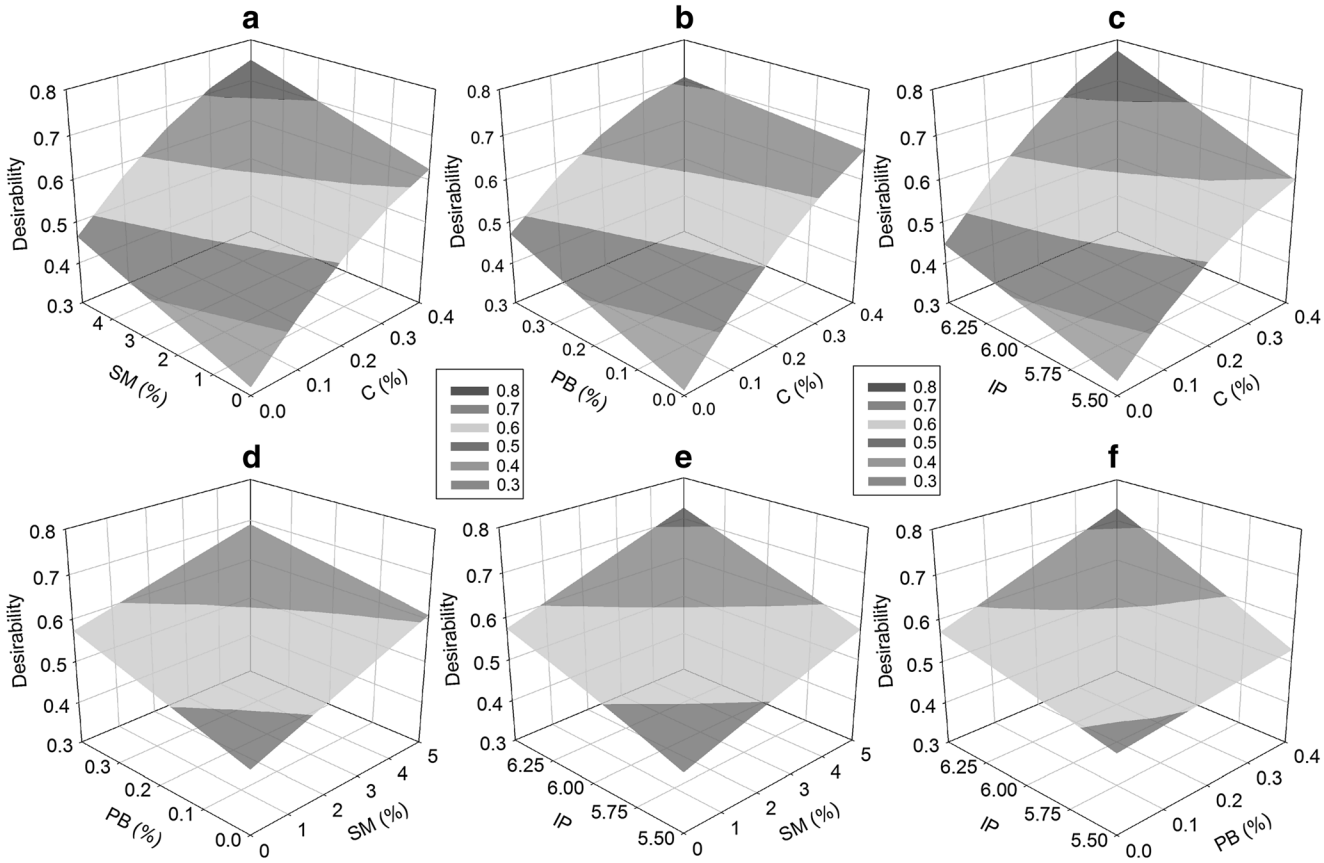

Fig. 4 Response surfaces showing desirability values for growth of CRL 778 strain, final pH, and lactic acid in quinoa/wheat ferment (30 °C, 24 h) under the influence of citrate (C), skim milk (SM), phosphate buffer (PB), and initial pH (IP). **a** C and SM, **b** C and PB, **c** C and IP, **d** SM and PB, **e** SM and IP, and **f** PB and IP

with 40 % *v/v* of QWF or QWF_{Op} increased their shelf life 24–48 h with respect to the control (without ferment or CP) which remained unspoiled only 4 days. The addition of CP 0.2 and 0.3 % could increase the shelf life of control breads up to 7–8 days at room temperature. Moreover, the addition of QWF:CP in different ratios (40:0.2, 40:0.3, and 20:0.3 %) could prevent bread spoilage for 15 days. Interestingly, the use of QWF_{Op} allowed diminishing the ferment concentration to the half, selecting a final ratio in ferment:CP of 20:0.2 %.

Discussion

The consumers' demand for food with high nutritional quality and free from chemical additives increases their needs to look

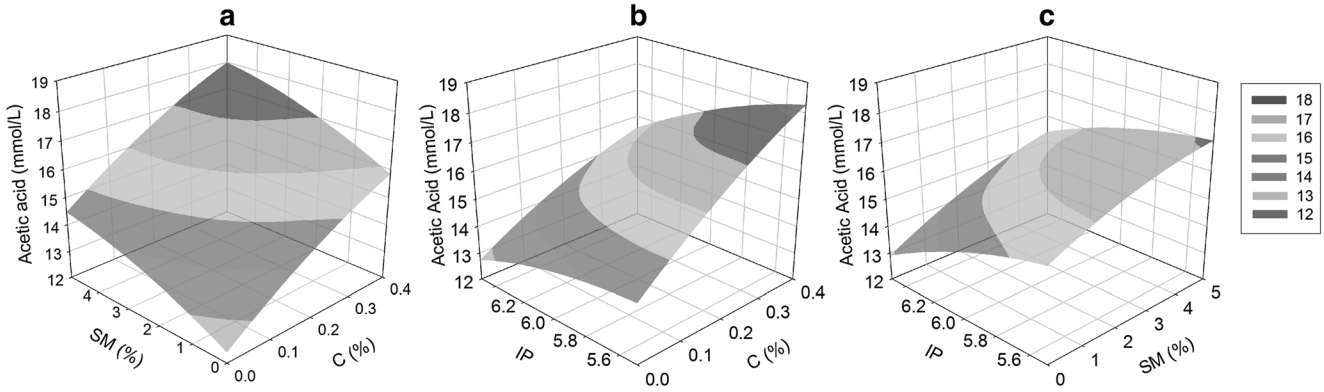

Fig. 5 Response surfaces showing the acetic acid production in quinoa/wheat ferment (30 °C, 24 h) under the influence of citrate (C), skim milk (SM), and the initial pH (IP). **a** C and SM, **b** C and IP, **c** SM and IP

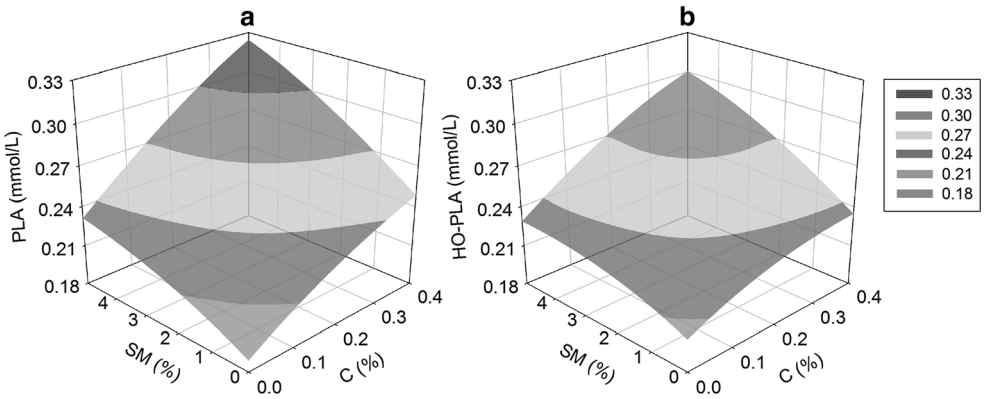

Fig. 6 Response surfaces showing the antifungal compound production in quinoa/wheat ferment (30 °C, 24 h) under the influence of citrate (C) and skim milk (SM). **a** PLA production, **b** OH-PLA production

for new products and preserving strategies, which have to be economically viable. The addition of quinoa to the ferment containing wheat flour (WF) increased its nutritional value as assessed by the higher amount of total amino acids observed in QWF (4.3 g/L) compared with that observed for wheat (0.6 g/L, WF), being this value similar to that reported previously for quinoa ferment (5.0 g/L, QF) (Dallagnol et al. 2013). Moreover, the amino acids profile also resembled to that obtained for QF. Interestingly, Leu concentration was 10 times higher in QWF than that reported for WF. This fact could be due to the proteolytic activity of quinoa proteases over wheat proteins since Leu, being the third more abundant amino acid in wheat (Rombouts et al. 2009), was not observed in WF at high quantities. Orn and Phe were also detected in higher quantities in QWF than in WF; these amino acids together with Leu are important precursors for aroma compounds during bread cooking (Schieberle 1990; Gassenmeier and Schieberle 1995).

At 30 % of quinoa flour in QWF_{Op}, the organoleptic characteristics of the corresponding breads seemed as good as those obtained with ferment WF, which could suggest a prevailing influence of the lactic acid on the sensitive attributes in the baked model (Gobbetti et al. 1995; Spier et al. 2007). Indeed, breads containing either QWF_{Op} or WF had a pleasant particular bitter taste and a higher volume than breads without lactic ferment. In order to determine a more precise sensory profile of the bread samples, a panel of trained assessors is need. Alternatively, the model of breads baked with the preservative ferments might include different levels of quinoa flour in their recipe.

Table 2 Biochemical characteristics of quinoa/wheat (30/70 %) ferment before (QWF) and after optimizing (QWF_{Op})

Parameters	Ferment		R^1=QWF_{Op}/QWF
	QWF	QWF_{Op}	
Growth (Δ log cfu/mL)	1.85±0.10	2.31±0.11	1.25
pH	3.58±0.06	3.68±0.11	1.03
Lactic acid (g/L)	17.43±1.82	27.13±0.04	1.56
Acetic acid (g/L)	0.84±0.03	1.03±0.01	1.22
PLA (g/L)	0.036±0.003	0.071±0.002	2.00
OH-PLA (g/L)	0.051±0.007	0.075±0.002	1.46

[1] Relationship between ferments that indicates the increase obtained with the optimization process

This work demonstrated that CP could be partially replaced by a ferment containing quinoa for extending shelf life of packed bread; *L. plantarum* CRL 778 could release the antifungal compounds PLA and OH-PLA in a mixed wheat/quinoa ferment containing 30 % of quinoa at similar concentrations to those observed for 100 % quinoa ferments (Dallagnol et al. 2013). This fact is partially due to the higher amount of antifungal precursors, Phe and Tyr, in QWF compared to WF, which is directly associated to the presence of quinoa (Fig. 1). However, PLA or OH-PLA did not increase in QWF containing percentages of quinoa greater than 40 %, even though Phe and Tyr concentrations were almost two times higher in 100 % quinoa ferments (Dallagnol et al. 2013). This fact indicates that other limiting factors are involved in the synthesis of these antifungal compounds. The α-keto acids are the amino group acceptors during transamination of Phe and Tyr, which is the bottleneck in the biosynthetic pathway of PLA and OH-PLA (Vermeulen et al. 2006). Indeed, the bioavailability in α-ketoglutarate is a key factor of the pathway since it is the major acceptor of amino groups in LAB (Tanous et al. 2002). α-Ketoglutarate is synthesized by deamination of Glu and Gln throughout the enzyme glutamate dehydrogenase (GDH) (Vermeulen et al. 2007). Since Glu and Gln were detected in all fermentations, the enzyme GDH responsible of deamination and the redox condition of the cell could be the cause of the low conversion of Tyr and Phe to PLA and OH-PLA. The presence of an electron acceptor is fundamental for Glu deamination by GDH. It was already established that citrate could be used by *L. plantarum* CRL 778 as an alternative electron acceptor when grown in a chemically defined medium (Dallagnol et al. 2011). In this work, we showed that the addition of citrate or skim milk to QWF produced a higher amount of PLA and OH-PLA. Vermeulen et al. (2006) obtained greater amounts of these antifungals in sourdough only when α-KG was added.

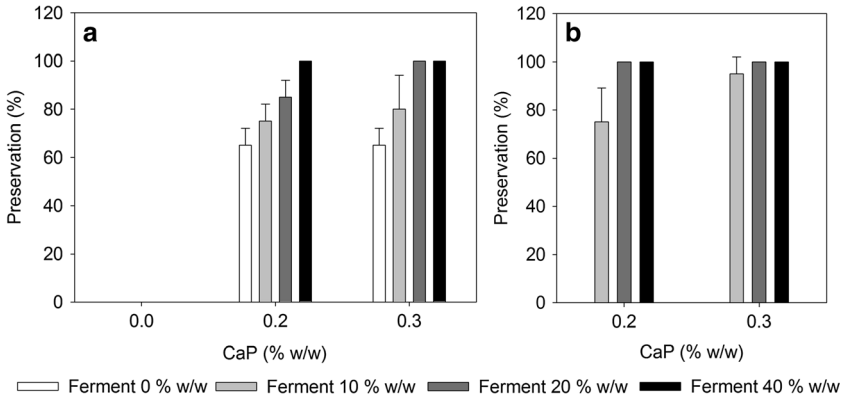

Fig. 7 Bio-preservative effect of QWF (**a**) and QWF_{Op} (**b**) in packaged breads made with calcium propionate (CP) and stored by 15 days at room temperature

Phenyllactic acid and, in lesser extent, OH-PLA are known as efficient antimicrobials, the first one having a fungicidal activity of 3.5 to >10 g/L, depending on the mold's species and medium pH (Ström et al. 2002; Lavermicocca et al. 2003). Other compounds such as lactic and acetic acids can prevent bread from spoilage although their inhibitory activity is lower than PLA. Gerez et al. (2009) reported that the concentrations necessary to inhibit 50 % conidia germination of *Aspergillus niger* at pH 3.5 were about 0.011, 1.1, and 16.2 g/L for PLA, acetic acid, and lactic acid, respectively. In response-surface models (RSM) to optimize QWF, high initial pH values and the addition of phosphate buffer improved lactate production. On the opposite, acetate production was greater with lower initial pH; this could be attributed to a higher citrate catabolism at low pH as it was established for LAB (Palles et al. 1998).

L. plantarum CRL 778 is a facultative heterofermentative strain that produced scarce acetic acid under the conditions evaluated. Co-fermentation with an obligate heterofermentative lactobacillus (*Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus brevis*) could be considered for increasing the amount of this acid in QWF_{op} and improving their antifungal effect and flavor (reviewed by Corsetti and Settanni 2007). The acetic acid is furthermore important as the growth of spoilage rope causing bacilli (*Bacillus subtilis*) is inhibited by high acetic acid concentrations. Besides, acetic acid is more volatile than lactic acid; thus, its impact on the flavor is more pronounced than that of lactic acid. In traditional, type I sourdough with continuous propagation (such as French and US sourdough process, and German rye sourdoughs), heterofermentative lactobacilli, especially *L. sanfranciscensis*, are dominating the fermentation (Corsetti 2013).

The use of chemical preservatives as CP has shown to be only partially effective on preventing bread spoilage causing 20 and 40 % annual production losses (Ryan et al. 2008; Gerez et al. 2010). Moreover, it has been demonstrated that CP is not sufficient to prevent spoilage for long storage periods (Lavermicocca et al. 2000; Ryan y col., 2008). In this work, we showed that the use of CP plus QWF_{Op} could increase the shelf life of bread. This effect can be attributed to several factors such as the production of PLA, OH-PLA, lactic, acetic, and propionic acids by *L. plantarum* CRL 778. The lower pH of the obtained breads using QWF_{Op} could also contribute to enhance PC action by lowering the pH of dough bread pH from 5.3–5.4 to 4.7–4.9 thus maintaining it near of the PC's pKa value.

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