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BEHAVIOR OF THE COMPLEX MICRO-ECOLOGY IN MAIZE AND RYE FLOUR AND MOTHER-DOUGH FOR *BROA* THROUGHOUT STORAGE

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ABSTRACT

Besides water, maize and rye flour are the main constituents of *broa* – a unique sourdough bread, manufactured following traditional protocols at the farm level in Portugal. Mother-dough, i.e., a piece of leavened dough kept aside from batch to batch under refrigeration conditions, constitutes the only starter culture used throughout breadmaking. Maize and rye flour, as well as mother-dough, were accordingly assayed for their microbiological profiles throughout storage time, to characterize the evolution in viability of the adventitious microorganisms: total viable counts, as well as viable yeasts, molds, gram-negative rods, gram-positive rods (endospore-forming and nonsporing) and gram-positive cocci (catalase^{+}and catalase^{-}). In general, all microbial groups exhibited an outstanding resistance to storage, so use of mother-dough appears technologically effective in this form of breadmaking, and an appropriate storage of flour does not convey any important changes to their microbiological profile.

PRACTICAL APPLICATIONS

Broa is a traditional sourdough bread made of maize (*Zea mays*) and rye (*Secale cereale*) flour. It is widely manufactured at farm level in Northern Portugal following ancient manufacturing procedures, and has earned the food specialty status (with an Appellation d'Origine Protégée label already granted). This research is expected to contribute to a better understanding of its microbiological dynamics (and related chemistry), thus effectively supporting health claims associated with its consumption and rational optimization of its

technological process – both of which, in turn, will help to expand its market and economic value. The practical purpose of this research is to study the behavior of microflora during the storage period of mother-dough and flour samples for *broa*. To date, no research work had specifically tackled on this topic, yet the importance of this traditional specialty bread in the Portuguese economy fully justifies allocation of resources to elucidate the effects of processing on the final product.

INTRODUCTION

Maize, and other cereals such as sorghum and millet, is typically employed in a variety of fermented cereal-based foods, especially in Africa, and also in the manufacture of alcoholic and non-alcoholic beverages, gruels and porridges, dump lings (used in stews) and fried products. Examples of said products are *ogi* and *agidi* – in Nigeria, *koko*, *akassa* and *kenkey* – in Ghana, *uji* – in East Africa, *ogi* and *mawè* – in Benin, *mahewu* – in Southern Africa, *pozol* – in Mexico, *poto-poto* – in Congo, *aseda*, *nasha*, *marisa*, *hullu-murr*, *asedat-damirga*, *nasha-beida* and *kisra-beida* – in Western Sudan, and *dosai*, *appam*, *rabadi* and *ambali* – in India (Antony *et al*. 1996; Abdalla *et al*. 1998; Salovaara 1998; Ampe and Miambi 2000; Annan *et al*. 2003; Osman *et al*. 2010). In Portugal, maize flour is used in combination with rye flour, salt and water to produce *broa* – a unique home- baked sourdough bread (Rocha and Malcata 1999, 2012; Rocha 2011).

Rye is, in turn, the most important cereal crop after wheat, rice and maize – and is used in breadmaking, especially in Central, Northern and Eastern Europe (Salovaara 1998; de Angelis *et al*. 2006); in Finland, rye sourdough bread actually accounts for about one-third of all bread consumption (Simonson *et al*. 2003). Finally, wheat sour- dough are mainly used in crackers, and in such specialties as San Francisco French bread – and are found chiefly in South European countries, whereas flat wheat breads are typical of Arabia, North Africa and the Middle East (Salovaara 1998).

Broa is a good example of how breadmaking is still much of an art. *Broa* is highly appreciated in the wide market for its distinct flavor and unique texture, and consists in a bread with an average weight of ca. 1.5 kg, although it can vary from ca. 1 to ca. 3.5 kg, with a circular to ellipsoidal format, a round top and a flat basis, and containing a crust of ca. 1 to 2 cm. In some sub-regions, *broa* is wrapped in cabbage leaves before baking. This type I-group sourdough bread is nowadays considered a gourmet speciality. Most chemical reactions therein are brought about by its adventitious microflora, which are passed from batch to batch using the mother-dough (or seed dough) as vector; mother-dough is simply a piece of leavened dough, taken at random from the previous batch after breadmaking, and which is intended to be used as (crude) starter. The continuous propagation of sourdough promotes spontaneous ecological selection of only some strains (Arendt *et al*. 2007).

The dominant microbial flora of various sourdough and mother-dough has been comprehensively studied. It is well- established that they are typically complex micro-ecosystems, in which a set of compatible strains of yeasts and LAB predominate, and thus play major roles, via both alcoholic and lactic fermentations (Corsetti *et al*. 1998). Growth of these microorganisms is favored by the environ- mental conditions prevailing during the fermentation of dough, i.e., relative low temperature and high water-activity (Faid *et al*. 1994), and their synergistic interactions contribute to the development of unique flavor and texture in the final product (Bennion 1967; Barber *et al*. 1983; Boraam *et al*. 1993; Collar

et al. 1994a,b; Almeida and Pais 1996a,b; Corsetti *et al*. 1998).

The use of sourdough in some wheat breads is intended for flavor improvement, whereas in rye breads it is necessary to confer suitable technological properties for baking afterwards (mainly arising from pH drop). The vast literature focused on sourdough fermentation has consistently emphasized the importance of sourdough towards improvement of volume and crumb structure, flavor, nutritional attributes and shelf life of bread (Arendt *et al*. 2007). The properties of the final bread depend critically on the biochemical phenomena during fermentation (that changes the carbohydrate, protein and lipid constituents of flour), which in turn depend on several endogenous factors – microorganisms and type of cereal(s), and on several exogenous parameters – extension and processing characteristics of milling, sifting and kneading, salt addition, amount and maturation level of mother-dough, and temperature and time of fermentation and baking steps (Arendt *et al*. 2007).

Empirical know-how for *broa* manufacture has been passed from generation to generation, and topical alterations have meanwhile become standard practice. This is the case of keeping the mother-dough in the refrigerator, inside a plastic bag, instead of keeping it in the wooden kneader (and often covered by salt). Since traditional *broa* is not manufactured on a daily basis, but its frequency depends rather on the current needs, the issue of how stability of the complex microflora in mother-dough evolves throughout storage arises. Based on these concerns, the microbiological profile of maize and rye flour, from their original form through the mixture as mother-dough, was monitored throughout storage for several days, using room temperature and refrigeration conditions. Thus, this research effort aimed at a better understanding of the phenomena that take place during storage of mother-dough for *broa*, and pursues a previous study on the characterization of the microbiological profile of flour and sourdough collected from several local producers of *broa* in two different periods of the year (Rocha and Malcata 2012).

Studies on the effect of storage of mother-dough under refrigeration upon their microflora are nonexistent in the scientific literature. Moreover, the evolution of dough micro-ecology including other groups of microorganisms rather than *Lactobacillus* and yeasts in the microbiological studies has not been tackled so far. Therefore, it is believed that this innovative approach may contribute to advance the state of the art of sourdough science.

MATERIALS AND METHODS

Traditional Manufacture of Sourdough

For (1-batch) traditional breadmaking – made *in loco* by a selected farmer in Cabeceiras de Basto county (Portugal), cereals were ground in a water-mill house, followed by sifting using a sieve of wire with a mesh of ca. 1 mm. Samples of flour were taken at this stage for analysis. To prepare sourdough (locally called *crescente*), 0.3 kg of mother-dough (MD, with ca. 6-day age) – i.e., a piece of ripened dough kept from a previous batch and locally called *isco* –, 4 L of warm water (ca. 50C), 2 kg of flour M and 1 kg of flour R were manually kneaded (ca. 5–10 min) (dough yield, DY = 233) and kept fermenting overnight (ca. 12 h) in a wooden kneader (first fermentation).

To prepare dough, 8 kg of maize flour was manually kneaded in the wooden kneader with 5.7 L of water (ca. 72C), plus 1.5 L of warm water with salt (ca. 100 g); after scalding and mixing the maize flour, 6 kg rye flour and the previous sourdough were gradually

added, and manually mixed (ca. 40 min) (DY =151). Fermentation (2nd fermentation) took place in the wooden kneader, covered with a clean towel, for ca. 2 h at room temperature (ca. 25C); after fermentation, a piece of dough (the renewed mother- dough) was left for the next batch, and an aliquot was taken for our analysis. The temperatures of dough after mixing and after fermentation were 36 and 30C, respectively. Monitoring of fermentation and baking was done empirically. The composition of mother-dough was ca. 59% (w/w) maize and 41% (w/w) rye flour, water at ca. 0.66 L/kg flour, and salt at ca. 5.9 g/kg dough. A complete flowchart of this general protocol is labeled as Fig. 1. Complementary description of traditional breadmaking procedures of *broa* may be found in Rocha and Malcata (2012) and Rocha *et al*. (2011).

Chemical Characteristics

The average values of some chemical parameters of maize, rye flour and *broa* are, respectively: 6.0, 6.1 and 5.1, for pH; 13.7, 13.1 and 44.1%, for moisture; 1.3, 1.5 and 1.2%, for ash; 0.127, 0.055 and 0.145%, for total acidity; 0.116, 0.096 and 0.10%, for chlorides; 2.4, 4.0 and 6.4%, for total sugars; 2.0, 2.0 and 1.8%, for fiber; 8.6, 9.3 and 5.6%, for total protein; and 4.6, 2.3 and 1.3%, for total fat. The average values of some physical parameters of maize and rye flour for *broa* production are, respectively: 286.3 and 188.1 s, for falling-number; 52.3 and 51.9%, for absorption; and 16.6 and 47.8%, for particle-size index (Rocha 2011). Complementary average of chemical values pertaining to different sources of traditional sourdough and *broa* samples are, respectively: 4.15 and 5.16, for pH; 12.97 and 7.65 mL NaOH 0.1 N/10 g_{sample}, for total titratable acidity; 0.42 and 0.13 g/100 g_{sample}, for *L*-lactic acid; 0.47 and 0.15 g/100 g_{sample}, for *D*-lactic acid; 0.12 and 0.06 g/100 g_{sample}, for acetic acid; 1.17 and 1.25%, for ashes; and 51.92 and 48.22%, for moisture (Rocha and Malcata 2012).

Sampling Procedures and Experimental Design

Samples were taken at random from (1-batch) regular feed- stocks of flour of M and R, as well as from MD, at the manufacturing stages mentioned above, i.e., the flour samples were taken immediately after milling, and mother- dough immediately after renewing. They were placed in sterile stomacher packages (Seward, London, U.K.), and immediately sent to our laboratory under refrigerated conditions.

Samples of M and R flour were kept (in plastic bags) under controlled temperature and relative humidity, in a Fitoclima S600 PLH chamber (ARALAB, Albarraque, Portugal), at 20C and 60% relative humidity. Aliquots (in duplicate) were taken at random at 0, 1, 2, 3, 7, 9, 14, 29 and 39 days, and subjected to analysis.

In an independent experiment, samples of Mr and Rr flour and MDr were kept (in plastic bags) under controlled refrigeration conditions (4C). Following the same procedure, aliquots (in duplicate) were taken at random at 0, 1, 2, 6 and 8 days, for maize and rye flour, and at 1, 2 and 6 days, for mother-dough.

All the samples (the above aliquots in duplicate) were subject to microbiological analysis using duplicate (independent microbial extraction in duplicate, followed by inoculations also in duplicate) and the effect of time and temperature of storage was studied.

Microbiological Assays

Most culture media were purchased from Biokar (Beauvais, France), Difco (Lawrence, KS), Lab M (Lancashire, U.K.) and Merck (Darmstadt, Germany), as appropriate. The pH of the culture media, measured with a Crison apparatus (Barcelona, Spain), was adjusted to the desired value at 25C, after dissolution of all (thermostable) components. All culture media, but violet red bile dextrose agar (VRBDA), were autoclaved after previous dissolution, under stirring, to boiling point. When required, complementary nonthermostable components were aseptically added to the culture media through a 0.22-μm membrane filter (Millipore, Bedford, MA) and duly stirred.

Duplicates of 10 g samples of maize flour, rye flour and mother-dough were suspended in 90 mL of sterile 2% (w/v) sodium citrate (Merck), aseptically homogenized in a beaker for 12 min, and kept under gentle agitation for an extra 8 min. The pH was measured at this stage. Serial decimal dilutions (for a total of eight concentrations) were then made using 0.1% (w/v) sterile peptone water Sigma (St. Louis, MO). Suspensions (original and following dilutions) were kept refrigerated at 4C until analyses were in order. Inoculation volumes of 20, 500 or 1,000 μL were used in duplicate, as appropriate. Therefore, four measurements were obtained for each time and temperature of storage and incubation conditions. Viable counts were determined via surface-colony count (Harley and Prescott 1990; Norrell *et al*. 1990; Seeley *et al*. 1991), and the results were expressed as log of colony-forming units (cfu)/g sample.

Total viable counts of vegetative forms were obtained by plating on tryptone soy agar (TSA, Lab M) and incubating at 30C for 1–2 days. Viable counts of (presumptive) yeast counts were obtained on yeast extract dextrose chloramphenicol agar (YEDCA, Lab M), supplemented with two vials/L X009 (chloramphenicol) (Lab M), and mold counts on rose-Bengal chloramphenicol agar base (RBCAB, Difco), supplemented with two vials/L Rose Bengal Antimicrobial Supplement C (chloramphenicol) (Difco), incubated at 30C for 48 h and at room temperature for 3–5 days. Viable counts of (presumptive) facultative anaerobic $Gram^-$ rods were obtained on: VRBDA (Merck), for Enterobacteriaceae counts; and MacConkey agar (Merck), for *Salmonella*, *Shigella*, *Yersinia* and coliforms (among others), incubated at 37C for 1 day. Viable counts of (presumptive) $Gram^-$ aerobic rods belonging to *Pseudomonas* genus were obtained on *Pseudomonas* agar base (PAB, Lab M), supplemented with 10.0 mL glycerol (Merck) and two vials/L X108 CFC (cephalothin, fucidin and cetrimide) (Lab M), and incubated at 30C for 1–2 days. Viable counts of (presumptive) endospore-forming $Gram^+$ rods were obtained on: Bacillus cereus medium (BCM, Lab M), supplemented with 100 mL/L X073 (sterile egg yolk emulsion) (Lab M) and two vials/L X074 (polymyxin B) (Lab M); and Reinforced clostridial medium (RCM, Lab M), supplemented with 100 μg/mL neomycin sulphate (Merck), for *Clostridium* counts, and incubated at 30C for 3 days. Viable counts of (presumptive) regular, nonsporing $Gram^+$ rods *Lactobacillus* (*Pediococcus* and *Leuconostoc*) were obtained on *Lactobacillus* de Man, Rogosa and Sharp agar (MRS, Lab M), and incubated at 30C for 3–5 days. Viable counts of (presumptive) $Gram^+$, $catalase^+$ cocci were obtained on Baird-Parker medium base (BPM, Lab M), supplemented with 50 mL/L X085 (sterile egg yolk tellurite emulsion) (Lab M) and 50 mg/L sulfamethazine (Merck), for *Staphylococcus* (*Micro- coccus*) counts, incubated at 37C for 2 days. Viable counts of (presumptive) $Gram^+$, $catalase^-$ cocci were obtained on:

M17 (Merck), for *Streptococcus* (*Lactococcus*), and incubated at 30C for 2–3 days; Kenner faecal streptococcal agar (KFS, Merck), supplemented with 10 mL/L (1%) *2,3,5*-triphenyltetrazolium (Merck), for *Streptococcus* (*Enterococcus*), and incubated at 37C for 2–3 days; Kanamycin esculin azide agar (KEAA, Merck), for *Enterococcus* (group D-streptococci), incubated at 37C for 2–3 days; and Mayeux, Sandine and Elliker agar (MSE, Biokar), for *Leuconostoc*, incubated at 30C for 2–3 days.

All culture media were incubated under aerobiosis, except MacConkey, M17, KFS and KEAA, –which were incubated under anaerobiosis, using a modified atmosphere of CO_2 + H_2 (GasPak Plus from BBL, Cockeysville, MD), and RCM – which was incubated under anaerobiosis, using a modified atmosphere of N_2:H_2:CO_2 (10:10:80, v/v, Gasin – Gases Industriais, Matosinhos, Portugal). All culture media were inoculated via the spread plate method but VRBDA – which was inoculated via the pour-plate method with overlay (Norrell *et al*. 1990; Seeley *et al*. 1991). All culture media selective for bacteria were supplemented with 150 mg/mL of cycloheximide (Sigma) to prevent yeast growth. Complementary description of the microbial methodologies is present in Rocha and Malcata (2012). Furthermore, all samples were subjected to pH determination, according to the AOAC official method 943.02.

Statistical Analyses

All experimental results were subjected to statistical analysis. Comparison of mean differences of the logarithm of viable counts (independently in M, R, Mr, Rr and MDr), within the fixed factor time, was via one-way analysis of variance (ANOVA), using IBM SPSS Statistics, v. 18.0 (IBM, Chicago IL). The associated *F*-test was complemented with Brown–Forsythe and Welch tests – which are robust tests of equality of means, when the homoscedasticity hypothesis is not satisfied. When the *F*-test led to significant differences, Tukey-HSD (honestly significant difference) post-hoc test was performed to compare differences between groups of the variable (time); this test is more sensitive when several paired comparisons are involved, whereas Bonferroni test is preferable for a small number of comparisons. An α-value of 0.05 was used as reference for the *F*- and post-hoc tests.

Since flour samples stored at different temperatures were milled and provided at different times, the effect of temperature (in the same type of sample) could not be studied. Nevertheless, the microbial characteristics among types of flour (maize and rye flour) were monitored for each temperature: the experimental results regarding storage of maize and rye flour at 20C (M and R) and at 4C (Mr and Rr) were subjected to a two-way ANOVA, using IBM SPSS Statistics, v. 18.0 (IBM). The fixed factors were: sample type – maize and rye flour at 20C and at 4C; and time of storage – 0, 1, 2, 3, 7, 9, 14, 29 and 39 days at 20C, and 0, 1, 2, 6 and 8 days at 4C. A full factorial model was used (with intercept), resorting to a type III-sum of squares. A complete 9 ×2 (at 20C) or 5 ×2 (at 4C) factorial design was accordingly implemented; the reference α-value of 0.05 was corrected via division by the number of tests performed in each effect.

RESULTS AND DISCUSSION

The microbial viable counts on M and R flour throughout storage at 20C and 60% relative humidity are depicted in Fig. 2a and 2b, respectively, and Table 1. The results pertaining to the viable counts on Mr and Rr flour stored under refrigeration are shown in Fig. 2c and 2d, respectively, and Table 2. The results covering storage under refrigeration of MDr for up to

6 days are presented in Fig. 3 and Table 2.

The statistical significance of the respective experimental results (i.e., microbial counts) obtained via one-way ANOVA and Tukey-HSD post-hoc tests are depicted in Tables 1 and 2. Furthermore, contrast estimates (mean differences) bearing statistical significance, as obtained in the two-way ANOVA encompassing comparison between the type of flour within time, are tabulated in Table 3, for maize and rye flour at both temperatures (20 and 4C). Finally, the so-called great averages of log (cfu/gsample) were calculated from the results obtained in each sample type throughout the entire period of storage, and tabulated in Tables 1 and 2.

Total Viable Counts

Total viable counts, on TSA, for maize and rye flour at 20C (Fig. 2a and 2b, respectively, and Tables 1 and 3) revealed, in general, no significant differences within the time period considered – although maize flour showed significant differences between 2, 7 and 14 days, corresponding to a 9% difference at most. The viable counts ranged in 6.8–7.4 and 7.2–7.7 log cfu/g in maize and rye flour at 20C, respectively. In addition, no significant differences between flour samples were observed, except lower values in maize flour in day 3 and from 14 to 29 days (Table 3). Therefore, in general, the storage period appeared not to have an important effect on the total viable counts. In other words, maize and rye flour maintain their general viable counts when stored at room temperature in adequate conditions of moisture.

The average of total viable counts on TSA was similar in both flour samples under refrigeration (Fig. 2c and 2d, and Tables 2 and 3), except at 6 days – when it was slightly higher in maize than in rye flour (Table 3). In the 8th day of the study, these values varied from 6.5 to 7.0 log cfu/g, and corresponding to differences of 2 and 7% in maize and rye flour, respectively (Fig. 2c and 2d, and Table 2).

Results of mother-dough (Fig. 3 and Table 2) showed that viable counts (on TSA) by 2 and 6 days were significantly higher – which may indicate that the microflora of mother-dough still is developing under refrigeration conditions, although in small rates. Nevertheless, the viable counts varied between 8.3 and 8.8 log cfu/g, thus corresponding to a maximum difference of a mere 6%.

The steadiness of the total viable counts observed (Figs. 2 and 3) anticipates the general maintenance within time of all specific groups of microorganisms here studied. The great average (i.e., the mean of the microbial counts obtained throughout the entire periods of study) on TSA in flour samples (M, R, Mr and Rr) (Tables 1 and 2) consubstantiate the relatively higher counts found in flour samples at 20 than at 4C; additionally, when comparing the samples stored under refrigeration (Mr, Rr and MDr) (Table 2), the effect of the fermentation in the development of the microflora becomes apparent. Furthermore, these great averages in M and R flour samples at 20C and mother- dough (MDr) (Tables 1 and 2) are consistent with the aver- ages obtained in a previous work encompassing the analysis of samples provided by 14 local producers of *broa* and in two different periods (Rocha and Malcata 2012).

Yeasts and Molds

Yeasts were incubated on YEDCA and molds on RBCAB. Yeast counts in both flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3) remained stable from 0 to 3 days, and then varied

significantly; in fact, such values ranged in 4.9–7.9 and 6.3–8.2 log cfu/g in maize and rye flour types, respectively – thus corresponding to maximum differences of 38 and 23%, respectively. These variations observed in yeast counts were the highest found among all culture media tested with (Fig. 2 and 3, and Tables 1 and 2). In turn, mold counts were 5.9–6.9 log cfu/g in both flour samples, and varied by 9 and 15% in maize and rye flour samples throughout the entire period. Yeasts from 7 to 29 days and mold from 1 to 9 days were significantly higher in rye than in maize flour – but were essentially similar in the remaining days (Table 3).

The inspection of the Figs. 2c and 2d, and Table 2, unexpectedly indicates the absence of presumptive yeasts in both flour samples under refrigeration. Regarding mold counts, significant differences corresponded to a maximum difference of 9%; additionally, mold counts were significantly higher in rye than in maize flour (Table 3) – with values of 6.2–6.4 log cfu/g in rye flour, and ranging between 4.0 and 4.4 log cfu/g in maize flour.

Yeast and mold counts in mother-dough stored at 4C varied in time between 6.9 and 8.0, and between 5.3 and 6.1 log cfu/g, respectively – thus undergoing variations of 13% in both cases (Fig. 3 and Table 2). Although in small concentrations, molds (Fig. 3 and Table 2) seem to find proper conditions to persist until mother-dough is used again in the next batch.

The typical maintenance of molds after fermentation (see great averages in Table 2) illustrates their faculty to grow at low temperatures and high relative humidity. The current yeasts counts on flour at 20C were significantly higher and in the case of MDr were lower (Tables 1 and 2) than those reported by Rocha and Malcata (2012).

Mother-dough is usually preserved (between sequential backsloppings) for days or weeks, at room temperature or at the refrigerator. Hence, rather than good gas producers, the dough yeasts are known for their viability under low temperatures and high acidic conditions (Almeida and Pais 1996a,b; Arendt *et al*. 2007). In effect, yeasts play a minor role upon decrease of pH in sourdough. Owing to the buffering capacity of the flour samples, this reduction is even lower in dough than in sugar broth-type matrixes (Barber *et al*. 1985). Yeasts have an important role towards leavening (i.e., the capacity to produce CO_2) in sourdough, but also contribute greatly to flavor and aroma production in the final bread. In the case of *broa*, the latest effects are even more important than leavening, because the leavening effect is not pronounced in breads based on maize and rye flour.

The endogenous yeasts present in sourdough are adapted to acidic environments, and their optimal growth temperature is lower than those for lactobacilli (Gänzle *et al*. 1998). At low temperatures, the acidification of sourdough by LAB is slower, thus favoring yeast activity and accordingly their leavening capacity. Nevertheless, low temperatures may also have a deleterious effect on yeast activity due to conditions favorable for acetic acid production (yeast leavening capacity is particularly affected by heterofermentative lactobacilli and other heterofermenter LAB). Actually, growth and leavening capacity of yeasts present in the sourdough is affected by the type of acid produced by *Lactobacillus* and other LAB, as well as by other substances released by these micro- organisms that inhibit yeasts (Häggman and Salovaara 2008a,b). On the other hand, the synergist interactions between yeasts and LAB are of first importance to the characteristics of sourdough: yeasts produce amino acids, peptides, vitamins and other growth factors necessary and stimulatory for LAB growth, whereas the acids and other substances produced by LAB inhibit multiplication of other competitive microorganisms – including pathogenic and spoilage organisms also present in flour

(Salovaara 1998). Typical yeasts isolated from sourdough can be found in several works such as Almeida and Pais (1996a,b), Barber and Báguena (1988), Barber *et al*. (1983), Häggman and Salovaara (2008a,b), Rocha and Malcata (1999) and Salovaara (1998).

Gram⁻ Rods

Viable counts of aerobic or facultative anaerobic Gram⁻ rods (Fig. 2a and 2b, and Tables 1 and 3), on VRBDA, PAB and MacConkey media ranged in 4.2–6.1 and 4.2–7.6 log cfu/g, in maize and rye flour at 20C, respectively – corresponding to changes within the range 11–36%. More significant differences were found in VRBDA than in PAB and MacConkey media. Higher viable counts on PAB were observed in rye than in maize flour, whereas on MacConkey medium they were higher in the period of 7–39 days. Rye led to higher viable counts on VRBDA than maize flour within 3–39 days, unlike observed at 0–2 days. Therefore, rye flour entertained typically higher levels of Gram⁻ rods than its maize counterpart (Table 3).

Regarding Gram⁻ rods in flour samples at 4C (Fig. 2c and 2d, and Tables 2 and 3), higher viable counts were found in rye flour on all culture media used but VRBDA (Table 3). Viable counts on these culture media varied from 3.3 to 4.8 and from 3.4 to 6.3 log cfu/g in maize and rye flour, respectively (Fig. 2c and 2d, and Table 2). Maximum percent differences in viable counts within time in maize and rye flour under refrigeration were, respectively: 28 and 6% on VRBDA, 8 and 7% on PAB, and 15 and 8% on MacConkey. As expected, flour may be an important source of such undesirable microorganisms, which will eventually be eliminated during fermentation and baking stages.

Observing the results of mother-dough (Fig. 3 and Table 2), it is important to emphasize the absence of Enterobacteriaceae grown on VRBDA, and the low viable numbers observed on MacConkey medium (i.e., 4.6–4.7 log cfu/g). This piece of evidence suggests that fermentation is important to decrease undesirable microorganisms in the raw-materials (beyond its technological role). *Pseudomonas* grown on PAB was found to have relatively high concentrations, i.e., between 6.9 and 7.2 log cfu/g – underling the importance to increase the fermentation time in breadmaking of *broa*. No significant variations (ranged from 2 to 3%) within time were found for all Gram⁻ rods.

The great average (log cfu/g) on VRBDA, PAB and MacConkey media (Tables 1 and 2) show the expected higher content of Gram⁻ rods in flour samples at 20 than at 4C – thus refrigeration is worthwhile to reduce Gram⁻ rods in these matrixes. Comparing with the results from Rocha and Malcata (2012), the current viable counts on PAB and MacConkey media found in mother-dough are higher.

The adverse Gram⁻ endogenous bacteria are present in initial flour samples and it is found that their growth was at the beginning of dough fermentation – before the highly competitive acid-tolerant yeasts and LAB became dominant (Röcken and Voysey 1995; de Vuyst *et al*. 2009). Therefore, the disappearance of Gram⁻ rods in mother-dough is favored as fermentation proceeds. Based on this, a suitable maturation time of mother-dough and sourdough is very important to take full advantage of ecological competition against undesirable microflora and thus eventually extend the shelf life of *broa*.

Gram$^+$ Rods

Bacillus grown on BCM medium from flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3) reached levels very close to those obtained for total viable counts, typically in the range 5.4–6.7 log cfu/g; furthermore, no significant variation (10%) was observed in maize flour, whereas only little variation was observed in rye flour (i.e., 19%); these values were identical in the two flour samples for most sampling days (Table 3). Regarding RCM, the viable counts on flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3) did not vary at all in maize flour (10%), as opposed to rye flour (ca. 28%), and similar results were attained within most of the 39 days. Therefore, this group of microorganisms remained similar in the two flour samples for most of the time (Table 3).

With respect to presumptive *Lactobacillus* grown on MRS from flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3), the viable counts ranged in 4.8–6.3 and 4.5–5.6 log cfu/g (and the corresponding variations were up to 23 and 18%) in maize and rye flour, respectively; maize actually unfolded higher values than rye flour, within 0–7 days (Table 3). Counts on this medium were essentially similar among the first four sampling days.

The counts of endospore-forming Gram$^+$ rods (grown on BCM and RCM) in flour samples at 4C were all found to be significant higher in rye than in maize flour (Fig. 2c and 2d, and Tables 2 and 3); in maize flour, all viable counts on these media by 8 days were significantly different from the remain period, whereas less frequent differences were observed in rye flour. On the other hand, (presumptive) viable numbers of *Lactobacillus* grown on MRS was significantly higher in maize flour at 4C (Table 3). In both flour samples at 4C, the viable counts on these two culture media varied between 3.9 and 6.1 log cfu/g, and minimum and maximum differences of 5 and 15% were found.

Endospore-forming Gram$^+$ rods grown on BCM were present in mother-dough (Fig. 3 and Table 2) to consider- able levels (i.e., 8.5–8.8 log cfu/g) – also comparable to total viable counts; viable counts on RCM were also found at important levels (i.e., 7.8–8.1 log cfu/g). Said viable count evolution revealed only small changes (4–5%) during time. The (presumptive) *Lactobacillus* viable counts grown on MRS in mother-dough at 4C (8.8 log cfu/g) were 10-fold higher than those of yeasts grown on YEDCA (Fig. 3 and Table 2). This ratio is in agreement with the typical ratios found in wheat (Barber *et al*. 1983) and rye (Häggman and Salovaara 2008a) sourdough. In addition, no significant changes (1%) were revealed during the whole period studied.

The great averages (log cfu/g) on BCM, RCM and MRS (Tables 1 and 2) show the higher viable counts found at higher temperatures (except in RCM counts for rye flour). When comparing the samples under refrigeration (i.e., flour and mother-dough), the growth of *Bacillus* and *Lactobacillus* during the fermentation process becomes apparent; unexpectedly, high viable numbers on RCM were found in mother-dough – which may be a consequence of a deficient modified atmosphere during incubation. More- over, when comparing the averages with those obtained in a previous work (Rocha and Malcata 2012), the viable counts on BCM were significantly higher in the current mother-dough, and the average of viable counts on MRS were similar in both studies. Thus, higher viable counts of endospore-forming Gram$^+$ rods were typically observed in this study.

These results showed that yeasts, *Bacillus* and LAB were the predominant microbial groups with respect to total viable counts. LAB are generally mesophilic and most strains grow at pH of 4.0–4.5; nevertheless, they can grow at temperatures from 5 to 45C, and be active in a large

range of pH values (3.2–9.6). In spontaneous dough fermentation, LAB dominate rapidly the Gram$^-$ bacteria, in particular, lactobacilli – which is apparent in our results (Röcken and Voysey 1995; de Vuyst *et al*. 2009).

The sourdough LAB are usually sensitive to drying preservation, as well as to acidity – so when sourdough are kept at room temperature, continuous acidification may eventually lead to the disappearance of certain species of this group of microorganisms (Corsetti and Settanni 2007). Thus, the use of refrigeration and plastic bags during storage of *broa* mother-dough between propagation steps is a good option – and which is corroborated with the current results.

The microbial flora of sourdough has been studied to some length focusing mainly on yeasts and *Lactobacillus* (Arendt *et al*. 2007). Our results showed that the drop of pH during dough fermentation had a crucial role towards the control of Gram$^-$ bacteria, but the ubiquitous endospore- forming Gram$^+$ rods persist. Therefore, synergetic interactions in sourdough systems are not restricted to yeasts and *Lactobacillus* but also include species of *Bacillus*.

The yeast and bacterial viable counts in sourdough vary according to the type of dough and process parameters. According to Barber *et al*. (1983), the expected order of magnitude of yeasts and *Lactobacillus* in sourdough are 10^6- 10^7 and 10^8–10^9 cfu/g, respectively. *Lactobacillus* and yeasts contents in Finnish sour rye ferments from bakeries (after 13–15 h of fermentation) and home bakers have been studied by Salovaara and Katunpää (1984) and Salovaara and Savolainen (1984); they found that *Lactobacillus* viable counts ranged from 2×10^6 to 4×10^8 cfu/g, and yeast counts ranged from 5×10^5 to 5×10^8 cfu/g in bakery samples, and between 1×10^4 and 1×10^9 cfu/g in home- baking samples. Barber and Báguena (1988) obtained the following viable counts in industrial and *in vitro* wheat sourdough: 10^5–10^8 and 10^4–10^8 cfu/g, for yeasts; and 10^5- 10^7 and 10^5–10^8 cfu/g, for *Lactobacillus*. Hence, the effect of the baking process upon the microbial results is apparent. The total average microbial counts of presumptive yeasts and lactobacilli in the current mother-dough was 7.6 and 8.8 log cfu/g, respectively – which are comparable with those above pertaining to whole-meal rye flour sourdough (Salovaara and Katunpää 1984; Salovaara and Savolainen 1984). Furthermore, the 10-fold log cycle found higher lactobacilli counts relative to yeast counts also observed in the 2 days rye dough prepared via backslopping, i.e., consecutive re-inoculation (Häggman and Salovaara 2008b). Furthermore, the yeast counts in our work were significantly higher than those obtained by Almeida and Pais (1996a), which is explained by the distinct fermentation time – and confirmed by higher pH values in our mother-dough. The most common lactobacilli found in sourdough were widely described in the literature, such as Ampe and Miambi (2000), Barber and Báguena (1988), Barber *et al*. (1983), Häggman and Salovaara (2008b), Rocha and Malcata (1999) and Salovaara (1998).

LAB and their interactions with yeasts in mother- and sourdough play important roles upon several organoleptic and textural features generated throughout sourdough fermentation – which is affected by composition of flour and manufacturing conditions (Gobbetti *et al*. 1994). Homofermentative LAB are responsible for development of a final bread with a good grain and elastic crumb, whereas heterofermentative LAB contributes much more to improve bread taste and promote leavening. Sourdough leavening is mainly determined by

CO_2 produced during fermentative activity by yeasts, contributing to open up the texture (Barber *et al*. 1983; Boraam *et al*. 1993). Therefore, the homo- or heterofermenter character of lactobacilli affects the quality of the final bread, namely the loaf volume (to a lesser extent than yeasts), and the aroma and taste (Barber *et al*. 1983).

Among other metabolites, lactic and acetic acids produced by LAB are of major importance to the final taste of bread, besides increasing its shelf life and avoiding mold spoilage (Corsetti *et al*. 1998). Apart from the typical sour taste given – which is desirable in sour breads, the acetic acid produced by heterofermentative LAB holds fungicidal properties, thus increasing the shelf life of bread, and also inhibiting the germination of endospores of *Bacillus* that may withstand baking temperatures (Corsetti *et al*. 1998; Salovaara 1998). On the other hand, lactic acid – the only end-product in homolactic fermentations and the major end-product in heterolactic fermentation, is softer toward flavor than acetic acid, but stronger than acetic acid in terms of decreasing pH, thus affecting the antimicrobial proper- ties of sourdough (Salovaara 1998).

Accordingly, the effectiveness of sourdough as a preservative against microbial spoilage of bread depends upon its composition of lactic and acetic acids and several other antimicrobial metabolites (such as hydrogen peroxide), which in turn is a function of the type and amount of LAB and other microorganisms present (including their species and strains), on top of composition type of flour and other fermentable substrates used, aeration, time and temperature of fermentation, the initial pH and buffering capacity and of a number of other baking conditions and breadmaking processes employed (Barber and Báguena 1988; Salovaara 1998).

According to Arendt *et al*. (2007), the pH of a ripened sourdough comprises values between 3.5 and 4.3. The drop of pH due to fermentation was apparent: average values of 6.3 and 6.5 were observed in flour samples and 4.1 in mother-dough. Beyond the antimicrobial effect, this is of foremost importance because acidification of the dough imparts changes upon the structure of components: e.g., the changes in the hydration capacity of gluten proteins influence the fermentation activity of microorganisms, as well as their enzymatic activity – and ultimately the quality of bread, viz. loaf volume, texture and aroma (Arendt *et al*. 2007).

Gram$^+$ Cocci

Relatively low presumptive *Staphylococcus* counts were obtained during storage of flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3): 4.8–5.7 and 3.8–4.2 log cfu/g in maize and rye flour, respectively; these values correspond to no significant difference (9%) in rye flour, but to a variation of 16% in maize flour; maize had always statistically higher viable numbers than rye flour (Table 3). Viable counts on BPM were similar between 0 and 3 days. Unlike those observed in flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3), presumptive *Staphylococcus* could not be found in both flour samples at 4C (Fig. 2c and 2d, and Tables 2 and 3) during the whole period. In mother-dough, low concentration of *Staphylococcus* (i.e., 4.0–4.4 log cfu/g) grown on BPM were found (Fig. 3 and Table 2); similarly to Gram$^-$ rods from mother-dough at 4C (Fig. 3 and Table 2), no significant difference (8%) within time was found for *Staphylococcus*. Finally, comparing the great averages (Tables 1 and 2) for M, R and MDr with those reported by Rocha and Malcata (2012), relative higher values were detected in MDr, whereas in flour samples the values

are close.

Gram^{+} catalase^{-} cocci counts from flour samples at 20C on M17, KFS, KEAA and MSE (Fig. 2a and 2b, and Tables 1 and 3) ranged in 4.9–6.4 and 4.5–7.0 log cfu/g, for maize and rye flour at 20C, respectively – corresponding to percent variability from 7 to 24, and from 14 to 29, respectively. Presumptive streptococci grown on M17 were significantly higher in rye than maize flour at 20C within the period of 7–39 days; streptococci and enterococci (grown on KFS and KEAA, respectively) were significantly higher in maize than rye flour in several samples; and leuconostocs (grown on MSE) were similar throughout the whole period, except on 0 and 2 days – when they were higher in maize than rye flour (Table 3).

Viable counts of Gram^{+} cocci were generally higher in rye than in maize flour at 4C (particularly those grown on M17 and MSE) (Table 3), but frequently no significant differences in viable counts were found throughout storage time in each flour type (Fig. 2c and 2d, and Table 2 and 3); indeed, only significant differences were found on KFS and MSE media. Gram^{+} catalase^{-} cocci accounted for 3.4–4.7 and 3.8–6.1 log cfu/g in maize and rye flour at 4C, respectively – and the maximum percent variability observed was only of 13%.

The benefits of the presence of LAB belonging to Gram^{+} cocci catalase^{-} (viz. *Streptococcus*, *Lactococcus*, *Enterococcus* and *Leuconostoc*) are apparent from inspection of Fig. 3 and Table 2 – where values ranged between 8.1 and 8.8 log cfu/g. Throughout the period investigated, no significant difference (1 to 6%) was observed in all culture media for this group of microorganisms.

The great average (log cfu/g) of viable counts on M17, KFS, KEAA and MSE culture media (in M, R, Mr, Rr and MDr) (Table 1 and 2) data point out for the higher viable counts found in flour samples at 20C that of the respective at 4C, as well as for the obvious growth of lactic acid cocci during fermentation. Furthermore, when comparing these great averages in M and R flour at 20C and MDr with those obtained by Rocha and Malcata (2012), one observed that the values here were generally similar on M17 but slightly higher on KFS, KEAA and MSE.

The presence of lactic acid coccaceae in mother-dough is apparent in our results, thus confirming the importance of other microorganisms belonging to the LAB group (rather than *Lactobacillus*) – e.g., *Enterococcus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* – to the biodiversity and the equilibrium of microbial consortia in mother and sour- dough within time. Although *Lactobacillus* strains are the most frequent and studied bacteria in sourdough, species of *Leuconostoc*, *Weissella*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Streptococcus* have been also identified. Additionally, while further studies are needed, it is thought that lactic acid cocci play important roles within distinct stages of dough fermentation: some are expected mainly to be present at the first stage of dough fermentation (e.g., *Leuconostoc* spp.), others are slow acid-producers, and others are able to survive in high acidic environments – thus emerging at the end of the dough fermentation process (Faid *et al*. 1994; Röcken and Voysey 1995; Corsetti and Settanni 2007; de Vuyst *et al*. 2009).

General Discussion

The observed viable counts in flour samples at 20C (Fig. 2a and 2b, and Tables 1 and 3) made apparent the contribution of flour samples upon the microbial diversity found in mother- and sourdough for *broa*. Yeasts reached maximal viable counts topically; additionally, the maximum variations among all the conditions and samples tested were

observed in yeast viable counts from flour samples at 20C. From the results above, it also became apparent that the microflora remained generally stable in both flour samples throughout the 39 days of storage, despite a few significant differences found. The pH steadiness (Fig. 2) was also a result of the low microbial activity of flour samples during storage. Therefore, in the absence of any abnormal external factors, the flour samples seem to reach a stationary biodiversity throughout storage period.

No important changes were observed in both maize and rye flours under refrigeration (at 4C) within the 8-day storage period (Fig. 2c and 2d, and Tables 2 and 3). Although the results pertaining to flour at different temperatures (Fig. 2, and Tables 1 and 2) cannot be fully compared, it was quite apparent that a slight higher variability in viable counts would likely arise at the highest temperature.

Regarding the storage of mother-dough under refrigeration (Fig. 3 and Table 2), differences of mean values were not statistically significant in most cases. In fact, through- out the sampling period, an average of 4.5% difference in viable counts of every culture medium was attained; this anticipates a possible fermentation at very slow growth rates occurring in mother-dough stored under refrigeration. Thus, one concluded that it is a good choice to keep the mother-dough stored under refrigeration between backslopping processes. The relative constancy of pH (Fig. 3) was also a clue for the low activity prevailing in the refrigerated mother-dough for *broa*. Additionally, the viable counts in this specific mother-dough has showed the relative short fermentation time employed; as a result, the number of undesirable microorganisms did not lower enough, chiefly the Gram^{-} rods and *Staphylococcus*. There- fore, to take full advantage of the fermentation process without compromising technological or logistic aspects, an extension of *broa* sourdough fermentation could be practiced.

Mother-dough accelerates the initial phase of fermentation, and promotes beneficial changes during breadmaking

– leading to a natural selection of a stable microbial consortium, dominated by LAB (*Lactobacillus* and lactic cocci), *Bacillus* and yeasts, and thus reducing to some extent the initial complex microflora present in dough. Although reliable and easy to handle, the use of a mother-dough leads frequently to deviations between batches; to avoid such a variation in empirical breadmaking processes, well-defined fermentation times and amounts of mother-dough (and of other ingredients) in the backslopping process should be implemented among local producers of *broa*.

The stabilization of the microbial of mother-dough during storage can also be increased by combining the refrigeration conditions to the use of sodium chloride. The use of sodium chloride (as happened in the older traditional process of *broa*) is expected to influence the microbial eco- system towards a desirable set of LAB and yeasts during fermentation (Röcken and Voysey 1995; de Vuyst *et al*. 2009), and a decrease in molds content.

The originality of this research effort will likely contribute to a better understanding of the phenomena that take place between sequential backslopping of seed- or mother- dough. Studies on the effect of long-term storage periods upon the microflora of mother- and sourdough are indeed very scarce. Our findings also showed the presence at important levels of other microorganisms rather than yeasts and lactobacilli during sourdough fermentation. In fact, these additional groups of microorganisms are still poorly characterized in sourdough.

CONCLUSIONS

No important differences on the microbiological profile were observed during the storage of mother-dough in the period of up to 1 week under refrigeration (at 4C); in addition, mother-dough microflora stills metabolically viable under this conditions. This realization is rather important, since many home-made manufactures of *broa* produce this food on an irregular basis, with spacing of a few days or even weeks between batches. Therefore, the storage of mother-dough in the refrigerator is a good option for the local farmers.

Flour has an important contribution to the microflora existing in dough – as apparent by the diversity of microorganisms found therein. However, the microbial evolution throughout storage for 39 days unfolded, in general, no important changes in the flour samples, i.e., a steadiness of the microbial counts was observed in the main (although some significant differences within the time period were observed). Thus, flour keep generally their microbial properties within storage period and their maintenance at refrigeration is not required.

NOMENCLATURE

B, *broa* (Bread); Catalase^{+}, catalase-positive; Catalase^{-}, catalase-negative; CFU, colony-forming units; DY, dough yield; Gram^{+}, Gram-positive; Gram^{-}, Gram-negative; LAB, lactic acid bacteria; M, maize flour; MD, mother-dough; MDr, mother-dough under refrigeration; Mr, maize flour under refrigeration; R, rye flour; Rr, rye flour under refrigeration.

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REFERENCES

ABDALLA, A.A., EL TINAY, A.H., MOHAMED, B.E. and ABDALLA, A.H. 1998. Effect of traditional processes on phytate and mineral content of pearl millet. Food Chem. *63*, 79–84.

ALMEIDA, M.J. and PAIS, C.S. 1996a. Characterization of the yeast population from traditional corn and rye bread doughs. Lett. Appl. Microbiol. *23*, 154–158.

ALMEIDA, M.J. and PAIS, C.S. 1996b. Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. Appl. Environ. Microbiol. *62*, 4401–4404.

AMPE, F. and MIAMBI, E. 2000. Cluster analysis, richness and biodiversity indexes derived from denaturating gradient gel electrophoresis fingerprints of bacterial communities demonstrate that traditional maize fermentations are driven by the

transformation process. Int. J. Food Microbiol. *60*, 91–97.

ANNAN, N.T., POLL, L., SEFA-DEDEH, S., PLAHAR, W.A. and JAKOBSEN, M. 2003. Volatile compounds produced by *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* in single starter culture fermentations of Ghanaian maize dough. J. Appl. Microbiol. *94*, 462–474.

ANTONY, U., SRIPRIYA, G. and CHANDRA, T.S. 1996. The effect of fermentation on the primary nutrients in foxtail millet (*Setaria italica*). Food Chem. *56*, 381–384.

ARENDT, E.K., RYAN, L.A.M. and BELLO, F.D. 2007. Impact of sourdough on the texture of bread. Food Microbiol. *24*, 165–174.

BARBER, S. and BÁGUENA, R. 1988. Microflora de la masa madre panaria. V. Aislamiento, identificación y evaluación de las propiedades funcionales de microorganismos de la masa madre. Ver. Agroquím. Tecnol. Aliment. *28*, 67–78.

BARBER, S., BÁGUENA, R., MARTÍNEZ-ANAYA, M.A. and TORNER, M.J. 1983. Microflora de masa madre panaria. I. Identificación y propiedades funcionales de microorganismos de masas madre industriales, elaboradas com harina de trigo. Ver. Agroquím. Tecnol. Aliment. *23*, 552–562.

BARBER, S., MARTÍNEZ-ANAYA, M.A. and BÁGUENA, R. 1985. Microflora de la masa madre panaria. III. Propiedades funcionales en masas elaboradas com harina de trigo, de cepas puras de *S. cerevisiae* y de las levaduras comerciales de las que proceden. Rev. Agroquím. Tecnol. Aliment. *25*, 447–457.

BENNION, E.B. 1967. *Breadmaking. Its Principles and Practice*, 4th Ed., Oxford University Press, London. 391 pp.

BORAAM, F., FAID, M. and LARPENT, J.P. 1993. Lactic acid bacteria and yeasts associated with traditional Moroccan sour-dough bread fermentation. Sci. Aliments *13*, 501–509.

COLLAR, C., MARTÍNEZ-ANAYA, M.A. and BARBER, C.B. 1994a. Interactive effects between microbial breadmaking starters and wheat flours on sour dough and bread quality. Rev. Esp. Ciencia Tecn. Aliment. *34*, 191–201.

COLLAR, E., BARBER, C.B. and MARTÍNEZ-ANAYA, M.A. 1994b. Microbial sour doughs influence acidification properties and breadmaking potential of wheat dough. J. Food Sci. *59*, 629–633, and 674.

CORSETTI, A. and SETTANNI, L. 2007. Lactobacilli in sourdough fermentation. Food Res. Int. *40*, 539–558.

CORSETTI, A., GOBBETTI, M., ROSSI, J. and DAMIANI, P. 1998. Antimould activity of sourdough lactic acid bacteria: Identification of a mixture of organic acids produced by *Lactobacillus sanfranciscensis*. Appl. Microbiol. Biotechnol. *50*, 253–256.

de ANGELIS, M., CODA, R., SILANO, M., MINERVINI, F., RIZELLO, C.G., DI CAGNO, R., VICENTINI, O.,

DE VICENZI, M. and GOBBETTI, M. 2006. Fermentation by selected sourdough lactic acid bacteria to decrease coeliac intolerance to rye flour. J. Cereal Sci. *43*, 301–314.

de VUYST, L., VRANCKEN, G., RAVYTS, F., RIMAUX, T. and WECKS, S. 2009. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microflora. Food Microbiol. *26*, 666–675.

FAID, M., BORAAM, F., ZYANI, I. and LARPENT, J.P. 1994. Characterization of sourdough bread ferments made in the laboratory by traditional methods. Zeits. Lebensm.-Unters. Forsch. *198*, 287–291.

GÄNZLE, M.G., EHRMANN, M. and HAMMES, W.P. 1998. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl. Environ. Microbiol. *64*, 2616–2623.

GOBBETTI, M., CORSETTI, A. and ROSSI, J. 1994. The sourdough microflora: Interactions between lactic acid bacteria and yeasts: Metabolism of carbohydrates. Appl. Microbiol. Biotechnol. *41*, 456–460.

HARLEY, J.P. and PRESCOTT, L.M. 1990. *Laboratory Exercises in Microbiology*, pp. 52–60, William C. Brown, Dubuque, IA.

HÄGGMAN, M. and SALOVAARA, H. 2008a. Effect of fermentation rate on endogenous leavening of *Candida milleri* in sour rye dough. Food Res. Int. *41*, 266–273.

HÄGGMAN, M. and SALOVAARA, H. 2008b. Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains. Lebensm. Wiss. Technol. *41*, 148–154.

NORRELL, S.A., CREAGER, J.G., BLACK, J.G. and DAVISON,

V.E. 1990. *Laboratory Manual for Microbiology: Principles and Applications*, pp. 18–36, Prentice-Hall, Englewood Cliffs, NJ.

OSMAN, M.A., RAHMAN, I.E.A., HAMAD, S.H. and DIRAR, H.A. 2010. Biochemical changes occurring during traditional Sudanese processing of Kisra bread. J. Food Agric. Environ. *8*, 102–106.

ROCHA, J.M. 2011. *Microbiological and Lipid Profiles of Broa: Contributions for the Characterization of a Traditional Portuguese Bread*, pp. 91–106, Instituto Superior de Agronomia, Universidade de Lisboa, Lisbon, Portugal. Ph.D thesis, 705 pp., http://hdl.handle.net/10400.5/3876 (accessed November 20 2015).

ROCHA, J.M. and MALCATA, F.X. 1999. On the microbiological profile of traditional Portuguese sourdough. J. Food Protect. *62*, 1416–1429.

ROCHA, J.M. and MALCATA, F.X. 2012. Microbiological profile of maize and rye flours, and sourdough used for the manufacture of traditional Portuguese bread. Food Microbiol. *31*, 72–88.

ROCHA, J.M., KALO, P.J. and MALCATA, F.X. 2011. Neutral lipids in free, bound and starch lipid extracts of flours, sourdough and Portuguese sourdough bread, determined by NP-HPLC-ELSD. Cereal Chem. *88*, 400–408.

RÖCKEN, W. and VOYSEY, P.A. 1995. Sour-dough fermentation in bread making. J. Appl. Bacter. Symp. *79*, 28S–48S.

SALOVAARA, H. 1998. Lactic acid bacteria in cereal-based products. In *Lactic Acid Bacteria – Microbiology and Functional Aspects* (S. Salminen and A. von Wright, eds.) pp. 115–137, Marcel Dekker, New York NY.

SALOVAARA, H. and KATUNPÄÄ, H. 1984. An approach to the classification of lactobacilli isolated from Finnish sour rye dough ferments. Acta Alim. Polon. *10*, 231–239.

SALOVAARA, H. and SAVOLAINEN, J. 1984. Yeast type isolated from Finnish sour rye starters. Acta Alim. Polon. *10*, 241–245.

SEELEY, J.H.W., VAN DEMARK, P.J. and LEE, J.J. 1991. *Microbes in Action. A Laboratory Manual of Microbiology*, pp. 37–58, W. H. Freeman, New York.

SIMONSON, L., SALOVAARA, H. and KORHOLA, M. 2003. Response of wheat sourdough parameters to temperature, NaCl and sucrose variations. Food Microbiol. *20*, 193–199.

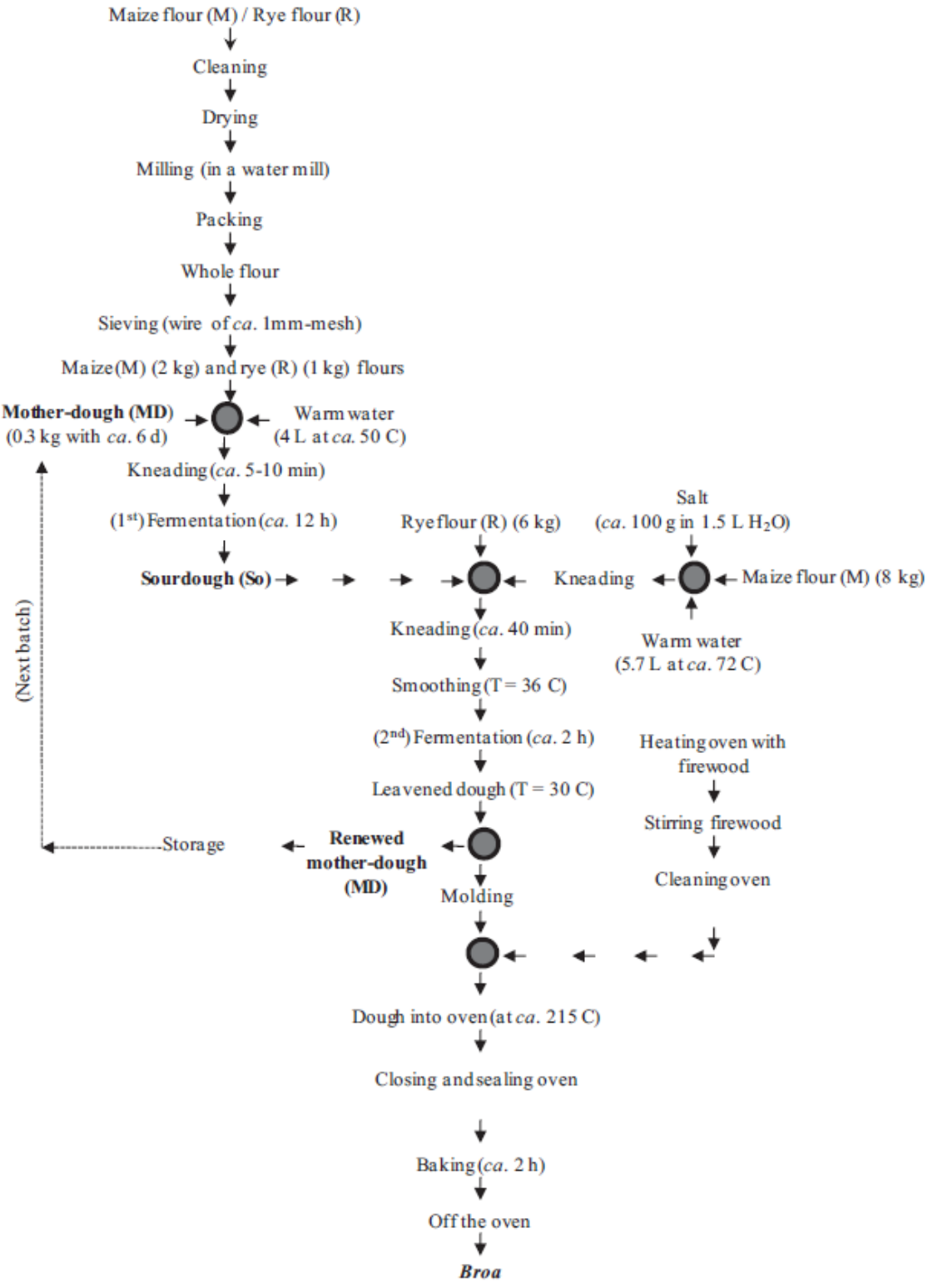

FIG. 1. FLOWCHART OF THE CLASSICAL PROTOCOL FOR BREADMAKING OF *BROA* (AND SPECIFIC CONDITIONS EMPLOYED *IN LOCO* BY THE FARMER FROM CABECEIRAS DE BASTO)

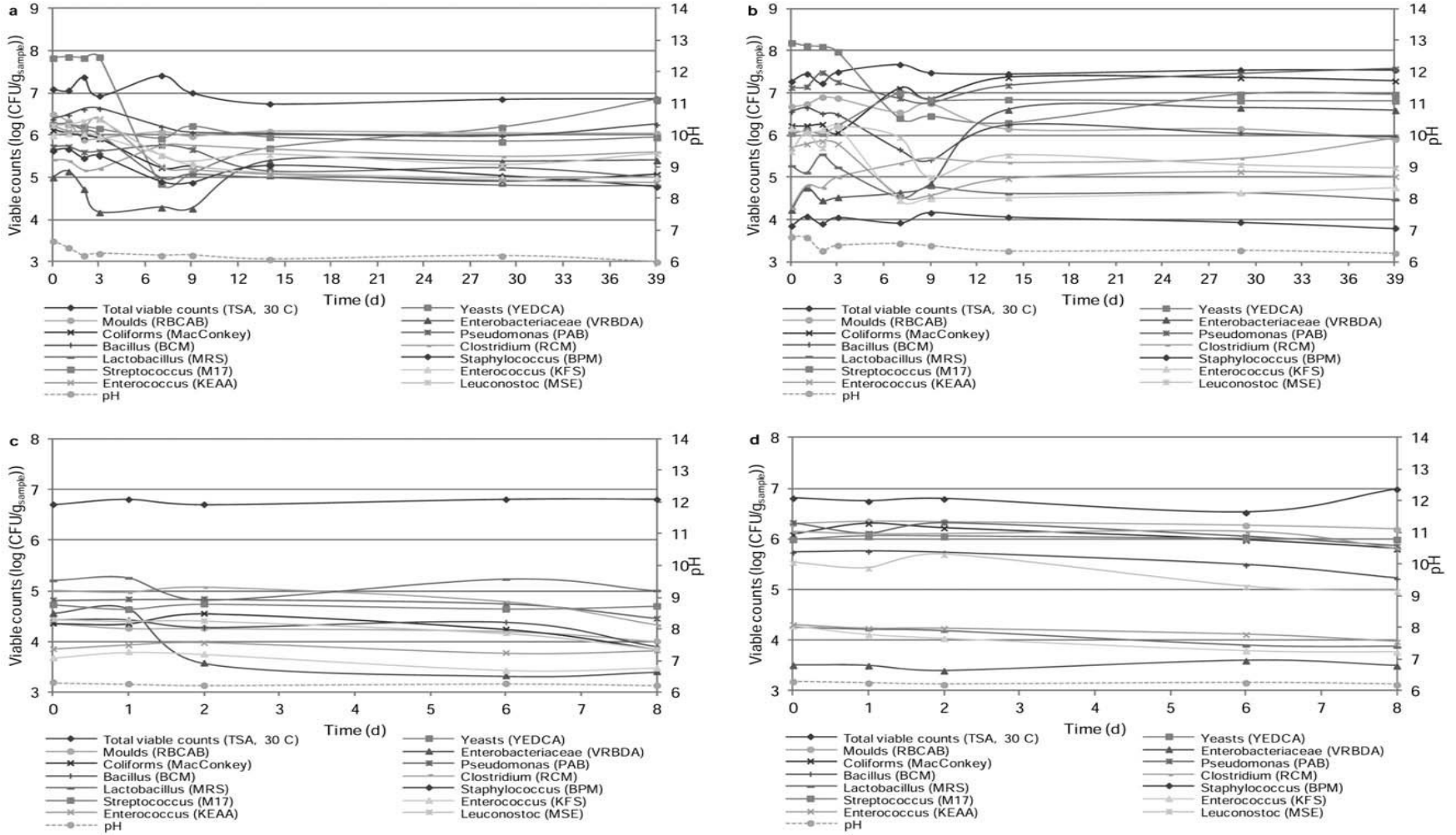

FIG. 2. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE, CFU/GSAMPLE) THROUGHOUT TIME AND pH, IN (A) MAIZE (M) AND (B) RYE (R) FLOUR AT 20C, AND IN (C) MAIZE (MR) AND (D) RYE (RR) FLOUR AT 4C Standard deviations and statistical results are depicted in Tables 1 and 2.

TABLE 1. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/GSAMPLE) THROUGHOUT TIME (0, 1, 2, 3, 7, 9, 14, 29 AND 39 DAYS) AND GREAT AVERAGE IN MAIZE (M) AND RYE (R) FLOUR AT 20C

	No. of days	0	1	2	3	7	9	14	29	39	R^2	Great average
Target microorganisms	Culture media	Maize flour at 20C (M)										
Total viable counts	TSA, 30C	7.10 ± 0.20	7.07 ± 0.30	7.38 ± 0.28^{s}	6.94 ± 0.34^{x}	7.42 ± 0.29^{ab}	7.01 ± 0.23^{ae}	6.75 ± 0.17	6.86 ± 0.16	6.87 ± 0.11	37.6	7.04 ± 0.23
Yeasts and molds	YEDCA, yeasts	$7.83 \pm 0.12^{d,e,f,g,h}$	$7.86 \pm 0.11^{k,l,m,n,o}$	$7.83 \pm 0.12^{q,r,s,t,u}$	$7.86 \pm 0.12^{v,w,x,y,z}$	$4.85 \pm 0.17^{ab,ac,ad}$	$5.10 \pm 0.00^{ae,af,ag}$	$5.70 \pm 0.00^{ah,ai}$	6.19 ± 0.41^{aj}	6.85 ± 0.12	98.0	6.67 ± 1.25
	RBCAB, molds	$6.50 \pm 0.23^{b,c,d,e,g,h}$	6.33 ± 0.29^{j}	5.90 ± 0.20	5.93 ± 0.23	6.08 ± 0.13	5.98 ± 0.10	6.10 ± 0.04	6.06 ± 0.08	6.05 ± 0.07	48.3	6.10 ± 0.19
Facultative anaerobic	VRBDA	$5.00 \pm 0.16^{c,d,e}$	$5.14 \pm 0.05^{j,k,l}$	$4.73 \pm 0.17^{p,s,t,u}$	$4.18 \pm 0.30^{x,y,z}$	$4.29 \pm 0.23^{ab,ac,ad}$	$4.27 \pm 0.35^{ae,af,ag}$	5.39 ± 0.06	5.38 ± 0.07	5.41 ± 0.10	86.0	4.86 ± 0.51
Gram-negative ($Gram^-$) rods	MacConkey	$6.12 \pm 0.34^{d,e,f,g,h}$	$6.04 \pm 0.26^{k,l,m,n,o}$	$5.98 \pm 0.08^{q,r,s,t,u}$	$5.92 \pm 0.04^{v,w,x,y,z}$	5.24 ± 0.26	5.27 ± 0.25	5.11 ± 0.36	4.92 ± 0.30	5.08 ± 0.20	75.5	5.52 ± 0.48
$Gram^-$ aerobic rods	PAB	$5.74 \pm 0.24^{f,g,h}$	$5.74 \pm 0.22^{m,n,o}$	$5.61 \pm 0.15^{s,u}$	$5.63 \pm 0.13^{x,z}$	$5.76 \pm 0.08^{ab,ac,ad}$	$5.67 \pm 0.11^{ae,ag}$	5.16 ± 0.23	5.24 ± 0.20	5.01 ± 0.23	66.4	5.51 ± 0.29
Endospore-forming	BCM	6.38 ± 0.28	6.48 ± 0.21	6.62 ± 0.13	6.63 ± 0.10	6.20 ± 0.37	6.06 ± 0.43	6.03 ± 0.33	5.98 ± 0.36	6.26 ± 0.30	29.2	6.29 ± 0.25
Gram-positive ($Gram^+$) rods	RCM	5.43 ± 0.11	5.40 ± 0.29	5.18 ± 0.40	5.21 ± 0.44	5.76 ± 0.07	5.77 ± 0.04	568 ± 0.16	5.51 ± 0.35	5.61 ± 0.24	27.3	5.50 ± 0.22
Regular, nonsporing $Gram^+$ rods	MRS	$6.25 \pm 0.15^{d,e,f,g,h}$	$6.26 \pm 0.23^{k,l,m,n,o}$	$6.13 \pm 0.14^{q,r,s,t,u}$	$6.02 \pm 0.02^{v,w,x,y,z}$	5.01 ± 0.13	5.09 ± 0.10	5.01 ± 0.11	4.83 ± 0.09	4.82 ± 0.08	95.9	5.49 ± 0.65
$Gram^+$, catalase-positive ($catalase^+$) cocci	BPM	$5.64 \pm 0.17^{d,e,g,h}$	$5.68 \pm 0.12^{k,l,n,o}$	$5.46 \pm 0.20^{q,r,t,u}$	$5.52 \pm 0.11^{v,w,y,z}$	4.91 ± 0.06	4.88 ± 0.04	5.28 ± 0.28^{ai}	5.04 ± 0.26	4.79 ± 0.15	77.5	5.24 ± 0.35
$Gram^+$, catalase-negative ($catalase^-$) cocci	M17	6.26 ± 0.11	6.26 ± 0.11	6.22 ± 0.17	6.16 ± 0.22	5.93 ± 0.29	6.23 ± 0.38	5.96 ± 0.07	5.85 ± 0.17	5.95 ± 0.20	25.6	6.09 ± 0.17
	KFS	$6.00 \pm 0.25^{e,f,g,h}$	$6.04 \pm 0.26^{l,m,n,o}$	$6.04 \pm 0.26^{r,s,t,u}$	$5.97 \pm 0.32^{w,x,y,z}$	5.53 ± 0.27	5.29 ± 0.43	5.12 ± 0.15	5.00 ± 0.25	5.00 ± 0.06	71.8	5.56 ± 0.46
	KEAA	$6.24 \pm 0.37^{d,e,f,g,h}$	$6.11 \pm 0.35^{k,l,m,n,o}$	$6.12 \pm 0.34^{q,r,s,t,u}$	$6.40 \pm 0.29^{v,w,x,y,z}$	5.27 ± 0.25	5.20 ± 0.38	5.09 ± 0.27	4.95 ± 0.27	4.89 ± 0.04	78.5	5.58 ± 0.62
	MSE	$6.24 \pm 0.20^{d,e,f,g,h}$	$6.26 \pm 0.23^{k,l,m,n,o}$	$6.33 \pm 0.24^{q,r,s,t,u}$	$6.37 \pm 0.26^{v,w,x,y,z}$	5.52 ± 0.23	5.37 ± 0.33	5.55 ± 0.30	5.30 ± 0.26	5.56 ± 0.19	73.3	5.83 ± 0.45

Target microorganisms	Culture media	Rye flour at 20C (R)										
Total viable counts	TSA, 30C	7.27 ± 0.25	7.45 ± 0.30	7.23 ± 0.29	7.49 ± 0.15	7.67 ± 0.08	7.48 ± 0.25	7.45 ± 0.19	7.54 ± 0.25	7.54 ± 0.28	7.60	7.46 ± 0.14
Yeasts and molds	YEDCA, yeasts	$8.18 \pm 0.08^{d,e,f,g,h}$	$8.11 \pm 0.06^{k,l,m,n,o}$	$8.09 \pm 0.12^{q,r,s,t,u}$	$7.97 \pm 0.04^{v,w,x,y,z}$	6.40 ± 0.00	6.45 ± 0.00^{ag}	$6.29 \pm 0.71^{ah,ai}$	6.97 ± 0.03^{aj}	6.96 ± 0.13	90.6	7.27 ± 0.81
	RBCAB, molds	6.68 ± 0.23^{h}	6.74 ± 0.18^{o}	$6.90 \pm 0.05^{s,t,u}$	6.88 ± 0.13^{z}	6.54 ± 0.47^{ad}	6.75 ± 0.30^{ag}	6.15 ± 0.17	6.15 ± 0.17	5.90 ± 0.62	51.3	6.52 ± 0.36
Facultative anaerobic	VRBDA	$4.23 \pm 0.13^{a,c,d,e,f,g,h}$	$4.75 \pm 0.10^{i,j,m,n,o}$	$4.45 \pm 0.13^{r,s,t,u}$	$4.53 \pm 0.10^{w,x,y,z}$	$4.64 \pm 0.10^{ab,ac,ad}$	$4.85 \pm 0.09^{ae,af,ag}$	6.60 ± 0.05	6.64 ± 0.06	6.58 ± 0.13	98.9	5.25 ± 1.03
Gram-negative ($Gram^{-}$) rods	MacConkey	$6.22 \pm 0.39^{d,f,g,h}$	$6.22 \pm 0.39^{i,k,m,n,o}$	$6.26 \pm 0.39^{q,r,s,t,u}$	$6.06 \pm 0.43^{v,w,x,y,z}$	7.11 ± 0.12	6.86 ± 0.31	7.36 ± 0.13	7.36 ± 0.24	7.28 ± 0.24	72.6	6.75 ± 0.55
$Gram^{-}$ aerobic rods	PAB	7.13 ± 0.39^{b}	$7.15 \pm 0.35^{k,l}$	$7.48 \pm 0.13^{q,r}$	7.26 ± 0.23	$6.87 \pm 0.12^{ac,ad}$	$6.77 \pm 0.17^{af,ag}$	7.18 ± 0.21	7.45 ± 0.30	7.57 ± 0.08	48.0	7.21 ± 0.27
Endospore-forming	BCM	$6.55 \pm 0.09^{d,e,g,h}$	$6.65 \pm 0.09^{k,l,m,n,o}$	$6.51 \pm 0.09^{q,r,t,u}$	$6.48 \pm 0.13^{v,w,y,z}$	$5.66 \pm 0.08^{ab,ac}$	$5.41 \pm 0.23^{ae,af,ag}$	6.24 ± 0.07	6.04 ± 0.26	5.96 ± 0.06	89.4	6.17 ± 0.43
Gram-positive ($Gram^{+}$) rods	RCM	$4.27 \pm 0.10^{a,b,c,d,e,f,g,h}$	$4.80 \pm 0.08^{k,l,m,n,o}$	$4.75 \pm 0.13^{q,r,s,t,u}$	$5.00 \pm 0.08^{y,z}$	5.33 ± 0.10^{ad}	5.45 ± 0.13^{ag}	5.35 ± 0.35^{ai}	5.45 ± 0.13^{aj}	5.93 ± 0.17	89.1	5.15 ± 0.49
Regular, nonsporing $Gram^{+}$ rods	MRS	$5.28 \pm 0.47^{d,f,h}$	5.11 ± 0.13	$5.55 \pm 0.41^{q,r,s,t,u}$	$5.24 \pm 0.28^{v,z}$	4.53 ± 0.25	4.76 ± 0.09	4.62 ± 0.13	4.63 ± 0.10	4.47 ± 0.33	61.8	4.91 ± 0.39
$Gram^{+}$, catalase-positive ($catalase^{+}$) cocci	BPM	3.85 ± 0.12	4.08 ± 0.13	3.90 ± 0.08	4.05 ± 0.13	3.93 ± 0.10	4.16 ± 0.12	4.06 ± 0.26	3.94 ± 0.20	3.80 ± 0.25	19.7	3.97 ± 0.12
$Gram^{+}$, catalase-negative ($catalase^{-}$) cocci	M17	$6.04 \pm 0.26^{d,e,f,g,h}$	$6.10 \pm 0.32^{i,j,k,l,m,n,o}$	$6.04 \pm 0.26^{p,q,r,s,t,u}$	$6.22 \pm 0.17^{v,w,x,y,z}$	7.00 ± 0.10	6.83 ± 0.22	6.84 ± 0.09	6.82 ± 0.08	6.81 ± 0.06	78.7	6.52 ± 0.41
	KFS	$6.16 \pm 0.12^{d,e,f,g,h}$	$6.08 \pm 0.04^{k,l,m,n,o}$	$6.12 \pm 0.15^{q,r,s,t,u}$	$6.22 \pm 0.17^{v,w,x,y,z}$	4.45 ± 0.07	4.50 ± 0.11	4.52 ± 0.24	4.64 ± 0.08	4.75 ± 0.08	97.4	5.27 ± 0.83
	KEAA	$5.70 \pm 0.36^{d,e,f,g,h}$	$5.79 \pm 0.14^{k,l,m,n,o}$	$5.85 \pm 0.16^{q,r,s,t,u}$	$5.79 \pm 0.08^{v,w,x,y,z}$	4.56 ± 0.25^{ac}	4.56 ± 0.25^{af}	4.97 ± 0.20	5.13 ± 0.09	5.02 ± 0.04	86.1	5.26 ± 0.53
	MSE	5.60 ± 0.53	$6.05 \pm 0.15^{l,n,o}$	5.69 ± 0.41	$6.18 \pm 0.40^{w,y,z}$	5.94 ± 0.28^{aa}	5.00 ± 0.25	5.52 ± 0.10	5.29 ± 0.21	5.22 ± 0.17	56.4	5.61 ± 0.40

Means (except for the great average) within a line with a superscript were statistically different from each other; statistical significance ($\alpha = 0.05$) and adjusted R^2 obtained for microbial counts (in each culture medium) within time in maize and rye flour at 20C, obtained via Tukey-HSD post-hoc tests of (9 × 1 factorial design) one-way ANOVA: *a*, 0 d × 1 d; *b*, 0 d × 2 d; *c*, 0 d × 3 d; *d*, 0 d × 7 d; *e*, 0 d × 9 d; *f*, 0 d × 14 d; *g*, 0 d × 29 d; *h*, 0 d × 39 d; *i*, 1 d × 2 d; *j*, 1 d × 3 d; *k*, 1 d × 7 d; *l*, 1 d × 9 d; *m*, 1 d × 14 d; *n*, 1 d × 29 d; *o*, 1 d × 39 d; *p*, 2 d × 3 d; *q*, 2 d × 7 d; *r*, 2 d × 9 d; *s*, 2 d × 14 d; *t*, 2 d × 29 d; *u*, 2 d × 39 d; *v*, 3 d × 7 d; *w*, 3 d × 9 d; *x*, 3 d × 14 d; *y*, 3 d × 29 d; *z*, 3 d × 39 d; *aa*, 7 d × 9 d; *ab*, 7 d × 14 d; *ac*, 7 d × 29 d; *ad*, 7 d × 39 d; *ae*, 9 d × 14 d; *af*, 9 d × 29 d; *ag*, 9 d × 39 d; *ah*, 14 d × 29 d; *ai*, 14 d × 39 d; and *aj*, 29 d × 39 d.

ANOVA, analysis of variance; BCM, *bacillus* cereus medium; BPM, Baird-Parker medium; KEAA, kanamycin esculin azide agar; KFS, Kenner faecal streptococcal agar; MRS, *Lactobacillus* de Man, Rogosa and Sharp agar; MSE, Mayeux, Sandine and Elliker agar; PAB, *Pseudomonas* agar base; RBCAB, Rose-Bengal chloramphenicol agar base; RCM, reinforced clostridial medium; TSA, tryptone soy agar; VRBDA, violet red bile dextrose agar; YEDCA, yeast extract dextrose chloramphenicol agar.

TABLE 2. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/GSAMPLE) THROUGHOUT TIME (0, 1, 2, 6 AND 8 DAYS) AND GREAT AVERAGE IN MAIZE (MR) AND RYE (RR) FLOUR, AND MOTHER-DOUGH (MDr) AT 4C

	No. of days	0	1	2	6	8	R^2	Great average
Target microorganisms	Culture media	Maize flour at 4C (Mr)						
Total viable counts	TSA, 30C	6.70 ± 0.00[a,c,d]	6.80 ± 0.00[e]	6.70 ± 0.00[h,j]	6.80 ± 0.00	6.80 ± 0.00	100.0	6.76 ± 0.06
Yeasts and molds	YEDCA, yeasts	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.00 ± 0.00
	RBCAB, molds	4.36 ± 0.08[d]	4.26 ± 0.20	4.26 ± 0.20[i]	4.20 ± 0.14	4.00 ± 0.00	33.4	4.22 ± 0.13
Facultative anaerobic Gram-negative	VRBDA	4.55 ± 0.11	4.64 ± 0.05[e,f,g]	3.57 ± 0.04	3.32 ± 0.29	3.40 ± 0.08	94.1	3.90 ± 0.64
(Gram⁻) rods	MacConkey	4.36 ± 0.16	4.35 ± 0.06[g]	4.55 ± 0.11[i]	4.24 ± 0.23[j]	3.85 ± 0.12	71.5	4.27 ± 0.26
Gram⁻ aerobic rods	PAB	4.82 ± 0.07[d]	4.83 ± 0.07[g]	4.84 ± 0.09[i]	4.75 ± 0.07	4.45 ± 0.30	44.3	4.74 ± 0.16
Endospore-forming Gram-positive	BCM	4.43 ± 0.10[d]	4.43 ± 0.10[g]	4.28 ± 0.10[i]	4.38 ± 0.15[j]	3.90 ± 0.12	75.2	4.28 ± 0.22
(Gram⁺) rods	RCM	5.00 ± 0.25[c,d]	4.96 ± 0.16[f,g]	5.07 ± 0.08[i]	4.78 ± 0.11	4.32 ± 0.33	61.4	4.83 ± 0.30
Regular, nonsporing Gram⁺ rods	MRS	5.20 ± 0.08	5.25 ± 0.13	4.83 ± 0.30	5.23 ± 0.22	5.00 ± 0.36	21.7	5.10 ± 0.18
Gram⁺, catalase-positive (catalase⁺) cocci	BPM	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.00 ± 0.00
Gram⁺, catalase-negative (catalase⁻) cocci	M17	4.73 ± 0.17	4.63 ± 0.13	4.74 ± 0.07	4.64 ± 0.08	4.69 ± 0.08	–4.3	4.68 ± 0.05
	KFS	3.67 ± 0.05[a]	3.80 ± 0.26[f]	3.75 ± 0.13	3.43 ± 0.13	3.48 ± 0.10	44.9	3.62 ± 0.17
	KEAA	3.85 ± 0.17	3.93 ± 0.15	3.97 ± 0.20	3.77 ± 0.15	3.82 ± 0.25	–6.8	3.87 ± 0.08
	MSE	4.43 ± 0.09	4.40 ± 0.16[f,g]	4.41 ± 0.28[h,j]	4.16 ± 0.23	3.85 ± 0.12	56.4	4.25 ± 0.25
Target microorganisms	Culture media	Rye flour at 4C (Rr)						
Total viable counts	TSA, 30C	6.81 ± 0.07	6.75 ± 0.07	6.79 ± 0.05	6.52 ± 0.35	6.99 ± 0.05	39.3	6.77 ± 0.17
Yeasts and molds	YEDCA, yeasts	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.00 ± 0.00
	RBCAB, molds	6.31 ± 0.05	6.35 ± 0.07	6.35 ± 0.09	6.27 ± 0.04	6.19 ± 0.11	30.0	6.30 ± 0.07
Facultative anaerobic Gram-negative	VRBDA	3.51 ± 0.26	3.50 ± 0.14	3.40 ± 0.14	3.60 ± 0.25	3.50 ± 0.14	–10.9	3.50 ± 0.07
(Gram⁻) rods	MacConkey	6.09 ± 0.10	6.31 ± 0.23[f,g]	6.22 ± 0.17[i]	5.98 ± 0.05	5.80 ± 0.05	60.0	6.08 ± 0.20
Gram⁻ aerobic rods	PAB	6.33 ± 0.24[d]	6.12 ± 0.15	6.33 ± 0.24[i]	6.05 ± 0.08	5.86 ± 0.07	46.7	6.14 ± 0.20
Endospore-forming Gram-positive	BCM	5.74 ± 0.07	5.75 ± 0.08[g]	5.73 ± 0.12[i]	5.49 ± 0.35	5.22 ± 0.17	51.2	5.59 ± 0.23
(Gram⁺) rods	RCM	6.14 ± 0.03	6.10 ± 0.08	6.10 ± 0.32	6.14 ± 0.19	5.81 ± 0.23	17.9	6.06 ± 0.14
Regular, nonsporing Gram⁺ rods	MRS	4.26 ± 0.22[d]	4.22 ± 0.17	4.19 ± 0.16	3.90 ± 0.18	3.88 ± 0.10	44.5	4.09 ± 0.19
Gram⁺, catalase-positive (catalase⁺) cocci	BPM	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.00 ± 0.00
Gram⁺, catalase-negative (catalase⁻) cocci	M17	5.99 ± 0.08	6.05 ± 0.06	6.05 ± 0.05	6.01 ± 0.05	6.00 ± 0.06	–2.1	6.02 ± 0.03
	KFS	4.29 ± 0.21[c,d]	4.12 ± 0.15	4.04 ± 0.26	3.80 ± 0.14	3.78 ± 0.10	51.0	4.01 ± 0.22
	KEAA	4.31 ± 0.12	4.24 ± 0.20	4.24 ± 0.20	4.12 ± 0.15	3.98 ± 0.15	24.2	4.18 ± 0.13
	MSE	5.53 ± 0.18[c,d]	5.42 ± 0.34[g]	5.69 ± 0.08[h,j]	5.06 ± 0.08	4.97 ± 0.04	69.3	5.34 ± 0.31
Target microorganisms	Culture media	Mother-dough at (MDr)						

Total viable counts	TSA, 30C	–	8.31 ± 0.23[a,b]	8.82 ± 0.07	8.64 ± 0.08	–	67.2	8.59 ± 0.26
Yeasts and molds	YEDCA, yeasts	–	6.87 ± 0.09[a,b]	7.98 ± 0.06	7.91 ± 0.05	–	98.6	7.58 ± 0.62
	RBCAB, molds	–	6.11 ± 0.10[b]	6.00 ± 0.42	5.33 ± 0.47	–	44.2	5.81 ± 0.43
Facultative anaerobic Gram-negative	VRBDA	–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	0.00 ± 0.00
(Gram$^-$) rods	MacConkey	–	4.71 ± 0.21	4.59 ± 0.05	4.60 ± 0.13	–	–1.5	4.63 ± 0.07
Gram$^-$ aerobic rods	PAB	–	6.87 ± 0.28	7.12 ± 0.34	7.24 ± 0.20	–	13.4	7.07 ± 0.19
Endospore-forming Gram-positive	BCM	–	8.64 ± 0.08	8.45 ± 0.30	8.81 ± 0.17	–	27.6	8.63 ± 0.18
(Gram$^+$) rods	RCM	–	8.14 ± 0.05[a,b]	7.76 ± 0.08	7.80 ± 0.00	–	91.6	7.90 ± 0.21
Regular, nonsporing Gram$^+$ rods	MRS	–	8.75 ± 0.08	8.75 ± 0.08	8.84 ± 0.09	–	3.8	8.78 ± 0.05
Gram$^+$, catalase-positive (catalase$^+$)	BPM	–	4.04 ± 0.26	4.38 ± 0.17	4.34 ± 0.13	–	31.4	4.25 ± 0.18
cocci								
Gram$^+$, catalase-negative (catalase$^-$)	M17	–	8.30 ± 0.02	8.32 ± 0.38	8.31 ± 0.04	–	–21.9	8.31 ± 0.01
cocci	KFS	–	8.12 ± 0.15	8.43 ± 0.24	8.24 ± 0.07	–	32.2	8.26 ± 0.16
	KEAA	–	8.39 ± 0.27	8.31 ± 0.23	8.28 ± 0.24	–	–17.2	8.33 ± 0.06
	MSE	–	8.28 ± 0.24[a,b]	8.77 ± 0.06	8.77 ± 0.16	–	64.0	8.60 ± 0.28

Means (except for the great average) within a line with a superscript were statistically different from each other; statistical significance ($\alpha = 0.05$) and adjusted R^2 obtained for microbial counts (in each culture medium) within time: (I) in maize and rye flour at 4C, obtained via Tukey-HSD post-hoc tests of (5 × 1 factorial design) one-way ANOVA: –, mean values nil in all observations: *a*, 0 d × 1 d; *b*, 0 d × 2 d; *c*, 0 d × 6 d; *d*, 0 d × 8 d; *e*, 1 d × 2 d; *f*, 1 d × 6 d; *g*, 1 d × 8 d; *h*, 2 d × 6 d; *i*, 2 d × 8 d; and *j*, 6 d × 8 d; and (II) in mother-dough at 4C, obtained via Tukey-HSD post-hoc tests of (3 × 1 factorial design) one-way ANOVA: –, mean values nil in all observations: *a*, 1 d × 2 d; *b*, 1 d × 6 d; *c*, 2 d × 6 d.

ANOVA, analysis of variance; BCM, *bacillus* cereus medium; BPM, Baird-Parker medium; KEAA, kanamycin esculin azide agar; KFS, Kenner faecal streptococcal agar; MRS, *Lactobacillus* de Man, Rogosa and Sharp agar; MSE, Mayeux, Sandine and Elliker agar; PAB, *Pseudomonas* agar base; RBCAB, Rose-Bengal chloramphenicol agar base; RCM, reinforced clostridial medium; TSA, tryptone soy agar; VRBDA, violet red bile dextrose agar; YEDCA, yeast extract dextrose chloramphenicol agar.

TABLE 3. STATISTICAL SIGNIFICANCE (A= 0.006/A= 0.01) OF CONTRAST ESTIMATES (MEAN DIFFERENCES) AND ADJUSTED R^2 OBTAINED FOR THE 9X2 / 5X2 FACTORIAL DESIGN BETWEEN TYPE OF FLOUR (B) IN EACH DAY (A) – (B AT A), FOR EACH DEPENDENT VARIABLE (CULTURE MEDIUM)

Contrast B at A	TSA, 30C	YEDCA, yeasts	RBCAB, molds	VRBDA	MacConkey	PAB	BCM	RCM	MRS	BPM	M17	KFS	KEAA	MSE
20C/4C	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)	(*B*1 × *B*2)
0 day (*A*1)/0 day (*A*1)	=/=	=/=	=/–1.95	0.78/1.04	=/–1.73	–1.39/–1.51	=/–1.31	1.16/–1.14	0.98/0.94	1.79/=	=/–1.27	=/–0.62	0.54/–0.47	0.65/–1.10
1 day (*A*2)/1 day (*A*2)	=/=	=/=	–0.42/–2.09	0.40/1.14	=/–1.96	–1.41/–1.29	=/–1.33	0.60/–1.13	1.15/1.03	1.60/=	=/–1.43	=/=	=/	=/–1.02
2 days (*A*3)/2 days (*A*3)	=/=	=/=	–1.00/–2.09	0.28/=	=/–1.67	–1.87/–1.49	=/–1.45	=/–1.04	0.58/0.63	1.56/=	=/–1.32	=/=	=/=	0.64/–1.29
3 days (*A*4)/6 days (*A*4)	–0.56/0.28	=/=	–0.95/–2.07	–0.35/=	=/–1.74	–1.63/–1.31	=/–1.11	=/–1.37	0.78/1.33	1.47/=	=/ 1.38	=/–0.38	0.61/=	=/–0.90
7 days (*A*5)/8 days (*A*5)	=/=	–1.55/=	–0.46/–2.19	–0.35/=	–1.87/–1.96	–1.11/–1.42	0.55/–1.32	=/–1.49	0.49/1.13	0.99/=	–1.07/–1.31	1.08/=	0.71/=	=/–1.12
9 days (*A*6)	=	–1.35	–0.77	–0.59	–1.59	–1.11	0.65	=	=	0.73	–0.60	0.79	0.63	=
14 days (*A*7)	–0.70	–0.59	=	–1.21	–2.26	–2.01	=	=	=	1.22	–0.88	0.61	=	=
29 days (*A*8)	–0.68	–0.77	=	–1.27	–2.44	–2.21	=	=	=	1.11	–0.97	=	=	=
39 days (*A*9)	–0.67	=	=	–1.18	–2.20	–2.55	=	=	=	0.99	–0.86	=	=	=
R^2	52.2/41.1	96.0/–	63.2/98.8	96.3/87.3	88.2/97.8	94.5/95.2	65.7/95.1	75.4/91.2	87.9/86.5	94.2/–	74.9/98.3	90.5/69.4	82.6/45.9	66.5/91.4

A refers to the sampling day (0 to 39 days / 0 to 8 days) and *B* to the type of sample (B1 – maize flour at 20C/4C, and B2 – rye flour at 20C/4C).

Notes: = , Mean differences not significantly different. The percent variation of the quantitative dependent variables explained by the factors (i.e., type of fermentation and time) in the model is given by R^2 – which is obtained by dividing the sum of squares between groups by the total sum of squares. Total viable counts on tryptone soy agar (TSA) for vegetative forms; yeasts on yeast extract dextrose chloramphenicol agar (YEDCA); molds on rose-Bengal chloramphenicol agar base (RBCAB); $Gram^-$ rods on violet red bile dextrose agar (VRBDA), *Pseudomonas* agar base (PAB) and MacConkey agar (MacConkey); $Gram^+$ rods on *Bacillus cereus* medium (BCM), reinforced clostridial medium (RCM), and on *Lactobacillus* de Man, Rogosa and Sharp agar (MRS); and $Gram^+$ cocci on Baird-Parker medium base (BPM), M17 agar (M17), Kenner faecal streptococcal agar (KFS), kanamycin esculin azide agar (KEAA) and Mayeux, Sandine and Elliker agar (MSE).