Journal of Food Quality

Journal of Food Quality ISSN 1745-4557

MICROBIAL ECOLOGY DYNAMICS IN PORTUGUESE *BROA* SOURDOUGH

JOÃO M. ROCHA[1,2,3,4]* and F. XAVIER MALCATA[4,5,6]

[1]Centro de Biotecnologia e Química Fina, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Arquiteto Lobão Vital, Apartado 2511, Porto, P-4202-401, Portugal
[2]Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Tapada da Ajuda, Lisboa, P-1349-017, Portugal
[3]Campus de Gualtar, Centro de Engenharia Biológica, Universidade do Minho, Braga, P-4710-057, Portugal
[4]Department of Chemical Engineering, University of Porto, Rua Dr. Roberto Frias, Porto, P-4200-465, Portugal
[5]Laboratory for Process Engineering, Environment, Biotechnology and Energy, Rua Dr. Roberto Frias, Porto, P-4200-465, Portugal

[6]Corresponding author.
TEL: (+351)968 017 411;
FAX: (+351)229 825 331;
EMAIL: fmalcata@fe.up.pt

Received for Publication September 26, 2015
Accepted for Publication August 23, 2016

*Present address: Centre of Molecular and Environmental Biology (CBMA), University of Minho, Campus Gualtar, P-4710-057 Braga, Portugal;
Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus Gualtar, P-4710-057 Braga, Portugal

10.1111/jfq.12244

ABSTRACT

This research effort aimed at a better understanding of microbial phenomena taking place during time in spontaneous sourdough fermentation for *broa*, a traditional Portuguese bread. Unlike most microbiological studies of sourdough, viable counts obtained were not limited to *Lactobacillus* and yeasts, but encompassed also molds, Gram-negative rods, endospore-(non)forming Gram-positive rods and catalase-positive/negative Gram-positive cocci. This less conventional approach unveiled the ubiquitous *Bacillus* genus throughout spontaneous *broa* sourdough fermentation. Presumptive yeasts, *Lactobacillus* and *Bacillus* were found to low levels after kneading, but became dominant by the end of regular (no aeration) fermentation. They apparently inhibit undesired rods and *Staphylococcus*, which were found to relatively high numbers after kneading. By 24 h of fermentation, lactic acid cocci accounted for an important fraction of biodiversity, and pH decreased significantly reaching about 4.1. Aeration accelerated the microbial dynamics. In terms of total viable counts, such a long-term fermentation appeared to reach a quasi-stationary state.

PRACTICAL APPLICATIONS

Microbial studies encompassing a large set of culture media and incubation conditions were previously undertaken in samples of maize and rye flours, and sourdoughs for *broa* obtained from 14 artisanal producers and in two seasons. Microbial counts and identification unfolded a complex wild microbiota, and fermentation played a major effect upon all microbial groups. Flours and mother-dough microbiota were characterized during storage afterward. A similar approach was followed here to ascertain the microbiological profile of a wide range of microorganisms throughout long-term sourdough fermentation, in attempts to answer the questions: how microorganisms evolve, and how they respond to increasing aeration. These studies provide an innovative way to look at sourdough systems and microbial dynamics therein. Results showed the importance of increasing fermentation time to take full advantage of ecological competition against undesirable microorganisms, and emphasized the need for further studies to reveal the role of *Bacillus* in sourdough fermentations.

INTRODUCTION

Sourdough has been used since ancient times in food processing; it is indeed one of the oldest biotechnological processes employed by Man. The use of sourdough in some wheat breads aims at flavor improvement, whereas in rye breads it is needed to confer suitable technological properties for baking (mainly arising from pH reduction). The vast literature regarding sourdough fermentation has consistently emphasized the importance of sourdough toward improvement of volume and crumb structure, flavor, nutritional attributes and shelf-life of bread. The dominant microbiota of various sourdoughs has been thus comprehensively studied, being typically complex micro-ecosystems with growth of microorganisms favored by low temperatures and high water activity prevailing during fermentation of dough (Faid *et al.* 1994; Corsetti *et al.* 1998; Arendt *et al.* 2007). Nevertheless, the literature on this topic has mainly focused on wheat sourdough fermentations, and specifically on yeasts and *Lactobacillus* (and less frequently on other lactic acid bacteria [LAB]) as dominant microbial flora (Corsetti *et al.* 1998; Arendt *et al.* 2007).

Bread and related products manufactured without baker's yeast resort to a sour ferment. Examples of such breads encompass Portuguese *broa* (Rocha *et al.* 2003; Rocha 2011; Rocha and Malcata 2012, 2015), Finnish sour-rye bread (Salovaara and Hatunpää 1984), German rye bread (Spicher and Werner 1988), San Francisco sourdough French bread (Kline and Sugihara 1971) and soda crackers (Sugihara 1985), wheat Italian panettone (Ottogalli *et al.* 1996), Italian pandoro (Zorzanello and Sugihara 1982) and colomba (Sugihara 1977), Iranian Sangak bread (Azar *et al.* 1977), and Sudanese Kisra (Abdalla *et al.* 1988; Osman *et al.* 2010), among other varieties found in Arabic and African countries and India, such as *lavash*, *injera*, *idli* and *dhokla* (Antony *et al.* 1996; Salovaara 1998).

Broa (Fig. 1) is a traditional type of Portuguese bread manufactured in the absence of a commercial starter culture. It is widely produced at artisan level by farmers of Northern Portugal, following ancient protocols empirically passed from generation to generation, and has earned the food specialty status of AOP. This bread holds a major economic importance, because of the large number of small farmers that depend on this extra source of income for survival; it also helps settling people in rural regions, thereby preventing rural exodus toward urban areas. *Broa* is obtained from maize (*Zea mays*) and rye (*Secale cereale*) flours, inoculated with a given amount of a previously fermented dough (or mother-dough). The *modus operandi* of artisan breadmaking of *broa* (Fig. 1) varies somehow (but not in essence) among local producers, namely type of milling (water-mill or electric mill), yield of milling and sifting, ratio between maize and rye flours, time and temperature of back-slopping and fermentation, amount of mother-dough added, manual kneading length and baking time (Rocha 2011; Rocha *et al.* 2011; Rocha and Malcata 2012, 2015). This diversity of protocols of *broa* breadmaking affect its adventitious microbiota (Rocha and Malcata 1999, 2012, 2015). For instance, the time mother-dough (Fig. 1) is kept prior to inoculation (i.e., between back-sloppings) varies considerably, since the frequency of bread production varies according to the needs of local farmers, coupled with its seasonal character (usually every other week in winter, and weekly during summer). Another important process parameter is time of fermentation of the starting sourdough (first fermentation) (Fig. 1): it is prepared by manual kneading maize and rye flours, warm water and mother-dough, and left overnight for a period ranging from about 6 to 18 h, variable from producer to producer, or even by the same producer (Rocha 2011). Finally, the duration of the second fermentation (Fig. 1) is also deemed important: dough is prepared with maize flour scaled with salted warm water, then rye flour and sourdough are progressively added and kneaded, and fermentation is allowed to take place for about 2 h, although it can take place for 1.5–3h (Rocha 2011).

An earlier survey of breadmaking processes employed by several local producers of *broa* (Rocha 2011), as well as the microbiological diversity found in a large number of traditional sourdoughs (Rocha and Malcata 2012) and, more recently, the study of the evolution in microbial ecology of the mother-dough (Fig. 1) during the storage period under refrigeration (Rocha and Malcata 2015), have raised several questions: (i) how fermentation time affects evolution of the microbial ecology of *broa* dough; (ii) how microbial ecology evolves throughout a long fermentation period and (iii) how sourdough (Fig. 1) microbiota responds to increasing aeration. In attempts to address these questions (while comprehensively covering a wide range of microbial groups), unleavened dough (of maize and rye flours), prepared according to artisanal procedures was analyzed for microbial counts throughout a long fermentation period (for up to 39 days), under controlled temperature and relative humidity. Furthermore, to study the effect of aeration, a sample of the same dough under continuous, gentle mixing was investigated for up to 14 days. Such a long fermentation period was undertaken to understand the behavior, and effects between batches upon existing complex microbiology throughout the long storage periods of mother-doughs (Fig. 1). Note that such long time windows are often times used by farmers between batches for *broa* production, thus representing in some cases the real storage time of the mother-dough (Fig. 1) kept (at room temperature or under refrigeration) between batches.

The originality of this research effort lies on its contribution to a better understanding of the phenomena during spontaneous sourdough fermentation. Studies on the effect

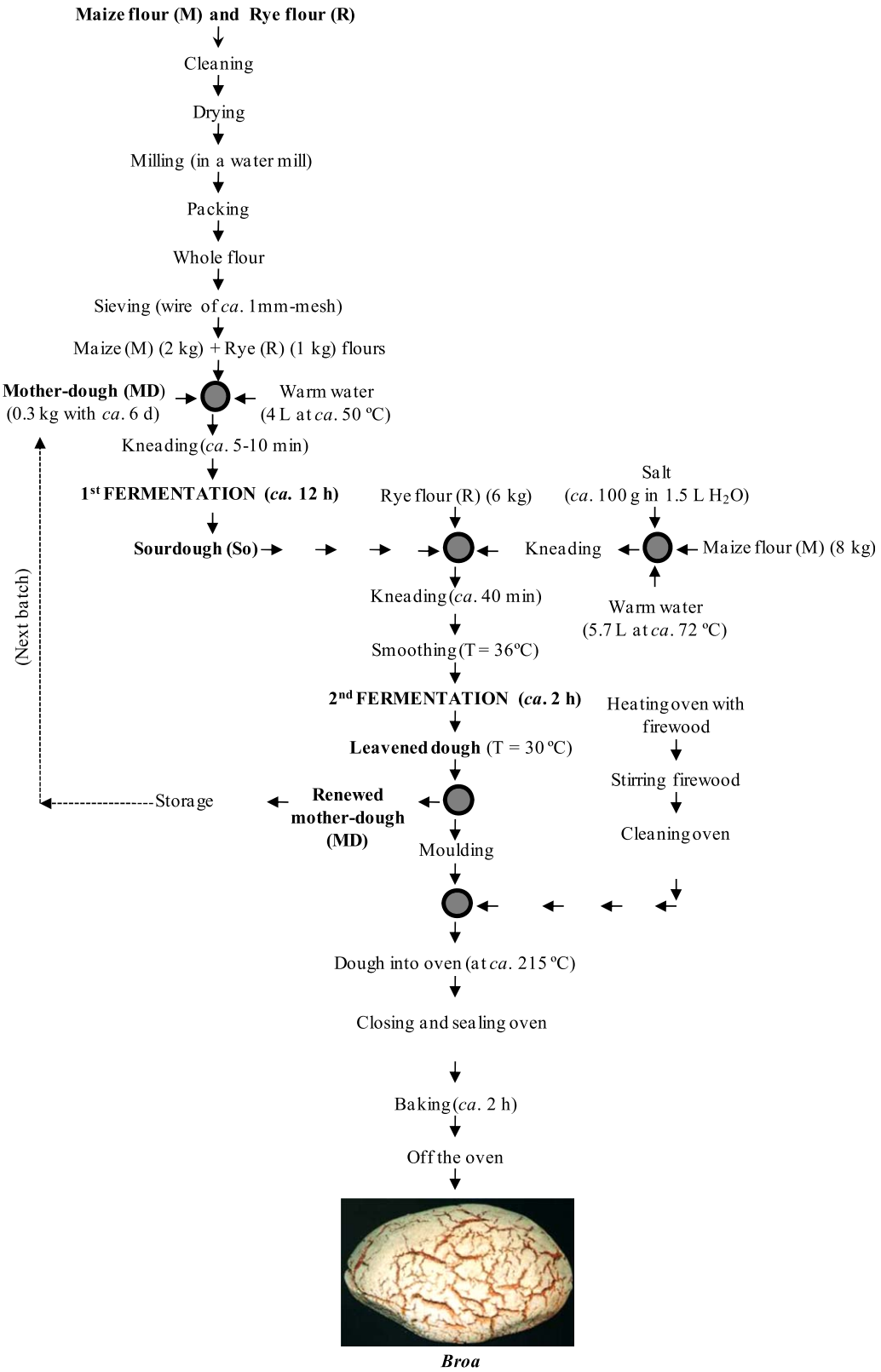

FIG. 1. FLOWCHART OF TRADITIONAL PORTUGUESE *BROA* SOURDOUGH BREAD (AND SPECIFIC EXPERIMENTAL CONDITIONS).

of long-term fermentation periods upon microbial ecology of sourdoughs are indeed scarce. Moreover, the joint study of microorganisms other than *Lactobacillus* and yeasts (viz. general mesophilic vegetative forms, molds, Gram-negative rods, endospore-forming and nonsporing Gram-positive rods, and catalase-positive and catalase-negative Gram-positive cocci) constitutes a distinct manner to ascertain microbial dynamics in sourdough, and reflects the importance of studies on the role of a broader range of microorganisms in sourdough.

MATERIALS AND METHODS

Traditional Manufacture of Sourdough

Traditional breadmaking procedures (Fig. 1) were employed *in loco* to prepare sourdough and *broa*, by a farmer from Cabeceiras de Basto (Portugal), as described in detail by Rocha and Malcata (2012, 2015); additional information of breadmaking technology of *broa* can be found in Rocha (2011) and Rocha *et al.* (2003, 2010a,b, 2011, 2012a,b); description of breadmaking of *broa* has also resorted to photographs *in situ* as per Rocha *et al.* (2003) and Rocha (2011).

A water-mill house was used for grinding and an 1-mm mesh sieve was employed. The maize and rye dough yield (DY) of sourdough (locally called *crescente*) (Fig. 1) was 233, and the mother-dough (locally called *isco*) (Fig. 1) added was about 6 days of age; the sourdough prepared was left for fermentation (first fermentation) (Fig. 1) overnight (ca., 12 h), at room temperature. The maize and rye dough prepared in the next day presented a DY of 151, and were left to ferment for about 2 h (second fermentation) (Fig. 1), at room temperature.

Experimental Design and Sampling

An aliquot from the single batch (1-batch) described above was taken after kneading, and prior to the 2-h (second) fermentation (Fig. 1). At this stage, the dough was composed by about 59% (w/w) maize and 41% (w/w) rye flours, 0.66 L_{water}/kg_{flour} and 5.9 g_{salt}/kg_{dough}. Physicochemical characteristics of maize and rye flours and sourdough can be found elsewhere (Rocha 2011; Rocha and Malcata 2012, 2015).

The aforementioned (1-batch) sample of type-I sourdough taken after kneading and prior to the 2 h fermentation (Fig. 1) was divided into two equal portions (of ca. 2.5 L), and kept in 3.5-L anaerobic jars (Oxoid, Basingstoke) in a Fitoclima-S600 PLH chamber with a ClimaPlus-400 controller (ARALAB, Albarraque, Portugal), under controlled temperature (20C) and relative humidity (60%). One half of said sourdough was left semi-closed (So), and the other (So-ag) left open under continuous stirring for enhanced aeration (ca., 20 rpm). Aliquots (in duplicate) of So were collected through fermentation at 0, 1, 2, 3, 7, 9, 14, 29 and 39 days, whereas aliquots (in duplicate) of So-ag were taken at 0, 1, 2, 3, 7, 9 and 14 days. The sourdough under stirring was fully dried by 14 days, so the experiment had to be discontinued by then. The samples were subject to microbiological analysis, and the effect of time (in So and So-ag sampling) as well as the effect of aeration (So versus So-ag) were studied.

Microbiological Enumeration

Microbial procedures were detailed by Rocha and Malcata (2012, 2015). Culture media and incubation conditions are tabulated in Table 1. Total viable counts were obtained after inoculation and incubation (in duplicate) on the appropriate culture media, according to Table 1. Therefore, four measurements were obtained for each fermentation time and incubation conditions. Results were expressed as logarithm of colony forming units (CFU) per gram of sample.

Statistical Methods

Effect of Time. Comparison of microbiological viable counts along time was independently done for So and So-ag samples (Table 2), via a one-way ANOVA using software IBM SPSS Statistics, v.22.0 (IBM, Chicago IL). The only factor considered was fermentation time (0, 1, 2, 3, 7, 9, 14, 29 and 39 days in So; and 0, 1, 2, 3, 7, 9 and 14 days in So-ag). The basic *F*-test in ANOVA was complemented with Brown-Forsythe and Welch tests. When *F*-test proved significant, Tukey-HSD *post-hoc* test was applied to ascertain differences between groups characterized by distinct fermentation times. An α-value (level of significance) of 0.05 was used as reference for the *F*- and *post-hoc* tests.

Effect of Aeration. To study the effect of aeration upon fermentation, So and So-ag samples were subjected to a two-way ANOVA (using SPSS) to ascertain the effect of aeration upon fermentation (Table 3). The parameters considered were: type of sample (So and So-ag); and fermentation time (0, 1, 2, 3, 7, 9 and 14 days). A full factorial model (with intercept) and a type-III sum of squares were used. A complete 7×2 factorial design was accordingly followed, by coding various contrasts. The α-value of 0.05, considered as reference for each *F*-test, was corrected for the multiple tests by dividing it by the number of tests (per family error-rate method).

RESULTS AND DISCUSSION

The statistical significance of the microbial counts (in Figs. 2a–5a, for So, and Figs. 2b–5b, for So-ag) was obtained

TABLE 1. GROWTH MEDIA, TARGET MICROORGANISMS AND INCUBATION CONDITIONS USED IN MICROBIOLOGICAL ANALYSES

Culture medium and supplement(s)	Target microorganisms	Incubation conditions
Total viable counts		
Tryptone soy agar (TSA, Lab M, Lancashire, UK); $pH_{final} = 7.0 \pm 0.2$	Total viable counts	30C; 1–2 days; Spread plate; Aerobiosis
Yeasts and molds		
Yeast extract dextrose chloramphenicol agar (YEDCA, Lab M); $pH_{final} = 6.6 \pm 0.2$ (not re-autoclaved); 2 vials/L X009 (Lab M)	Yeast counts	30C; 2 days, Spread plate; Aerobiosis
Rose-Bengal chloramphenicol agar base (RBCAB, Difco, Lawrence KS, USA), $pH_{final} = 7.2 \pm 0.2$; 2 vials/L rose Bengal Antimicrobial Supplement C (Difco)	Mold counts	Room temperature; 3–5 days; Darkness; Spread plate; Aerobiosis
Facultative anaerobic Gram-negative rods		
Violet red bile dextrose agar (VRBDA, Merck, Darmstadt, Germany), $pH_{final} = 7.4 \pm 0.2$ (not autoclaved)	Enterobacteriaceae counts	37C; 1 day; Pour-plate with overlay; Anaerobiosis
MacConkey agar (Merck); $pH_{final} = 7.1 \pm 0.2$	*Salmonella*, *Shigella*, *Yersinia* and others, and coliform bacteria	37C; 1 day; Spread plate, Anaerobiosis
Gram-negative aerobic rods		
Pseudomonas agar base (PAB, Lab M), $pH_{final} = 7.1 \pm 0.2$; 10.0 mL glycerol (Merck) (before autoclaving); 2 vials/L X108 CFC (Lab M)	*Pseudomonas* counts	30C, 1–2 days; Spread plate; Aerobiosis
Endospore-forming Gram-positive rods		
Bacillus cereus medium (BCM, Lab M); $pH_{final} = 7.2 \pm 0.2$; 100 mL/L X073 (Lab M); 2 vials/L X074 (Lab M)	*Bacillus* counts	37C; 1–2 days; Spread plate; Aerobiosis
Reinforced clostridial medium (RCM, Lab M); $pH_{final} = 6.8 \pm 0.2$; 100 μg/mL neomycin sulfate (Merck)	*Clostridium* counts	30C; 3 days; Spread plate; Anaerobiosis ($N_2 + H_2 + CO_2$, 10:10:80, v/v)
Regular, nonsporing Gram-positive rods		
de Man, Rogosa and Sharp agar (MRS, Lab M); $pH_{final} = 6.2 \pm 0.2$ (at 47C)	*Lactobacillus* (*Pediococcus* and *Leuconostoc*) counts	30C; 3–5 days; Spread plate; Aerobiosis
Gram-positive, catalase-positive cocci		
Baird-Parker medium base (BPM, Lab M); $pH_{final} = 6.8 \pm 0.2$; 50 mL/L X085 (Lab M); 50 mg/L sulphamethazine (Merck)	*Staphylococcus* (*Micrococcus*) counts	37C; 2 days; Spread plate; Aerobiosis
Gram-positive, catalase-negative cocci		
M17 (Merck); $pH_{final} = 7.2 \pm 0.2$	*Streptococcus* (*Lactococcus*) counts	30C; 2–3 days; Spread plate; Anaerobiosis
Kenner fecal streptococcal agar (KFS, Merck); $pH_{final} = 7.2 \pm 0.2$; 10 mL/L (1%) TTC (Merck)	*Streptococcus* (*Enterococcus*) counts	37C; 2–3 days; Spread plate; Anaerobiosis
Kanamycin esculin azide agar (KEAA, Merck); $pH_{final} = 7.1 \pm 0.2$	*Enterococcus* (group D-streptococci) counts	37C; 2–3 days; Spread plate; Anaerobiosis
Mayeux, Sandine and Elliker agar (MSE, Biokar, Beauvais, France); $pH_{final} = 6.9 \pm 0.2$	*Leuconostoc* counts	30C; 2–3 days; Spread plate; Aerobiosis

independently for each type of fermentation [sourdough obtained under regular conditions, i.e., fermentation without agitation (So), and sourdough obtained under gentle agitation (So-ag)] via 1-way-ANOVA and Tukey-HSD *post-hoc* tests, and is depicted in Table 2. Contrast estimates (mean differences) bearing a statistical significance, obtained in the 2-way ANOVA design encompassing the type of fermentation (So versus So-ag), are shown in Table 3.

pH

As a result of microbial action (Figs. 2–5, and Table 2), a major pH drop occurred in the first 24 h under both fermentation conditions, reaching about 4.1 as apparent in Fig. 2. The drop of pH in the first day plays an important role upon control of sourdough microbiota, and eventually upon the final taste and texture of *broa*, since it affects the hydration capacity of several constituents, enzymatic activities and such properties of the final bread as loaf volume, texture and aroma (Barber *et al.* 1982, 1987; Arendt *et al.* 2007). According to results presented below, the drop of pH during sourdough fermentation is also of major importance toward control of Gram-negative rods (Fig. 3), although the endospore-forming Gram-positive rods are likely to persist (Fig. 4). The pH decline in such a short initial time-window of 24 h is chiefly associated with the fast growth of Gram-

TABLE 2. STATISTICAL ANALYSIS OF THE EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/G_{SAMPLE}) AND GRAND AVERAGE ON DIFFERENT CULTURE MEDIA IN SOURDOUGH (SO) AND SOURDOUGH UNDER AGITATION (SO-AG) THROUGHOUT TIME

	N° days (d)	0	1	2	3	7	9	14	29	39	R^2	Grand average
Target microorganisms	Culture media	Sourdough (So)										
Total viable counts	TSA, 30C	$7.20 \pm 0.34^{a,b,c,d,e,f,g,h}$	$9.68 \pm 0.26^{m,o}$	$10.01 \pm 0.23^{p,q,r,s,t,u}$	8.97 ± 0.20	9.22 ± 0.17	9.10 ± 0.32	8.85 ± 0.37	$9.20 \pm 0,39$	8.93 ± 0.44	84.4	9.02 ± 0.78
Yeasts and molds	YEDCA, yeasts	$6.71 \pm 0.17^{a,b,f,g,h}$	$8.31 \pm 0.23^{j,k,l,m,n,o}$	$8.79 \pm 0.16^{p,q,r,s,t,u}$	$6.24 \pm 0.28^{x,y,z}$	$6.36 \pm 0.28^{ab,ac,ad}$	$6.46 \pm 0.32^{ae,af,ag}$	$7.64 \pm 0.05^{ah,ai}$	11.25 ± 0.05^{aj}	12.13 ± 0.10	99.0	8.21 ± 2.18
	RBCAB, molds	$6.31 \pm 0.24^{b,c,d,e,f}$	$5.75 \pm 0.50^{j,k,l,m,n,o}$	$5.02 \pm 0.17^{q,r,s,t,u}$	$4.65 \pm 0.29^{v,w,x,y,z}$	$7.88 \pm 0.40^{ac,ad}$	$7.72 \pm 0.49^{af,ag}$	$7.70 \pm 0.05^{ah,ai}$	6.15 ± 0.17	6.10 ± 0.26	92.5	6.36 ± 1.18
Facultative anaerobic Gram-negative rods	VRBDA	$5.88 \pm 0.20^{a,b,c,d,e,f,g,h}$	$4.52 \pm 0.55^{j,k,l,m,n,o}$	$4.38 \pm 0.39^{p,q,r,s,t,u}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.0	1.64 ± 2.50
	MacConkey	$6.60 \pm 0.19^{a,b,c,d,e,f,g,h}$	$7.49 \pm 0.67^{j,k,l,m,n,o}$	$7.82 \pm 0.24^{p,q,r,s,t,u}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.5	2.43 ± 3.67
Gram-negative aerobic rods	PAB	$5.88 \pm 0.17^{a,b,c,d,e,f,g,h}$	$8.97 \pm 0.32^{i,j,k,l,m,n,o}$	$6.63 \pm 0.36^{p,q,r,s,t,u}$	$4.91 \pm 0.11^{v,w,x,y,z}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.8	2.93 ± 3.64
Endospore-forming Gram-positive rods	BCM	$7.61 \pm 0.42^{a,b,c,d,e,g,h}$	$10.28 \pm 0.52^{i,j,k,m,n,o}$	$9.50 \pm 0.18^{p,s,t,u}$	$8.56 \pm 0.18^{v,w,x}$	$9.54 \pm 0.37^{ab,ac,ad}$	$9.76 \pm 0.12^{ae,af,ag}$	$7.51 \pm 0.14^{ah,ai}$	8.36 ± 0.16	8.36 ± 0.16	99.5	8.83 ± 0.98
	RCM	$4.37 \pm 0.30^{b,c,d,e,f,g,h}$	$4.26 \pm 0.25^{i,j,k,l,m,n,o}$	$2.73 \pm 0.28^{p,q,r,s,t,u}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.3	1.26 ± 1.95
Regular, nonsporing Gram-positive rods	MRS	$7.97 \pm 0.02^{a,b,c,d,e,f}$	$9.77 \pm 0.33^{j,m,n,o}$	$9.69 \pm 0.33^{p,s,t,u}$	$9.10 \pm 0.20^{w,x,y,z}$	$9.57 \pm 0.21^{ab,ac,ad}$	$10.12 \pm 0.15^{ae,af,ag}$	$6.32 \pm 0.03^{ah,ai}$	7.70 ± 0.00	8.10 ± 0.42	96.3	8.70 ± 1.26
Gram-positive, catalase-positive cocci	BPM	$4.72 \pm 0.16^{a,b,c,d,e,f,g,h}$	$3.87 \pm 0.35^{i,j,k,l,m,n,o}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.5	0.96 ± 1.91
Gram-positive, catalase-negative cocci	M17	$7.80 \pm 0.20^{a,b,c,d,e,f,g,h}$	$9.24 \pm 0.20^{j,k,l,m,n,o}$	$9.42 \pm 0.17^{p,s,t,u}$	$8.62 \pm 0.09^{v,w,x,y,z}$	$9.76 \pm 0.07^{ab,ac,ad}$	$9.70 \pm 0.25^{ae,af,ag}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.9	6.06 ± 4.58
	KFS	$7.93 \pm 0.15^{a,b,d,e,f,g,h}$	$9.85 \pm 0.54^{i,j,k,l,m,n,o}$	$9.18 \pm 0.14^{p,s,t,u}$	$8.39 \pm 0.30^{x,y,z}$	$8.67 \pm 0.14^{ab,ac,ad}$	$8.85 \pm 0.14^{ae,af,ag}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.7	5.88 ± 4.44
	KEAA	$8.19 \pm 0.17^{a,b,c,d,e,f,g,h}$	$9.77 \pm 0.48^{i,j,k,l,m,n,o}$	$9.22 \pm 0.17^{p,q,r,s,t,u}$	$6.04 \pm 0.26^{w,x,y,z}$	$6.29 \pm 0.21^{aa,ab,ac,ad}$	$7.26 \pm 0.22^{ae,af,ag}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.8	5.20 ± 4.08
	MSE	$7.84 \pm 0.13^{a,b,c,d,e,f,g,h}$	$9.95 \pm 0.37^{i,j,k,l,m,n,o}$	$9.30 \pm 0.20^{r,s,t,u}$	$8.89 \pm 0.24^{x,y,z}$	$9.00 \pm 0.25^{ab,ac,ad}$	$8.68 \pm 0.21^{ae,af,ag}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	99.8	5.96 ± 4.51
		Sourdough under agitation (So-ag)										
Total viable counts	TSA, 30C	$6.44 \pm 0.11^{a,b}$	$9.63 \pm 0.23^{g,h,i,j,k}$	$7.94 \pm 0.28^{l,m,n,o}$	6.43 ± 0.23	6.46 ± 0.26	6.48 ± 0.24	6.26 ± 0.43	–	–	95.1	7.09 ± 1.26
Yeasts and molds	YEDCA, yeasts	$6.82 \pm 0.16^{a,b,c,d,e}$	$7.97 \pm 0.17^{g,h,i,j,k}$	$7.58 \pm 0.09^{l,m,n,o}$	8.64 ± 0.16^{r}	8.66 ± 0.20^{t}	8.54 ± 0.10^{u}	7.08 ± 0.15	–	–	99.1	7.90 ± 0.76
	RBCAB, molds	$5.05 \pm 0.14^{a,b}$	$6.53 \pm 0.13^{h,i,j,k}$	$6.38 \pm 0.29^{l,m,n,o}$	5.58 ± 0.39^{p}	4.69 ± 0.55	5.27 ± 0.20	5.20 ± 0.24	–	–	18.2	5.53 ± 0.69
Facultative anaerobic Gram-negative rods	VRBDA	$6.01 \pm 0.11^{b,c,d,e,f}$	$5.71 \pm 0.57^{g,h,i,j,k}$	$3.55 \pm 0.33^{l,m,n,o}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	86.0	2.18 ± 2.83
	MacConkey	$6.62 \pm 0.16^{c,d,e,f}$	$5.93 \pm 0.85^{h,i,j,k}$	$6.05 \pm 0.14^{l,m,n,o}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	80.7	2.66 ± 3.32
Gram-negative aerobic rods	PAB	$6.01 \pm 0.38^{a,b,c,d,e,f}$	$5.06 \pm 0.82^{g,h,i,j,k}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	98.1	1.58 ± 2.72
Endospore-forming Gram-positive rods	BCM	$6.32 \pm 0.11^{a,b,c,d}$	$9.81 \pm 0.09^{g,h,i,j,k}$	$7.64 \pm 0.17^{l,m,n,o}$	5.56 ± 0.22^{q}	$5.51 \pm 0.35^{s,t}$	6.04 ± 0.26	5.98 ± 0.05	–	–	99.8	6.69 ± 1.55
	RCM	$4.35 \pm 0.26^{a,b,c,d,e,f}$	$4.64 \pm 0.12^{g,h,i,j,k}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	97.8	1.28 ± 2.20
Regular, nonsporing Gram-positive rods	MRS	$8.24 \pm 0.27^{a,c,f}$	$9.97 \pm 0.20^{g,h,i,j,k}$	7.80 ± 0.02^{o}	$7.32 \pm 0.18^{p,q,r}$	8.03 ± 0.41^{t}	7.97 ± 0.37^{u}	5.26 ± 0.30	–	–	99.7	7.80 ± 1.40
Gram-positive, catalase-positive cocci	BPM	$4.46 \pm 0.19^{b,c,d,e,f}$	$4.50 \pm 0.12^{g,h,i,j,k}$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	–	98.7	1.28 ± 2.19
Gram-positive, catalase-negative cocci	M17	$7.80 \pm 0.22^{a,b,c,d,e,f}$	$9.75 \pm 0.10^{g,h,i,j,k}$	$7.84 \pm 0.25^{l,m,n,o}$	5.97 ± 0.20^{r}	6.09 ± 0.10^{t}	5.90 ± 0.09^{u}	5.22 ± 0.17	–	–	97.7	6.94 ± 1.59
	KFS	$7.85 \pm 0.16^{a,c,d,e,f}$	$9.53 \pm 0.18^{g,h,i,j,k}$	$7.63 \pm 0.06^{l,m,n,o}$	5.53 ± 0.22^{r}	5.44 ± 0.32^{t}	5.31 ± 0.44^{u}	4.32 ± 0.49	–	–	97.7	6.52 ± 1.85
	KEAA	$7.79 \pm 0.34^{a,c,d,e,f}$	$9.58 \pm 0.12^{g,h,i,j,k}$	$7.51 \pm 0.14^{l,m,n,o}$	8.68 ± 0.12^{r}	8.64 ± 0.17^{t}	8.47 ± 0.33^{u}	5.08 ± 0.03	–	–	97.4	7.96 ± 1.44
	MSE	$7.94 \pm 0.21^{a,c,d,e,f}$	$9.59 \pm 0.14^{g,h,i,j,k}$	$7.61 \pm 0.11^{l,m,n,o}$	5.60 ± 0.28^{r}	5.49 ± 0.33	5.43 ± 0.38	4.93 ± 0.12	–	–	98.9	6.65 ± 1.74

Notes: Grand average was obtained as the mean of the microbial counts obtained throughout the entire periods of study. Means (except for the grand 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 viable counts (in each culture medium) within time (*A*) in sourdough (So), obtained via Tukey-HSD post-hoc tests of (9 × 1 factorial design) 1-way-ANOVA: ——, nil mean values; *a*, 0 days (A1) × 1 day (A2); *b*, 0 days (A1) × 2 days (A3); *c*, 0 days (A1) × 3 days (A4); *d*, 0 days (A1) × 7 days (A5); *e*, 0 days (A1) × 9 days (A6); *f*, 0 days (A1) × 14 days (A7), *g*, 0 days (A1) × 29 days (A8); *h*, 0 days (A1) × 39 days (A9); *i*, 1 day (A2) × 2 days (A3); *j*, 1 day (A2) × 3 days (A4); *k*, 1 day (A2) × 7 days (A5); *l*, 1 day (A2) × 9 days (A6); *m*, 1 day (A2) × 14 days (A7); *n*, 1 day (A2) × 29 days (A8); *o*, 1 day (A2) × 39 days (A9); *p*, 2 days (A3) × 3 days (A4); q, 2 days (A3) × 7 days (A5); *r*, 2 days (A3) × 9 days (A6); *s*, 2 days (A3) × 14 days (A7); *t*, 2 days (A3) × 29 days (A8); *u*, 2 days (A3) × 39 days (A9); *v*, 3 days (A4) × 7 days (A5); *w*, 3 days (A4) × 9 days (A6); ×, 3 days (A4) × 14 days (A7); *y*, 3 days (A4) × 29 days (A8); *z*, 3 days (A4) × 39 days (A9); *aa*, 7 days (A5) × 9 days (A6); *ab*, 7 days (A5) × 14 days (A7); *ac*, 7 days (A5) × 29 days (A8); *ad*, 7 days (A5) × 39 days (A9); *ae*, 9 days (A6) × 14 days (A7); *af*, 9 days (A6) × 29 days (A8); *ag*, 9 days (A6) × 39 days (A9); *ah*, 14 days (A7) × 29 days (A8); *ai*, 14 days (A7) × 39 days (A9); and *aj*, 29 days (A8) × 39 days (A9). Means (except for the grand 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 viable counts (in each culture medium) within time (A) in sourdough under agitation (So-ag), obtained via Tukey-HSD post-hoc tests of (7 × 1 factorial design) 1-way-ANOVA: *a*, 0 days (A1) × 1 day (A2); *b*, 0 days (A1) × 2 days (A3); *c*, 0 days (A1) × 3 days (A4); *d*, 0 days (A1) × 7 days (A5); *e*, 0 days (A1) × 9 days (A6); *f*, 0 days (A1) × 14 days (A7); *g*, 1 day (A2) × 2 days (A3); *h*, 1 day (A2) × 3 days (A4); *i*, 1 day (A2) × 7 days (A5); *j*, 1 day (A2) × 9 days (A6); *k*, 1 day (A2) × 14 days (A7); *l*, 2 days (A3) × 3 days (A4); *m*, 2 days (A3) × 7 days (A5); *n*, 2 days (A3) × 9 days (A6); *o*, 2 days (A3) × 14 days (A7); *p*, 3 days (A4) × 7 days (A5); *q*, 3 days (A4) × 9 days (A6); *r*, 3 days (A4) × 14 days (A7); *s*, 7 days (A5) × 9 days (A6); *t*, 7 days (A5) × 14 days (A7); and *u*, 9 days (A6) × 14 days (A7).

TABLE 3. STATISTICAL SIGNIFICANCE ($A = 0.007$) OF CONTRAST ESTIMATES (MEAN DIFFERENCES) AND ADJUSTED R^2 OBTAINED FOR THE 7 × 2 FACTORIAL DESIGN BETWEEN TYPE OF FERMENTATION (*B*) WITHIN TIME (*A*), IN EACH DEPENDENT VARIABLE (CULTURE MEDIUM)

Contrast B at A	TSA, 30C ($B_1 \times B_2$)	YEDCA, yeasts ($B_1 \times B_2$)	RBCAB, molds ($B_1 \times B_2$)	VRBDA ($B_1 \times B_2$)	Mac Conkey ($B_1 \times B_2$)	PAB ($B_1 \times B_2$)	BCM ($B_1 \times B_2$)	RCM ($B_1 \times B_2$)	MRS ($B_1 \times B_2$)	BPM ($B_1 \times B_2$)	M17 ($B_1 \times B_2$)	KFS ($B_1 \times B_2$)	KEAA ($B_1 \times B_2$)	MSE ($B_1 \times B_2$)
0 days (A_1)	−0.76	=	−1.27	0.13	=	=	−1.29	=	=	−0.26	=	=	=	=
1 day (A_2)	=	=	0.78	1.20	−1.56	−3.91	=	0.38	=	0.62	0.51	=	=	=
2 days (A_3)	−2.08	−1.21	1,35	−0.83	−1.77	−6.63	−1.86	−2.73	−1.89	=	−1.58	−1.56	−1.71	−1.70
3 days (A_4)	−2.54	2.41	0.93	=	=	−4.91	−3.01	=	−1.78	=	−2.65	−2.86	2.63	−3.30
5 days (A_5)	−2.76	2.30	−3.19	=	=	=	−4.03	=	−1.54	=	−3.67	−3.24	2.35	−3.51
9 days (A_6)	−2.62	2.08	−2.45	=	=	=	−3.71	=	−2.15	=	−3.80	−3.54	1.21	−3.25
14 days (A_7)	−2.59	−0.56	−2.51	=	=	=	−1.54	=	−1.06	=	5.22	4.32	5.08	4.93
R^2	96.3	95.7	91.8	99.0	99.2	99.3	97.7	99.5	97.0	99.6	99.6	98.8	99.1	99.2

A refers to sampling day (0–14 days) and *B* to type of fermentation (B_1 – with agitation, and B_2 – without agitation).
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 model is given by R^2 – which is obtained by dividing the sum of squares between groups by the total sum of squares.

negative rods and, to a larger extent, to LAB (i.e., regular nonsporing Gram-positive rods and Gram-positive catalase-negative cocci), as observed in Figs. 3–5 and Table 2.

Furthermore, aeration had no significant effect upon pH ($\alpha = 0.01$). By 14 days, pH attained 3.5 under both fermentation conditions (Fig. 2), which is consistent with Arendt *et al.* (2007) who claimed a typical pH in the range 3.5–4.3 by the end of the fermentation process of sourdoughs.

It is worth noting that the pH values typically found in wheat and rye sourdough breads are not reached in traditional *broa*: average values of pH obtained in complementary chemical analyses (data not shown) pertaining to sourdough and *broa* samples provided by several traditional producers were 4.15 and 5.16, respectively. The main reason for such high values found in *broa*'s sourdough and in the final bread (*broa*) is the shorter (first and second) fermentation time employed, and (to a lesser extent) the significantly higher buffer capacities of maize and rye flours relatively to wheat flours.

Total Viable Counts

In regular (no agitation) fermentation (Fig. 2a and Table 2), total viable counts on TSA increased significantly ($\alpha = 0.01$) up to 2 days, whereas under aeration (Fig. 2b and Table 2) it took only 1 day to attain said maxima. A significant decrease ($\alpha = 0.01$) in total viable counts was observed between 2 and 3 days (Fig. 2a and Table 2), probably due to the major drop of pH observed in the first 24 h and the consequent decline in microbiota diversity. Total counts stabilized after 3 days in both cases (Fig. 2a,b and Table 2), and from 3 days on they reached values comparable to the grand average of sourdoughs obtained from 14 regional producers and in two different periods of the year (Rocha and Malcata 2012). These results encompassing total viable counts are consistent with the microbial profiles obtained for different group of microorganisms studied (Figs. 3–5 and Table 2). Additionally, comparison of total viable counts from both fermentations revealed that significantly lower values ($\alpha = 0.05$) were obtained when aeration was present, except by 1 day when no significant differences could be detected (Table 3). In terms of total viable counts, such a long-term fermented sourdough appeared to be a quasi-stationary system during most of the period monitored.

Yeasts and Molds

Yeasts (on YEDCA) grew significantly on the first day, and from 9 days on under regular fermentation conditions (Fig. 2a and Table 2). Evolution of yeast counts revealed a gap of 1 day between fermentations (i.e., a lag period was observed when fermentation took place without aeration), with reference to the first 3 days (Fig. 2a,b, and Table 2). Under aeration (Fig. 2b and Table 2), yeast counts tended to

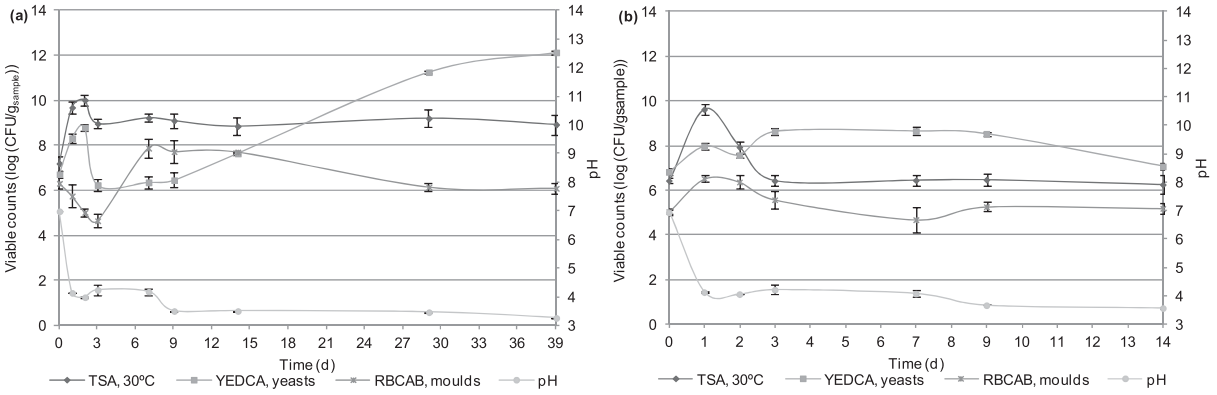

FIG. 2. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/G_{SAMPLE}) ON DIFFERENT CULTURE MEDIA FOR TOTAL VIABLE COUNTS, YEASTS AND MOLDS, AND PH IN (**A**) SOURDOUGH (SO) AND (**B**) SOURDOUGH UNDER AGITATION (SO-AG) THROUGHOUT TIME. TOTAL VEGETATIVE MESOPHILIC FORMS ON TRYPTONE SOY AGAR (TSA); AND YEASTS AND MOLDS ON YEAST EXTRACT DEXTROSE CHRORAMPHENICOL AGAR (YEDCA) AND ROSE-BENGAL CHLORAMPHENICOL AGAR BASE (RBCAB), RESPECTIVELY. STATISTICAL RESULTS ARE DEPICTED IN TABLE 2.

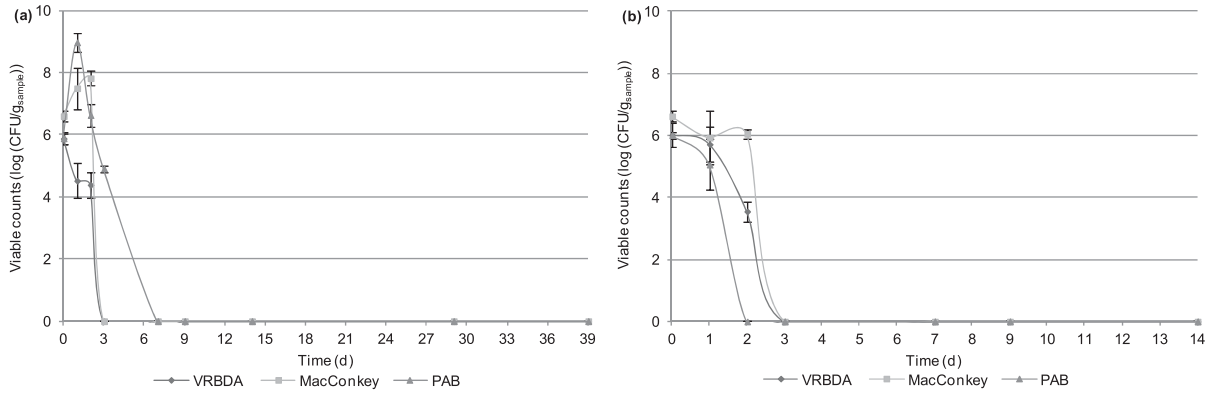

FIG. 3. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/G_{SAMPLE}) ON DIFFERENT CULTURE MEDIA FOR GRAM-NEGATIVE RODS IN (**A**) SOURDOUGH (SO) AND (**B**) SOURDOUGH UNDER AGITATION (SO-AG) THROUGHOUT TIME. FACULTATIVE ANAEROBIC GRAM-NEGATIVE RODS ON VIOLET RED BILE DEXTROSE AGAR (VRBDA) AND MACCONKEY AGAR (MACCONKEY); GRAM-NEGATIVE RODS ON *PSEUDOMONAS* AGAR BASE (PAB). STATISTICAL RESULTS ARE DEPICTED IN TABLE 2.

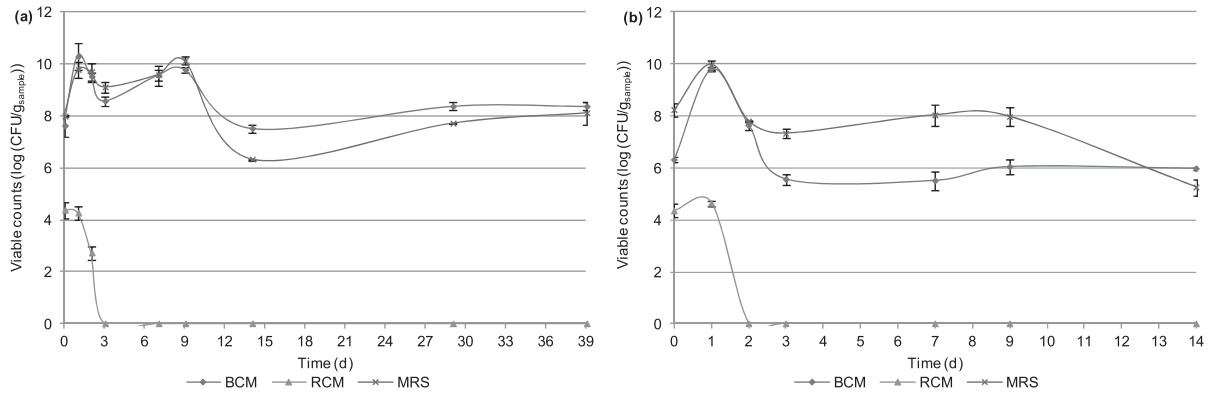

FIG. 4. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/G_{SAMPLE}) ON DIFFERENT CULTURE MEDIA FOR GRAM-POSITIVE RODS IN (**A**) SOURDOUGH (SO) AND (**B**) SOURDOUGH UNDER AGITATION (SO-AG) THROUGHOUT TIME. VEGETATIVE ENDOSPORE-FORMING GRAM-POSITIVE RODS ON *BACILLUS CEREUS* MEDIUM (BCM) AND REINFORCED CLOSTRIDIAL MEDIUM (RCM); AND REGULAR, NONSPORING GRAM-POSITIVE RODS ON DE MAN, ROGOSA AND SHARP AGAR (MRS). STATISTICAL RESULTS ARE DEPICTED IN TABLE 2.

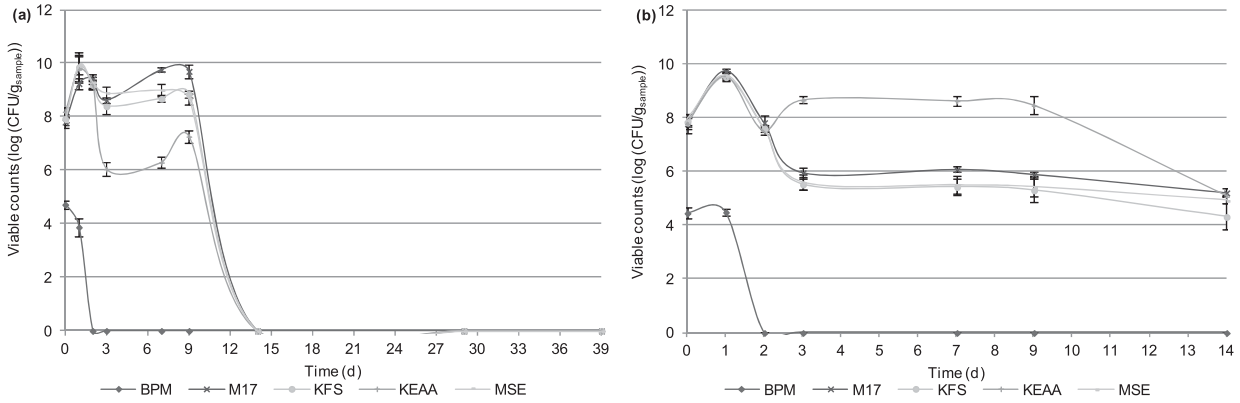

FIG. 5. EVOLUTION OF LOGARITHM OF TOTAL VIABLE COUNTS (AVERAGE ± STANDARD DEVIATION, CFU/G_{SAMPLE}) ON DIFFERENT CULTURE MEDIA FOR GRAM-POSITIVE COCCI IN (**A**) SOURDOUGH (SO) AND (**B**) SOURDOUGH UNDER AGITATION (SO-AG) THROUGHOUT TIME. GRAM-POSITIVE, CATALASE-POSITIVE COCCI ON BAIRD–PARKER MEDIUM BASE (BPM); AND GRAM-POSITIVE, CATALASE-NEGATIVE COCCI ON M17 AGAR (M17), KENNER FECAL STREPTOCOCCAL AGAR (KFS), KANAMYCIN ESCULIN AZIDE AGAR (KEAA) AND MAYEUX, SANDINE AND ELLIKER AGAR (MSE). STATISTICAL RESULTS ARE DEPICTED IN TABLE 2.

significantly stabilize ($\alpha = 0.05$) from 3 to 9 days, as happened in regular fermentation (Fig. 2a and Table 2); however, unlike regular fermentation, a small decrease took place by 14 days, so their viable numbers became identical to those at 0 days. Furthermore, yeast counts under aeration were significantly higher ($\alpha = 0.05$) during this period (by 3–9 days), and essentially the same as at the beginning of fermentation, specifically 0–1 days (Table 3).

Distinct deleterious effects of acetic and lactic acids upon yeasts (Häggman and Salovaara 2008) may possibly account for the significant growth of yeasts surprisingly observed from 9 days on (Fig. 2a and Table 2), as well as a complex range of local synergistic interactions between yeasts and LAB (Barber *et al.* 1983, 1987; Barber and Báguena 1988; Gobbetti *et al.* 1995; Almeida and Pais 1996b; Gobbetti 1998). According to our results, yeasts apparently adapted to the prevailing acidic environment (Gänzle *et al.*, 1998). Description of typical yeasts isolated from *broa* sourdough may be found elsewhere (Almeida and Pais 1996a; Rocha and Malcata 1999).

Regarding mold counts on RBCAB, maximum values were attained at an intermediate stage of 7–14 days in regular fermentation (Fig. 2a and Table 2), although molds are expected to be inhibited throughout fermentation due to outgrowth by (more rapidly multiplying) yeasts and bacteria; by the end of fermentation (29–39 days), mold counts were essentially the same as at the beginning. Under agitation (Fig. 2b and Table 2), mold counts tended to keep their values from 7 days on; unlike happened during 1–3 days, significantly lower counts than in regular fermentation were observed (Table 3), which can be justified by the adverse effect of mixing upon mold growth.

Gram-Negative Rods

Under regular conditions (Fig. 3a and Table 2), counts on VRBDA decreased significantly on the first day (which were similar to those by 2 days), and vanished by 3 days; counts on PAB increased significantly from 0 to 1 days, and then decreased significantly until depletion by 7 days; and counts on MacConkey increased significantly on the first day, but vanished from the third day on. Similarly to regular fermentation, under agitation (Fig. 3b and Table 2), viable counts on VRBDA and MacConkey media vanished by 3 days. However, the profile observed under the two fermentations was distinct, during the first 3 days, in terms of viable counts on VRBDA, PAB and MacConkey media; in addition, and unlike regular fermentation, the maximum values in these 3 culture media were observed by 0 days under additional aeration (Fig. 3a,b and Tables 2 and 3).

Compared with regular fermentation, viable counts on VRBDA medium were initially higher under aeration and lower by 2 days ($\alpha = 0.05$); and on MacConkey medium, they were lower by 1–2 days but similar at 0 days (Table 3). Viable counts on PAB medium were significantly lower ($\alpha = 0.05$) under agitation for 14 days (Table 3). Furthermore, vanishing of *Pseudomonas* on PAB medium was beneficially observed earlier when aeration was applied (Fig. 3a,b, and Table 2). Rocha and Malcata (1999) identified *broa* sourdough microorganisms grown on VRBDA and PAB media.

In a mixture of flour and water allowed to ferment for 1 day, Gram-negative endogenous bacteria initiate their metabolism with formation of gas and acidic odors, and a concomitant small drop in pH is observed. The competitive acid-tolerant yeasts and LAB reach rapidly viable numbers above those of the other adventitious microbiota initially

present in the flours, thus justifying the current results. LAB eventually becomes the dominating microbiota, so the dough becomes more acidic. The pH of a spontaneous ripen sourdough is typically 3.6–3.9, and the stability of sourdoughs depends on cooperation of specific species of yeasts and LAB (Röcken and Voysey 1995; de Vuyst *et al.* 2009). While bread is thermally treated through baking (thus contributing to its microbiological safety), our results (Fig. 3a,b, and Table 2) outlined the important role of fermentation toward inhibition of Gram-negative rods (grown on VRBDA, PAB and MacConkey), including potentially pathogenic and spoilage microorganisms originally present in flours (Salovaara 1998).

Among traditional producers of *broa*, the first spontaneous sourdough fermentation (i.e., first propagation step) (Fig. 1) is frequently very short in duration, and sometimes even neglected (Rocha 2011). Although maize flour for *broa* production is normally scalded, and further baking of dough (i.e., submission to strict heat treatment) brings about favorable effects toward food safety, the recommended period for *broa* spontaneous sourdough fermentation prior to dough manufacture (first fermentation), or even the recommend duration of both fermentations (i.e., the first and second fermentations) (Fig. 1) may still be questioned in attempts to take full ecological advantage against undesirable microbiota, and thus eventually increase shelf-life and safety of product. According to our results (Fig. 2a–5a and Table 2), a period of 2–3 days is indeed recommended for the first fermentation. An eventual extension of the second fermentation may also be recommended, although the effects on the final bread characteristics and the acceptance by consumers must be studied over increasing time of fermentation. In fact, typical Portuguese breads on the local market do not entertain acidity levels comparable to those found in sourdough breads from Central and Northern Europe. The artisanal homemade manufacture of *broa* occurs usually every other week or weekly, and even monthly (and, consequently, the same happens to the mother-dough between back-sloppings); hence, this extension of time for the first fermentation would not bring any extra processing requirements, such as need to increase fermentation vessel capacity or energy consumption (with concomitant extra costs), or time lost by farmers (to whom *broa* breadmaking represents a complementary source of income). While to our knowledge no public case exists of food poisoning arising from consumption of *broa*, our results showed that fermentation constitutes a critical control point, and increasing the *broa* fermentation period would be a good manufacturing practice to increase food safety.

Gram-Positive Rods

Viable counts on MRS by the end of the regular fermentation period (i.e., at 29 and 39 days) were essentially the same as at 0 days; in addition, *Lactobacillus* grew rapidly, especially between 0 and 1 day (Fig. 4a, and Table 2). In spite of significant similarity observed within the first day of fermentation (i.e., rapid multiplication), growth of *Lactobacillus* (on MRS) was significantly lower in fermentation under continuous aeration from 2 days on (Table 3); furthermore, different profiles were observed in the two fermentations (Fig. 4a,b and Table 2). It appears that *Lactobacillus* remained at high levels for a longer period under regular fermentation: no differences were indeed observed by 1–2 days under regular fermentation, whereas agitation decreased such numbers (Fig. 4a,b and Tables 2 and 3).

Microbial counts of typical *Lactobacillus* (and lactic acid Coccaceae) in regular fermentation (Fig. 4a and Table 2) were 10- to 100-fold those of their yeast counterparts Fig. 2a and Table 2), in agreement to what happens with wheat (Barber *et al.* 1983) and rye (Häggman and Salovaara 2008) sourdoughs. According to said results (Fig. 4a and Table 2), a 1 day-fermentation period was needed to reach sufficiently high counts of *Lactobacillus* and a significant decrease of pH, which represents an important breakthrough to be considered when attempting to choose an appropriate time for the (first) fermentation of sourdough for *broa*.

The role of sourdough fermentation upon inhibition of food spoilage and pathogenic microorganisms is mainly attributed to the LAB present therein, whereas homofermenters have a greater inhibitory effect against coliforms than heterofermenters. LAB can inhibit growth of accompanying microorganisms (contributing to their predominance and to regulation of microbial interactions in such complex systems) via production of several compounds, namely organic acids (in particular, acetic and lactic acids), hydrogen peroxide, carbon dioxide, ethanol and diacetyl. On the other hand, the relative insensitivity of some yeast strains contributes to their stable interactions with LAB in sourdoughs (Gobbetti and Corsetti 1997; Gobbetti 1998; Messens and de Vuyst 2002; Corsetti and Settanni 2007). Furthermore, LAB bacteriocins and bacteriocin-like inhibitory substances (produced by different LAB genera) are likely active against a large variety of Gram-positive bacteria (including spores of *Bacillus* and *Clostridium*), whereas Gram-negative bacteria are usually resistant, as well as yeasts and fungi (Gobbetti and Corsetti 1997; Caplice and Fitzgerald 1999; Messens and de Vuyst 2002; Corsetti and Settanni 2007; Settanni and Corsetti 2008). LAB, belonging to *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* genera, are also known to limit mycotoxinogenic mold growth (Dalié *et al.* 2010).

After having remained constant for 1 day *Clostridium* counts decreased significantly and eventually vanished by 3 days, under regular fermentation conditions (Fig. 4a and Table 2). Moreover, the same 1 day-advance in fermentation behavior was observed under agitation (Fig. 4a,b and Table 2); however, and unlike regular fermentation, a decrease

occurred in these viable counts from 0 to 1 day (Fig. 4b and Table 2). Under agitation, viable counts on RCM were significantly higher ($\alpha = 0.05$) by 1 day, and lower by 2 days, but were essentially similar throughout the remaining period (Table 3).

A large number of specific viable *Clostridium* species should be present in food to cause poisoning symptoms, while dangerous exotoxin-producer *Clostridium* species are more frequent in other foods where severe heat processing (as happens in bread) is not employed at all (Harrigan and McCance 1976). In fact, thermal baking and relative low pH values hamper their development throughout storage of *broa*. The effect of *broa* spontaneous sourdough fermentation toward *Clostridium* depletion is apparent in Fig. 4a and Table 2.

Bacillus (on BCM) were found to increase in number, and essentially remain at relatively high levels throughout this long-term fermentation (Fig. 4a and Table 2). Maximum viable counts were obtained by 1 day in both fermentations (Fig. 4a,b and Table 2). In regular fermentation, minima values were observed initially ($t = 0$ day) and by 14 days (Fig. 4a and Table 2). Under both fermentation conditions, *Bacillus* species (Fig. 4a,b and Tables 2 and 3) exhibited similar trends over 14 days, but were always significantly lower ($\alpha = 0.05$) under stirring (Table 3), except for the absence of differences by 1 day. The most abundant species of Gram-positive rods genera in sourdough for *broa* were reported by Rocha and Malcata (1999).

Unlike Gram-negative rods, the relatively high viable counts of endospore-forming Gram-positive rods (grown on BCM) observed throughout the whole period under scrutiny (Fig. 4a and Table 2) revealed the almost inevitability of having such microorganisms present during spontaneous sourdough fermentations. *Bacillus* spp. may grow over a wide range of pH values (from 2 to 11), which explains their ubiquitous presence in nature (Claus and Berkeley 1986).

Based on the data hereby generated, *Bacillus* are present to important levels in sourdoughs and, therefore, their role in spontaneous sourdough fermentation (rather than their potential health risks) has been clearly underestimated. In our view, said findings constitute an important contribution to sourdough science, and unfold a new challenge; more specifically, there is a need to better specify the role of ubiquitous *Bacillus* species in sourdough fermentations. Some species (e.g., *Bacillus subtilis*, *Bacillus coagulans* and *Bacillus cereus*) are broadly known to cause food spoilage, e.g., ropiness in bread and related foods, and are even potentially harmful to humans (Doyle 1989). According to Salovaara (1998), acids produced by LAB inhibit germination of endospores of *Bacillus* spp., which may, however, survive baking temperatures.

Therefore, the acidification via spontaneous sourdough fermentation appears important to effectively prevent malfermentation and bread spoilage, mainly as a result of poor hygienic practices and conditions prevailing in the bakery equipment. At pH levels typical of sourdoughs, the growth and activity of spoilage and ropiness microorganisms (e.g., some strains of *B. subtilis*, *Bacillus licheniformis*, *Bacillus megaterium* and *B. cereus*, as well as some species of *Clostridium*) may be prevented. Representative strains of *B. subtilis* and *B. licheniformis* may cause food-borne illness if present to levels over 10^5 CFU/g. Although food-borne illnesses related to consumption of ropy bread are unlikely to happen (due to the slimy manifestation of the crumb), loaves with relatively high counts of those species and no apparent rope symptoms may cause diarrhoea and vomiting (Röcken and Voysey 1995; Messens and de Vuyst 2002; Corsetti and Settanni 2007; Settanni and Corsetti 2008).

Despite claims that the levels of bacilli observed by the first 24 h of spontaneous sourdough fermentation do not constitute a real problem for *broa* breadmaking and consumption, their potential risks demand special caution; since *Bacillus* are present to high levels during spontaneous sourdough fermentation, their technological role upon spontaneous sourdough fermentation should not be neglected, unlike has happened to date. Moreover, extension of fermentation period would be a good manufacturing practice to be implemented in small-scale traditional breadmaking of *broa*, in attempts to increase food safety, thus avoiding potential (even remote) risks related to some groups of microorganisms. This realization is particularly important because short fermentation periods are frequent, thus resulting in *broa* exhibiting acidity well below that found in most sourdough breads. The implementation of such a procedure would not bring relevant logistic or technological constraints to the rural households where such a type of bread is manufactured.

Gram-Positive Cocci

In both fermentations (So and So-ag), *Staphylococcus* viable counts on BPM decreased significantly since the first day, and disappeared rapidly after 2 days (Fig. 5a,b and Table 2). Viable counts on BPM remained essentially the same by 0–1 days under agitation, but decreased during regular fermentation; furthermore, significantly lower values ($\alpha = 0.05$) at 0 day and higher at 1 day were attained under aeration (Table 3). The dynamics of disappearance of *Staphylococcus* (grown on BPM) was faster than that of Gram-negative rods (Fig. 3a and Table 2).

In both fermentations, Gram-positive catalase-negative cocci (Fig. 5a,b and Table 2) grew significantly in number within the first 24-h, but they also decreased rapidly afterward. Distinct behaviors resulted in both fermentations (Fig. 5a,b and Table 2). Under regular fermentation (Fig. 5a and Table 2), they remained at relatively high values until

9 days-fermentation but vanished afterward (14 days), whereas minimum viable counts were reached by this time under aeration (Fig. 5b and Table 2). In most culture media used, this group of microorganisms reached the maximum by 1 or 2 days under regular fermentation (Fig. 5a and Table 2), or by 1 day under agitation conditions (Fig. 5b and Table 2). This significant growth of Gram-positive catalase-negative cocci and their permanence for a relative long period confirm their importance in spontaneous sourdough fermentation, and justify their use in commercial starter cultures. On the other hand, viable counts of Gram-positive catalase-negative cocci in both fermentations were as follows (Fig. 5a,b, and Tables 2 and 3): mostly similar ($\alpha = 0.05$), by 0 and 1 days; unlike in KEAA medium, one observed significantly lower values ($\alpha = 0.05$) under agitation by 2 to 9 days on M17, KFS and MSE media; and (as stated before) counts were negligible by 14 days under regular fermentation, unlike happened under aeration. Description of typical Gram-positive cocci found in sourdough for *broa* may also be found in Rocha and Malcata (1999).

In spontaneous dough fermentation, LAB (which are the major contributors to acidification of dough) dominate rapidly over Gram-negative bacteria, in particular those belonging to *Lactobacillus* genus (Röcken and Voysey, 1995; de Vuyst *et al.* 2009). LAB are generally mesophilic but can grow under a wide range of temperatures (from 5 to 45C). In addition, most LAB strains grow at pH values ranging from 4.0 to 4.5, while some grow at pH values of 3.2 or 9.6 (Caplice and Fitzgerald 1999). Nevertheless, our Gram-positive catalase-negative cocci counts showed tended to disappear in long term fermentations. In fact, drying and acidic conditions prevailing during mother-dough storage may lead to LAB depletion in sourdough (Messens and de Vuyst 2002; Corsetti and Settanni 2007).

The relevant foregoing results of Gram-positive cocci (Fig. 5a,b and Table 2) emphasized the effect of fermentation time upon mother-dough microbiota. As stated before, mother-dough is preserved in home-bakeries and used on a weekly (or even monthly) basis, according to farmer's needs and the current season of year. Hence, an irregular use of mother-dough (besides short dough fermentation periods) may favor decrease of its biodiversity with regard to lactic cocci (viz. *Enterococcus*, *Pediococcus*, *Lactococcus* and *Leuconostoc*). In a previous study (Rocha and Malcata 2015), no significant differences in viable counts of Gram-positive cocci were observed throughout a storage period of mother-dough for 6 days at 4C.

General Discussion

After manual kneading and under regular fermentation conditions (Figs. 2a and 5a; Table 2), dough generally showed low viable counts of total microorganisms, yeasts, Bacillaceae and *Lactobacillus*, but high viable counts of *Staphylococcus* and Enterobacteriaceae. Furthermore, high total viable counts were reached by 2 days of fermentation, although *Staphylococcus* vanished by this time. Enterobacteriaceae and *Clostridium* vanished by 3 days, Pseudomonadaceae by 7 days and Gram-positive catalase-negative cocci by 14 days. Pseudomonadaceae, *Lactobacillus*, endospore-forming Gram-positive rods and Gram-positive catalase-negative cocci grew significantly within the first 24 h.

Lower counts of total viable microorganisms, *Bacillus* and *Lactobacillus* were typically obtained under increased aeration (Figs. 2b and 5b; Table 2), whereas higher yeast counts were observed from 3 to 9 days. In the first 3 days, however, evolution of total viable counts, yeasts, *Pseudomonas* and *Clostridium* were faster under agitation, with disappearance of Enterobacteriaceae, *Clostridiu*m and *Staphylococcu*s concomitantly observed. Regarding lactic acid Gram-positive cocci (grown on M17, KFS, KEAA and MSE media), a distinct behavior was found according to fermentation type (Table 3): significantly higher viable counts were attained throughout 2–9 days under regular fermentation, with vanishing by 14 days (unlike observed under aeration).

Since sourdough is an intermediate product, the role of the associated microorganisms may to advantage be assessed via their impact upon the final bread and other baked goods. The microbial growth and activity in sourdoughs are in general dependent on endogenous factors – i.e., those determined by cereal constituents (e.g., carbohydrate, nitrogen, vitamin, mineral and enzyme profiles), and on exogenous factors or processing parameters – such as temperature and time of fermentation, dough yield, redox potential, addition of sodium chloride, use of a sourdough starter or indigenous microorganisms, and number of propagation steps (Arendt *et al.* 2007).

The metabolic interactions between sourdough microorganisms enable them to use sources of substrates that would otherwise not be available; furthermore, the capacity to ferment other metabolites as a function of the prevailing ecological environments is also frequent. According to Gobbetti and Corsetti (1997), aeration may also induce changes in the metabolism of microorganisms; for instance, they verified that a fructose-negative strain of *L. sanfranciscensis* can co-ferment fructose in the presence of glucose and maltose. The same authors stated that similar changes in the sugar metabolism of obligate heterofermentative lactobacilli may also be induced by aeration, or by addition of other suitable electron acceptors instead of fructose (e.g., citrate or malate).

As stated earlier, sourdough under stirring became fully dry by 14 days; therefore, it is expected that the decreasing water activity dramatically affects microbial growth therein. Such an extended stirring period would not be practical at all, especially because manufacture of *broa* at artisanal and household scale is entirely handmade.

When comparing results in both fermentations (Figs. 2–5; Tables 2 and 3), a faster microbiota evolution was apparent under agitation. This finding may be of great importance, since fermentation represents a major processing step toward elimination of undesirable microorganisms. Although mechanized stirring may hardly be applicable in *broa* manufacture, an increase in manual kneading would certainly be feasible.

Final Considerations

Sourdough fermentation is usually required to bring about a few baking improvements, mostly arising from acidification during fermentation: increased loaf volume, shorter mixing time, increased mineral bioavailability, better machinability, more elastic behavior, enhanced anti-stalling features, higher sensory scores, longer shelf-life and improved nutritional value (Martínez-Anaya 1994; Arendt *et al.* 2007). However, presence of microorganisms other than LAB and yeasts has systematically been overlooked in the past, as well as the effect of extra aeration upon sourdough acidification.

Type-I sourdough for Portuguese *broa* is traditionally prepared by trial and error and at irregular periods, so a wide variation between batches is expected. To obtain a more stable sourdough and a final *broa* characterized by a higher and more consistent quality (i.e., improved taste, extended shelf-life, and enhanced resistance against microbial spoilage), control of selected breadmaking steps is a must. Our results suggested that inoculation with mother-dough at more regular intervals, and extension of fermentation time are feasible routes to address this goal; such approaches do not disturb the classical manufacture protocols at farm level. Regulation of acidity of *broa* (recall that pH 3.5–4.0 is usually accepted as optimum for sourdough breads) is thus of major importance to obtain a compromise between a pleasant acidic taste and a reasonable anti-bacterial and anti-mold performance (Martínez-Anaya 1994; Corsetti and Settanni 2007); and extra stirring for some time may help in this particular, as it fastens microbial metabolism, and consequently changes the microecology prevailing in dough.

CONCLUSIONS

The evolution of the micro-ecology during a long-fermentation period (hardly tackled so far in the literature), was studied in sourdough for *broa*, a Portuguese traditional bread made of maize and rye flours. Our results showed that a pH decrease, mainly due to metabolic activity of LAB, occurs in the first day of either type of fermentation conditions; pH further decreased 0.5 units by 14 days. Results also showed that spontaneous sourdough fermentation (i.e., the first fermentation prior to dough preparation) by a period of 2–3 days is recommended as a compromise between disappearance of microorganisms and sufficient multiplication of beneficial microorganisms, as well as to allow dough reach the acidity typical of sourdough breads. On the other hand, keeping mother-dough without renewal for a long time caused Gram-positive cocci belonging to LAB to essentially vanish.

Regarding fermentation under continuous, gentle mixing, our results showed that faster evolution of dough microbiota is promoted by aeration. One should specifically draw attention to the role of other groups of microorganisms, such as ubiquitous endospore-forming Gram-positive rods *Bacillus*, in spontaneous sourdough fermentations further to lactobacilli and yeasts.

NOMENCLATURE

Ag	agitation
B	*broa* (bread)
CFU	colony-forming units
DY	dough yield
LAB	lactic-acid bacteria
M	maize flour;
MD	mother-dough
R	rye flour
So	sourdough (sourdough obtained under regular fermentation conditions, i.e., without agitation/aeration)
So-ag	sourdough under agitation

ACKNOWLEDGMENTS

Several members of the Regional Directorate of Agriculture of Entre-Douro-e-Minho (DRAEDM, Portugal) and several local farmers are hereby gratefully acknowledged for their cooperation, in supplying samples for analysis. Financial support for author J.M.R. was provided by the fellowship PRAXIS XXI (ref. PRAXIS XXI/BD/16060/98), administered by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by author F.X.M. Partial financial support was from PAMAF (IED program, through research grant "*Pão de milho: caracterização do processo tradicional de produção e melhoramento tecnológico*," ref. PAMAF-1022), administered by Ministério da Agricultura, Desenvolvimento Rural e Pescas (Portugal) and coordinated by F.X.M. The author J.M. Rocha acknowledges the Centre of Molecular and Environmental Biology (CBMA) from the Department of Biology (DB) of University of Minho (UM), Portugal, where currently he is assistant researcher in the research line "Eco-labels for farmers, industry and consumer benefits", from the project "EcoAgriFood: Innovative green products and processes to promote Agri-Food BioEconomy", Operation NORTE-01-0145-FEDER-000009, with financial funding

allocated by European Regional Development Fund (FEDER) under the scope of Northern Regional Operational Programme – North 2020 and the financial support from the European Social Fund (FSE). The author J.M. Rocha also thanks the FCT Strategic Project of UID/BIO/04469/2013 unit, the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462) and the project "BioInd – Biotechnology and Bioengineering for improved Industrial and Agro-Food processes", REF. NORTE-07-0124-FEDER-000028, co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte) [Northern Regional Operational Framework Program, ON.2], QREN, FEDER. Author J.M. Rocha also gratefully acknowledges the Centre of Biological Engineering (CEB) and the University of Minho (UM), Portugal, for the research grants (Ref. # uminho/bpd/49/2014 and Ref. # uminho/bpd/53/2015) under the Integrated Framework Program ON.2 BIO2Norte – Biotechnology and Bioengineering for the Quality of Life and Sustainable Development (N-01-07-01-24-01-09). Finally, revision of this research manuscript was partially revised during a period at the Department of Chemical Engineering (DEQ), Faculty of Engineering, University of Porto (FEUP), Portugal, therefore author acknowledges the utilization of laboratory premises to perform this project. The authors have no conflicts of interest whatsoever to declare.

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.

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.

AZAR, M., TER-SARKISSIAN, N., GHAVIFEK, H., FERGUSON, T. and GHASSEMI, H. 1977. Microbiological aspects of Sangak bread. J. Food Sci. Tech. (India) *14*, 251–254.

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

BARBER, S., MARTÍNEZ-ANAYA, M.A. and GONZÁLEZ-CAUDELÍ, C. 1982. pH y propriedades funcionales de la masa panaria. Rev. Agroquím. Tecnol. Aliment *22*, 575–588.

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. Rev. Agroquím. Tecnol. Aliment *23*, 552–562.

BARBER, S., MARTÍNEZ-ANAYA, M.A., BÁGUENA, R. and TORNER, M.J. 1987. Microflora de la masa madre panaria. IV. Propiedades funcionales, en masas elaboradas com harina de trigo, de microorganismos puros – levaduras y lactobacilos – aislados de masas madre industriales. Rev. Agroquím. Tecnol. Aliment *27*, 107–119.

CAPLICE, E. and FITZGERALD, G.F. 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. *50*, 131–149.

CLAUS, D. and BERKELEY, R.C.W. 1986. Genus *Bacillus*. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2, (P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, eds.) pp. 1105–1139, Williams & Wilkins, Baltimore.

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.

DALIÉ, D.K.D., DESCHAMPS, A.M. and RICHARD-FORGET, F. 2010. Lactic acid bacteria – potential for control of mould growth and mycotoxins: a review. Food Control *21*, 370–380.

DOYLE, M.P. 1989. *Foodborne Bacterial Pathogens* (pp. 2–26), Marcel Dekker, New York, http://www.rsc.org/ebooks/archive/free/. Accessed 01-05-2011.

FAID, M., BORAAM, F., ZYANI, I. and LARPENT, J.P. 1994. Characterization of sourdough bread ferments made in the laboratory by traditional methods. Z. Lebensm. Unters. Forsh. *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. 1998. The sourdough microflora: interactions of lactic acid bacteria and yeasts. Trends Food Sci. Technol. *9*, 267–274.

GOBBETTI, M. and CORSETTI, A. 1997. *Lactobacillus sanfrancisco*, a key sourdough lactic acid bacterium: a review. Food Microbiol. *14*, 175–187.

GOBBETTI, M., SIMONETTI, M.S., CORSETTI, A., SANTINELLI, F., ROSSI, J. and DAMIANI, P. 1995. Volatile compounds and organic acid production by mixed wheat sour dough starters: influence of fermentation parameters and dynamics during baking. Food Microbiol. *11*, 625–630.

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

HARRIGAN, W.F. and MCCANCE, M.E. 1976. *Laboratory Methods in Food and Dairy Microbiology*, pp. 10–16, 25–31, 59–65, 106–108, 155–156, 214, Academic Press, London.

KLINE, L. and SUGIHARA, T.F. 1971. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the Souring activity. Appl. Microbiol. *21*, 459–465.

MARTÍNEZ-ANAYA, M.A. 1994. Review: factors influencing the quality of wheat sourdough processes, and the use of chemometrics in quality assessment. Rev. Esp. Ciencia Tecn. Aliment *34*, 469–493.

MESSENS, W. and DE VUYST, L. 2002. Inhibitory substances produced by *Lactobacillus* isolated from sourdoughs – a review. Int. J. Food Microbiol. *72*, 31–43.

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.

OTTOGALLI, G., GALLI, A. and FOSCHINO, R. 1996. Italian bakery products obtained with sourdough: characterization of the typical microflora. Adv. Food Sci. *18*, 131–144.

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. PhD thesis, 705 pp, http://hdl.handle.net/10400.5/3876.

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. and MALCATA, F.X. 2015. Behavior of the complex micro-ecology in maize and rye flour and mother-dough for broa throughout storage. J. Food Qual. *39*, 218–233.

ROCHA, J.M., BRÁS, A., TRIGUEIROS, J.J.B.L. and MALCATA, F.X. 2003. *Pão de milho: Caracterização do produto tradicional de produção e melhoramento tecnológico* [Bread of maize: Characterization of the traditional product and technological improvement], (ESB-UCP, ed.), 76 pp., Porto.

ROCHA, J.M., KALO, P.J. and MALCATA, F.X. 2010a. Neutral lipids in non-starch lipid and starch lipid extracts from Portuguese sourdough bread. Eur. J. Lipid Sci. Tech. *112*, 1138–1149.

ROCHA, J.M., KALO, P.J., OLLILAINEN, V. and MALCATA, F.X. 2010b. Separation and identification of neutral cereal lipids by normal phase high-performance liquid chromatography, using evaporative light-scattering and electrospray mass spectrometry for detection. J. Chromatogr. a *1217*, 3013–3025.

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.

ROCHA, J.M., KALO, P.J. and MALCATA, F.X. 2012a. Composition of neutral lipid classes and content of fatty acids throughout sourdough breadmaking. Eur. J. Lipid Sci. Tech. *114*, 294–305.

ROCHA, J.M., KALO, P.J. and MALCATA, F.X. 2012b. Fatty acid composition of non-starch and starch neutral lipid extracts of Portuguese sourdough bread. J. Am. Oil Chem. Soc. *89*, 2025–2045.

RÖCKEN, W. and VOYSEY, P.A. 1995. Sour-dough fermentation in bread making. J. Appl. Bacteriol. 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.

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

SETTANNI, L. and CORSETTI, A. 2008. Application of bacteriocins in vegetable food preservation. Int. J. Food Microbiol. *121*, 123–138.

SPICHER, G. and WERNER, N. 1988. Proteolytic activity of sourdough bacteria. Appl. Microbiol. Biotechnol. *28*, 487–492.

SUGIHARA, T.F. 1977. Non-traditional fermentations in the production of baked goods. Bakers Dig. *51*, 76–78.

SUGIHARA, T.F. 1985. Microbiology of breadmaking. In: *Microbiology of Fermented Foods*, Vol. 1 (B.J.B. Wood, ed.) pp. 249–261, Elsevier Applied Science, London.

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.

ZORZANELLO, D. and SUGIHARA, T.F. 1982. The technology of pandoro production – italian christmas cake. Bakers Dig. *56*, 12–15.