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Microbiological profile of maize and rye flours, and sourdough used for the manufacture of traditional Portuguese bread

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ABSTRACT

A thorough microbiological study of maize and rye flours, and sourdoughs obtained therefrom for eventual manufacture of *broa* – a dark sour bread typical in Northern Portugal, following artisanal practices, was carried out. Towards this purpose, samples were supplied by 14 artisanal producers, selected from 4 sub-regions, during two periods of the year. Total viable counts, as well as viable mesophilic and thermophilic microorganisms, yeasts and molds, Gram$^-$ rods, endospore-forming and non-sporing Gram$^+$ rods, and catalase$^+$ and catalase$^-$ Gram$^+$ cocci were assayed for. The comprehensive experimental dataset unfolded a unique and rather complex wild microflora in flours and sourdoughs throughout the whole region, which did not discriminate among sub-regions or seasons, or flour source for that matter. However, fermentation played a major role upon the numbers of the various microbial groups: the viable counts of yeasts, lactobacilli, streptococci, lactococci, enterococci and leuconostocs increased, whereas those of molds, Enterobacteriaceae, Pseudomonadaceae, staphylococci and micrococci decreased.

1. Introduction

Broa – a type of bread that still follows ancient manufacturing protocols and is widely manufactured at the farm level in Northern Portugal, is highly appreciated in the wide market for its distinct flavour and unique texture. The empirical know-how involved in the manufacture process has been transmitted from generation to generation, but scientific knowledge on the underlying microbiology is still incipient.

Maize, and such other cereals as sorghum and millet are often used for fermented cereal-based foods other than bread, especially in Africa – *e.g.*, beverages, gruels and porridges, dumplings used in stews, and fried products (Salovaara, 1998). In Portugal, maize flour is used in combination with rye flour to produce *broa*, a home-baked sourdough bread, still following ancient protocols. Instead of inoculating with a commercial starter culture, dough for *broa* is obtained by mixing maize and rye flours with water and salt, and soured via a given small amount of previously fermented dough – which is kept and passed from batch to batch, thus serving as a reservoir of adventitious microorganisms with a starter role. *Broa* consists in bread units 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.* 1–2 cm in thickness. In the manufacture of *broa*, sourdough is normally prepared one day prior to baking by adding maize and rye flours, water and mother dough in a wooden kneader. In the next morning, water with salt is usually warmed-up (sometimes to boiling point) prior to addition to the weighed maize flour, and briefly kneaded by hand; rye flour is subsequently added to the water, being in general present at 0.66 L kg^{-1} flour. Before manufacture, the flours are passed through sieves with a mesh of *ca.* 1 mm. The rye flour content of *broa* may vary between *ca.* 15 and 50% – which is typically added after maize flour being soaked with warm water and kneaded for a short period. At this point, the temperature of dough in the wooden kneader has already decreased, so the previous sourdough is gradually mixed; the

Abbreviations: B, *Broa* (bread); Catalase$^-$, catalase-negative; Catalase$^+$, catalase-positive; CFU, colony-units forming; FQ, fermentation quotient; Gram$^-$, Gram-negative; Gram$^+$, Gram-positive; LAB, lactic acid bacteria; M, maize flour; Oxidase$^-$, oxidase-negative; Oxidase$^+$, oxidase-positive; PCA, principal component analysis; R, rye flour; So, Sourdough; TTA, total titratable acidity; W, wheat flour.

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manual kneading process in the wooden kneader takes usually 30 min, after which it is allowed to stand for fermentation to occur for 1 up to 2 h, at room temperature. The clay wall oven is heated with firewood up to *ca.* 250 °C – and the dough is baked there during 30 min–3 h. The frequency of bread production varies according to the needs of local farmers, coupled with a seasonal character – usually every other week during winter, and weekly during summer.

The microbial flora of other sourdoughs has been studied to some length focusing mainly on yeasts and LAB (Arendt et al., 2007); our specific matrix supports, however, a unique environment in which wild strains of microorganisms grow, with yeasts and LAB also predominating. As a consequence, most chemical reactions therein are brought about by that wide and complex set of adventitious microflora, via synergistic interaction toward development of an acid taste. In general, a number of LAB metabolites and the drop in pH affect positively the texture of bread and shelf-live (Arendt et al., 2007). Furthermore, the continuous propagation of sourdough promotes spontaneous ecological selection (Almeida and Pais, 1996a, 1996b; Arendt et al., 2007).

Sourdough breads have enjoyed increasing popularity as convenient, nutritious, stable, natural and healthy foods. Hence, consumption of sourdough bread has steadily increased throughout Europe, especially central Europe and Scandinavia; this entails a major opportunity to expand the *broa* market. Manufacture of *broa* plays important roles from both economic and social standpoints, yet a long way is yet to be tracked before such a specialty food will be officially certified via an *Appéllation d'Origine Protégée* (AOP) label. Scientifically-sound characterization of *broa* will contribute to improve its quality, and to more effectively support health claims associated with its consumption, coupled with rational optimization of its manufacture process; this will help expand market niches in number and sizes, and consequently its economic value as a food commodity or specialty.

To our knowledge, the study of Portuguese traditional sourdough bread has been restricted to the work by Almeida and Pais (1996a, 1996b) – encompassing the yeast population, and Rocha and Malcata (1999) – encompassing both bacteria and yeasts. This research effort revisited the characterization of the microecology prevailing throughout the *broa* breadmaking process – *viz.* maize and rye flours, and sourdough; it was aimed at a better understanding of the phenomena that take place during fermentation. Microflora other than yeasts and lactobacilli – which at present are still poorly characterized in sourdoughs, were also included in the present work. To abide to consumer protection efforts and expand commercialization of *broa*, assessment of variability of the microflora of flours and sourdoughs within the geographical location of manufacture, and of its seasonality was also addressed.

2. Materials and methods

2.1. Sampling and chemicals for microbiological analysis

Samples were taken at random from flours of regional maize (M), rye (R) and wheat (W) prior to breadmaking, as well as from sourdough (So) immediately before manufacture of dough for *broa*; they were placed in sterile stomacher packages (Seward, London, UK), and immediately sent to our laboratory under refrigerated conditions. The samples of sourdough – prepared 1 day prior to baking, were taken after the first fermentation and labeled as sourdough: in general, the sourdough is prepared by manual mixing and kneading of mother dough, water, maize and rye flours, and kept overnight (12–18 h) in the wooden kneader. Normally, regional maize and rye flours are ground in a water-mill, sieved, and stored inside large bags in the mill house, for up to 2 days.

The materials were provided by 14 local certified farmers, within the 4 more representative sub-regions (Alto Minho, Basto, Vale do Sousa and Avintes). Two different counties among each sub-region, and at least 2 producers from each county were chosen: Paredes de Coura and Melgaço, for Alto Minho sub-region; Cabeceiras de Basto and Celorico de Basto, for Basto sub-region; Lousada and Penafiel, for Vale do Sousa sub-region; and Avintes, for Porto region. Furthermore, samples from each farmer were collected in two different periods of the year – in the so called cold (c) and warm (w) seasons, which corresponded to autumn-winter and to spring-summer, respectively. Traditional breadmaking procedures were followed *in loco* – as described in Rocha et al. (2011), for preparation of sourdough and baking of *broa* (B).

2.2. Culture media and microbiological enumeration

The culture media (Table 1) were purchased from Biokar (Beauvais, France), Difco (Lawrence KS, USA), Lab M (Lancashire, UK) and Merck (Darmstadt, Germany). *Herellea* agar (HA), *Alcaligenes* nutrient agar yeast extract (ANAYES), trypticase soy yeast extract starch agar (TSYES), fermentation medium agar (FM), plate count agar with furazolidone (PCAF) and lactic streak agar (LSA) culture media were reconstituted. The composition of HA was as follows: 40.0 g L^{-1} tryptone soy agar (Biokar), 10.0 g L^{-1} lactose (Fluka, Buchs, Switzerland), 10.0 g L^{-1} D-(+)-maltose monohydrate (Fluka), 1.25 g L^{-1} bacteriological bile (Biokar), 16.0 g L^{-1} bacteriological agar type *E* (Biokar) and 20.0 mL L^{-1} 0.10%(w/v) bromocresol purple. The composition of ANAYES was: 32.0 g L^{-1} nutrient agar (Biokar), 3.0 g L^{-1} yeast extract (Biokar) and 8.0 g L^{-1} bacteriological agar type *E* (Biokar). The composition of TSYES was: 30.0 g L^{-1} tryptone soy agar (Biokar), 2.0 g L^{-1} yeast extract (Biokar) and 1.0 g L^{-1} soluble starch (Sigma). The composition of FM was: 10 g L^{-1} anhydrous D-(+)-glucose (Fluka), 10.0 g L^{-1} of pancreatic digest of casein (Biokar), 1.0 g L^{-1} yeast extract (Biokar), 16.0 g L^{-1} bacteriological agar type *E* (Biokar) and 40.0 ml L^{-1} 0.1% bromocresol purple solution. The composition of PCAF was: 23.5 g L^{-1} PCA (Biokar) and 0.020 g L^{-1} furazolidone (Sigma). Finally, the composition of LSA was: 15.0 g L^{-1} bacteriological agar type *E* (Biokar), 10.0 g L^{-1} carboxymethylcellulose sodium salt (Fluka), 10.0 g L^{-1} tri-calcium dicitrate tetrahydrate (Fluka), 5.0 g L^{-1} bacteriological meat extract (Biokar), 5.0 g L^{-1} papaic digest of soybean meal (Biokar), 5.0 g L^{-1} casein-meat peptone (polypeptone) (Biokar), 5.0 g L^{-1} yeast extract (Biokar), 1.5 g L^{-1} lactose (Fluka), 1.5 g L^{-1} L-arginine HCl monohydrochloride (Fluka) and 0.020 g L^{-1} 0.1%(w/v) bromocresol purple (Fluka).

Supplements were added to several culture media, according to Table 1. All selective culture media used for bacteria were supplemented with cycloheximide (Sigma Chemical, St. Louis MO, USA) to prevent growth of yeasts. Anaerobic conditions in MacConkey, M17, LSA, KFS and KEAA were obtained via incubation in anaerobic jars (Oxoid, Basingstoke, Hampshire, UK), with GasPak Plus™ (BBL, Cockeysville MD, USA). In RCM, the modified oxygen-free atmosphere of $N_2 + H_2 + CO_2$ (10:10:80, v/v) (Gasin, Matosinhos, Portugal) in the anaerobic jars was obtained as described by Harrigan and McCance (1976).

Quantification of viable numbers of yeasts and bacteria was by plating on the corresponding selective culture media and incubating as summarized in Table 1. Duplicates of 10 g-samples of regional maize (M), rye (R), wheat (W) flour or sourdough (So) 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 another 8 min. Serial decimal dilutions (*i.e.*, dilution to $1{:}10^i$, $2 \leq i \leq 8$ or $2 \leq i \leq 4$, for the vegetative and sporulated forms, respectively) were then made on 0.1%(w/v) sterile peptone water (Sigma Chemical, St. Louis MO, USA). Suspensions were kept refrigerated (at 4 °C) until necessary. Spore