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Microflora distribution and species ratio of Tunisian fermented doughs for bakery industry

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Thirty samples of fermented wheat dough microflora, collected from different Tunisian bakeries, were characterised. Forty per cent of the samples contained approximately 10^6 cfu/g of mesophilic aerobic bacteria (MAB). Lactic acid bacteria (LAB) and yeasts dominated the microflora of these samples. They varied from 10^5 to 10^8 cfu/g. The LAB/yeasts ratio arising from microbial counts were varied between 1/1 and 200/1. More than 50% of the analysed samples were deprived of *Enterococcus*. The content of contaminating microflora like coliforms and mesophilic *Bacillus* ranged from 10^2 to 10^4 cfu/g. The ratio between LAB and coliforms were estimated to about $10^3/1$ for 26% of the analysed samples. This ratio is more important between LAB and mesophilic *Bacillus*. The LAB/mesophilic *Bacillus* ratio was about 10^4 for 45% of the analysed samples. However, *Micrococcaceae* were absent in all samples. This study could be used with further information to establish the cooperative effect existing between LAB and yeasts during food fermentation and to point out an eventual antagonism effect between LAB and spoilage microflora during bakery processing as showed for other fermented foods. The coliforms-like contaminating microflora serves as indicator on the hygienic quality of Tunisian bakery industry.

Key words: Fermented dough microflora, species ratio, lactic acid bacteria, yeasts, antagonism, bakery industry, bread quality, hygiene practices.

INTRODUCTION

The bakery products in Tunisia are based on the fermentation of wheat flours by means of natural and added baker's yeasts. Indeed, dough is usually prepared by mixing and kneading flour, water, salt and an adequate amount of commercial granulated or powdered yeast preparation (Meuser and Valentin, 2004; Hammes et al., 2005). In other cases this fermentation takes place without addition of micro-organisms and it is known as spontaneous one and is guided by the natural microflora of flour (Onno and Roussel, 1994; Hubert, 1996). In fact, the natural microflora of raw cereals is composed of bacteria, yeasts and fungi. The bacteria are mainly mesophilic and include gram-negative aerobes (e.g. *Pseudomonas*) and facultative anaerobes (*Enterobacteriaceae*) as well as gram-positive lactic acid bacteria (LAB). Undesirable *Staphylococcus aureus*, *Bacillus*

cereus and other pathogenic bacteria, may be present (De Vuyst and Neysens, 2005).

During the bread making process, naturally present LAB are involved in dough acidification, flavour development and hygienic quality of bread. Yeasts contribute essentially to the leavening of the dough due to their ability to produce carbon dioxide (Faucher et al., 1999), but also the development of flavour by the production of organic acids and other metabolic activities.

These micro-organisms are usually originating from flour, from the other ingredients (water, salt, yeast preparation, fruits, vinegar etc) (Catzeddu et al., 2006) or from the environment and the process utensils and implements. However, this typical microflora is modified by that present in the outside environment. All over the world, microbial attacks on bread cause very important losses in baking industry (Menteş et al., 2005). Bread can become contaminated with moulds or bacteria. *Bacillus* ssp. are the main organisms causing ropiness of bread and they arises from the contamination of yeast preparation, flour, etc (Şimşek et al., 2006; Yapici and Barut, 2003). On the

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other hand, *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus* are enterotoxigenic bacteria introduced because of the lack of hygiene during the production, transportation and marketing (Yapici and Barut, 2003; Şimşek et al., 2006). The antimicrobial substances produced by certain LAB can help to control the growth of spoilage and pathogenic organisms (Messens and De Vuyst, 2002; Şimşek et al., 2006).

Studies of the normal ecosystem of dough were mentioned by several reports (Galli et al., 1988; Infantes and Tourneur, 1991; Malineau and Arnoux, 1996; De Vuyst et al., 2002; Hammes et al., 2005). These previous works showed that the lactic microflora is composed by a complex-microbial association, with dominance of *Lactobacillus*, *Leuconostocs* and *Pediococcus*. Some authors (Gobbetti, 1998; Giraffa, 2003; Catzeddu et al., 2006) showed that *Enterococcus* sp. are occasionally found or used in sourdough process and for the processing of other fermented foodstuffs. Several species of yeasts were also found in fermented dough such as *Saccharomyces cerevisiae* which is considered as the major yeast species (Corsetti et al., 2001).

Furthermore, the dough ecosystem and interaction between micro-organisms were found to have consequential effects on the distribution of species and finally on the quality of the derived bread products (Barber and Baguena, 1988). In order to obtain preliminary evaluation on the hygiene practices implemented by Tunisian bakery industries and on the implication of the indigenous microflora of the flour in determining the quality of bread products, the microflora of Tunisian fermented dough samples will be investigated in this paper in the light of its microbiological group's distribution and evaluate the ratio between dominant micro-organisms.

MATERIALS AND METHODS

Sampling method

Thirty samples of fermented wheat dough were collected from different bakeries in the region of Tunis. The yeast inoculation ratio reported of the flour mass was of 1%. The collected samples were then kept under refrigeration (4°C) for no more than 24 h before microbiological analyses.

Microflora determination and enumeration

Ten grams of each sample were homogenized with 90 ml of sterile peptone-salt solution (1 g/l peptone, 5 g/l NaCl and 2 ml/l Tween 80) and mixed (Stomacher 400 PROLABO). After serial dilution, in sterile peptone-salt solution, samples were plated out on selected agar media. Total lactic acid bacteria counts were determined by using MRS agar medium (Man, Rogosa and Sharpe) (Man et al., 1960) containing cycloheximide (200 µg/ml) to inhibit the yeasts growth (Hardy, 1982; Lacerda et al., 2005; Şimşek et al., 2006). Plates were incubated at 37°C for 48 h. After growth, the colonies were purified by plating on MRS agar.

Gram-positive and catalase-negative strains were preliminarily clustered on the basis of morphological aspects, CO₂ production from glucose, arginine degradation and growth in MRS broth at

10°C and 15°C. Carbohydrate fermentation profiles were determined using API 50 CH strips and API 50 CHL medium (API system, Biomérieux, France). The API pattern data were examined numerically and compared to type strain fermentative patterns in previous study (M'hir et al., 2005). Viable yeasts were enumerated on Sabouraud agar medium with chloramphenicol (500 µg/ml) to inhibit bacteria growth. Incubations were performed for 48 - 72 h at 30°C. Isolates were examined through carbohydrates assimilation tests (API ID 32 C, Biomérieux) (results not shown). Enumeration of *Enterococcus* sp. was determined by using BEA medium (Bile Esculin Agar) and incubations were carried out at 37°C for 48 h.

Enumeration of mesophilic aerobic bacteria and contaminating microflora such as coliforms, mesophilic *Bacillus* and *Micrococcaeae* was performed by plating on appropriate selective agar media as reported by Faucher et al. (1999). The enumeration result was expressed as the number of colony forming units (cfu) by gram of analysed samples. For the purpose of this study, sampling and analysis were done in triplicate and the results presented are in the range of the found values.

RESULTS AND DISCUSSION

Enumeration of the different genera

In this work, the microflora of thirty natural fermented doughs was studied. The samples were examined for the presence of LAB, yeasts, mesophilic aerobic bacteria (MAB), mesophilic *bacillus*, coliforms and *Micrococcaeae* (Figure 1). MAB varies from 10² to 5.10⁸ cfu/g. 40% of the samples have MAB counts of about 10⁶ cfu/g. In fact, this group of micro-organisms indicates the microbiological quality and indicate the active flora favourable for dough fermentation (Figure 2a)

In all samples of fermented dough, counts of LAB ranged between 3 x 10⁵ and 3 x 10⁸ cfu/g (Figure 2a). Similar results have been reported (Barber and Baguena, 1988; Infantes and Tourneur, 1991; Faucher et al., 1999). Ricciardi et al. (2005) showed that LAB counts ranged from 10⁷ and 10⁸ cfu/g. Indeed, the great variability in the number and type of the found species, depends on several factors including the degree of dough hydration, the type of cereal used and the leavening temperature (De Vincenzi et al., 1994), as well as factors resulting from substrates present in the cereal fraction and from endogenous and microbial enzymes (De Vuyst and Neysens, 2005). The LAB developing in the dough may originate from selected natural contaminants of the flour (De Vuyst and Neysens, 2005; Vogel et al., 1999). LAB is so important in bakery processing because of their contribution in producing organic acids and developing organoleptic properties of bread, especially its flavour (Figure 2b).

The yeast counts are less numerous, this population ranging from 5 x 10⁴ to 4 x 10⁷ cfu/g (Figure 2b). These counts are lower than those reported by Faucher et al. (1999) who found values between 8 x 10⁶ and 1 x 10⁸ cfu/g. Matthias and Vogel (2005) reported that cereal fermentations are dominated by specifically adapted LAB occurring at number >10⁸ cfu/g, which may be in co-existence or possibly in symbiosis with typical yeasts

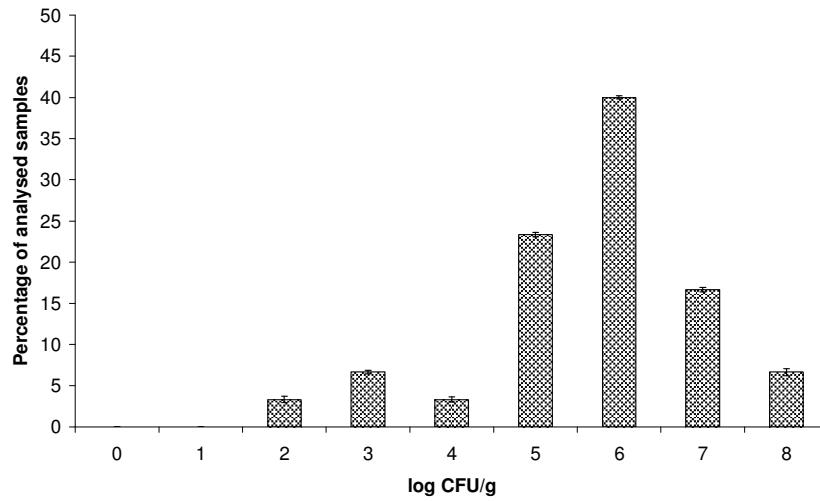
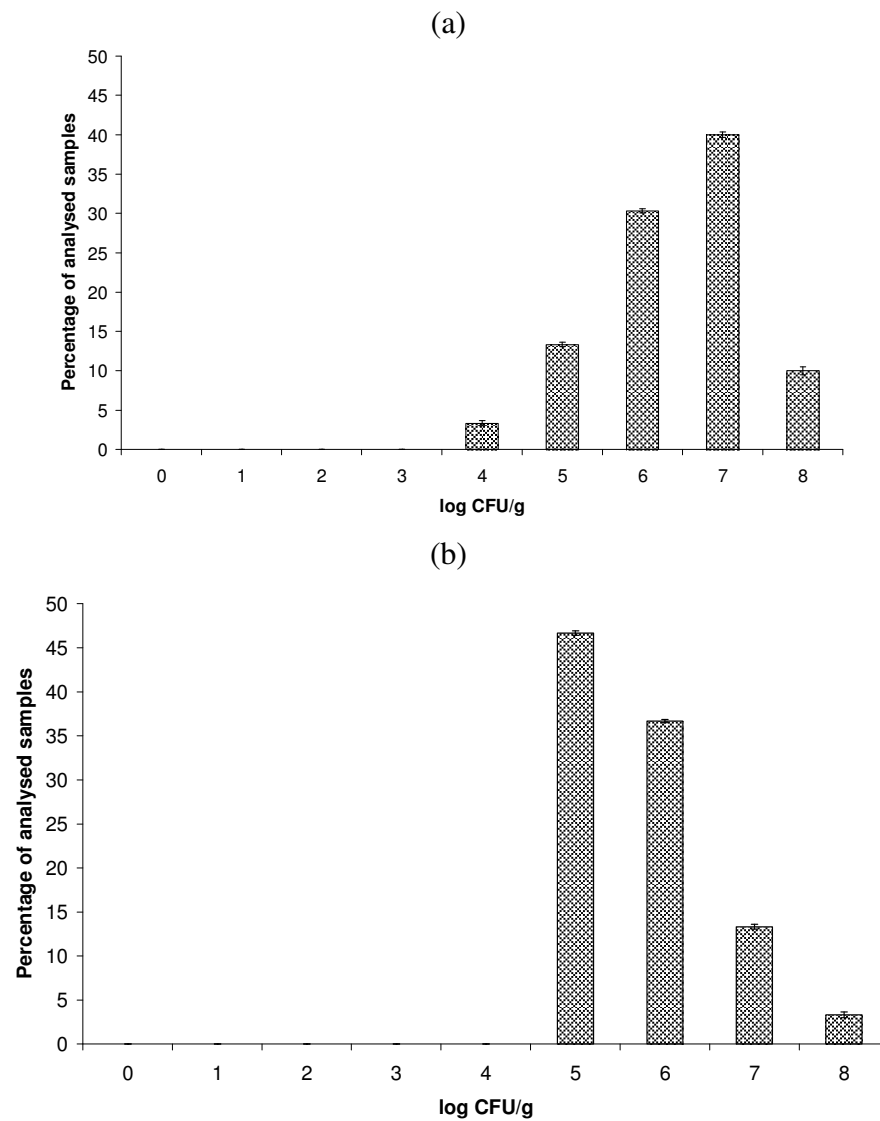


Figure 1. Mesophilic aerobic bacteria (MAB) distribution in the total analysed samples of wheat doughs.



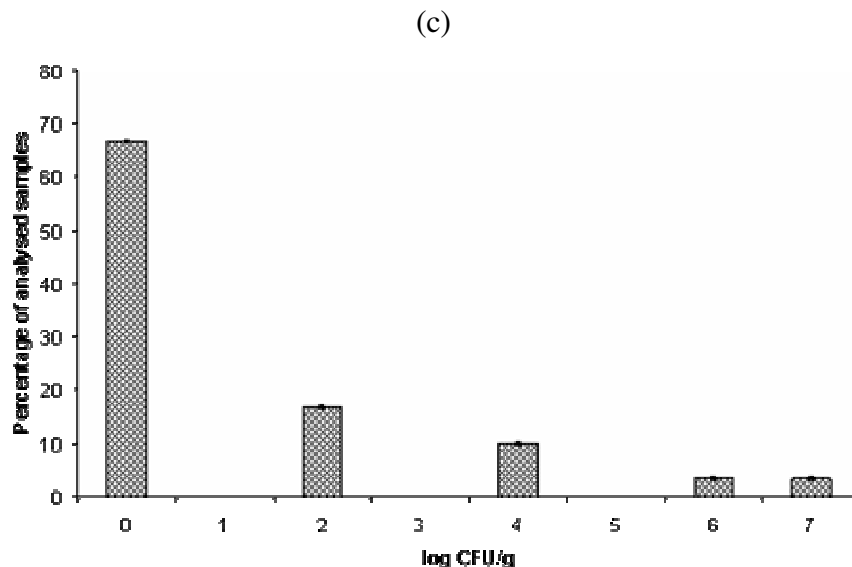


Figure 2. Lactic acid bacteria (LAB) (a), yeasts (b) and *Enterococcus* (c) distribution in the total analysed samples of wheat dough.

whose numbers depends especially on the inoculation ration of the dough. It was shown by Hammes et al. (2005) that, regardless their inoculum's origin, yeasts in dough may originate from the flour or the other ingredients of the dough and from the bakery environment. It should be emphasised that *S. cerevisiae* is not found in the raw materials; its occurrence in dough may be explained by the application of baker's yeast in most daily bakery practice in order to accelerate the leavening process (Faucher et al., 1999; De Vuyst and Neysens, 2005).

Enterococcus sp. was also present in the different analyzed samples. They ranged from 10^2 to 10^6 cfu/g (Figure 2c). However, more than 50% of the analyzed samples are deprived of *Enterococcus*. This bacterial genus is used in several fermented foods to improve their maturations (Gobbetti, 1998; Hammes and Gänzle, 1998; De Vuyst and Neysens, 2005). Girrafa (2003) showed that *Enterococcus* sp. are occasionally found or used in the processing of some fermented dairy products. In another recent study, Foulquié Moreno et al. (2006) reported that *enterococci* are used in some countries as probiotics (Figure 2c).

Interactions between species

In order to characterise eventual interactions between species such as antagonism or cooperation and to point out some eventual favourable results on the quality of bread products, we determined the quantitative ratio between both populations (LAB:yeasts). This ratio was found to vary between 1:1 and 200:1 (Figure 3). In the

literature, and for fermented dough, this ratio generally varied between 50:1 and 100:1 (Ottagalli et al., 1996; Gobbetti, 1998). Faucher et al. (1999) showed that this quantitative ratio (LAB:yeasts) varied between 4:1 and 300:1. Pepe et al. (2004) showed a quantitative ratio varying between 10:1 and 100:1. It is generally considered that in sourdoughs, the ratio of LAB to yeast should be about 100:1 for optimal leavening and acidification activities (Rehman et al., 2006). In good bakery practice, a sponge should contain metabolically active LAB at 10^8 - 10^9 cfu/g and yeasts at 10^6 - 10^7 cfu/g, responsible for acidification and leavening action of dough, respectively (De Vuyst and Neysens, 2005; Rehman et al., 2006).

The great variability in the number (LAB/yeasts) depends on several factors including the inoculation ration, the degree of dough hydration, the type of cereal used and the leavening temperature (De Vuyst and Neysens, 2005) (Figure 3). The weak values of the ratio observed in the Figure 3 (since 45% of the samples analyzed present a ratio equal to 10) could be explained by the high inoculation ration of the yeasts during dough preparation. As a general rule, LAB is the dominant organisms in dough and in many cases that co-exist with yeasts which are also present in elevated numbers (Vogel et al., 1999). In addition, the metabolic interactions between these two populations (LAB and yeasts) permit to improve the taste of the bakery products by the formation of volatile aromatic compounds and aroma precursors (De Vuyst and Neysens, 2005). The association of yeasts and lactic acid bacteria (mixed microflora) are often encountered or used in the production of beverages and fermented foods (Gobbetti, 1998).

Concerning the contaminating microflora, it was consti-

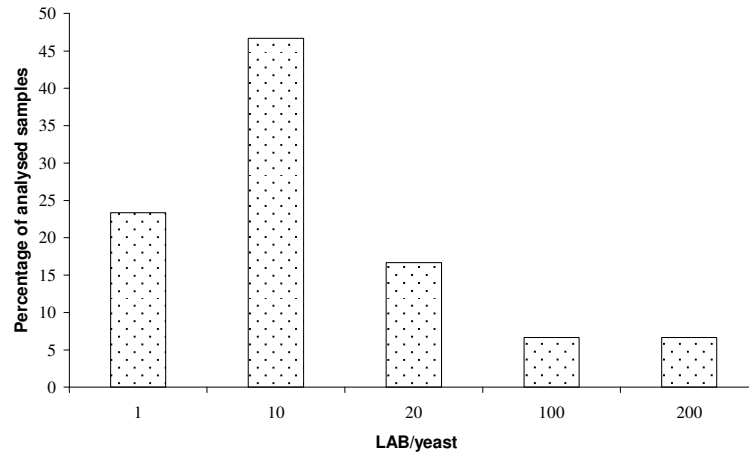


Figure 3. Ratio of lactic acid bacteria and yeasts (LAB/yeast).

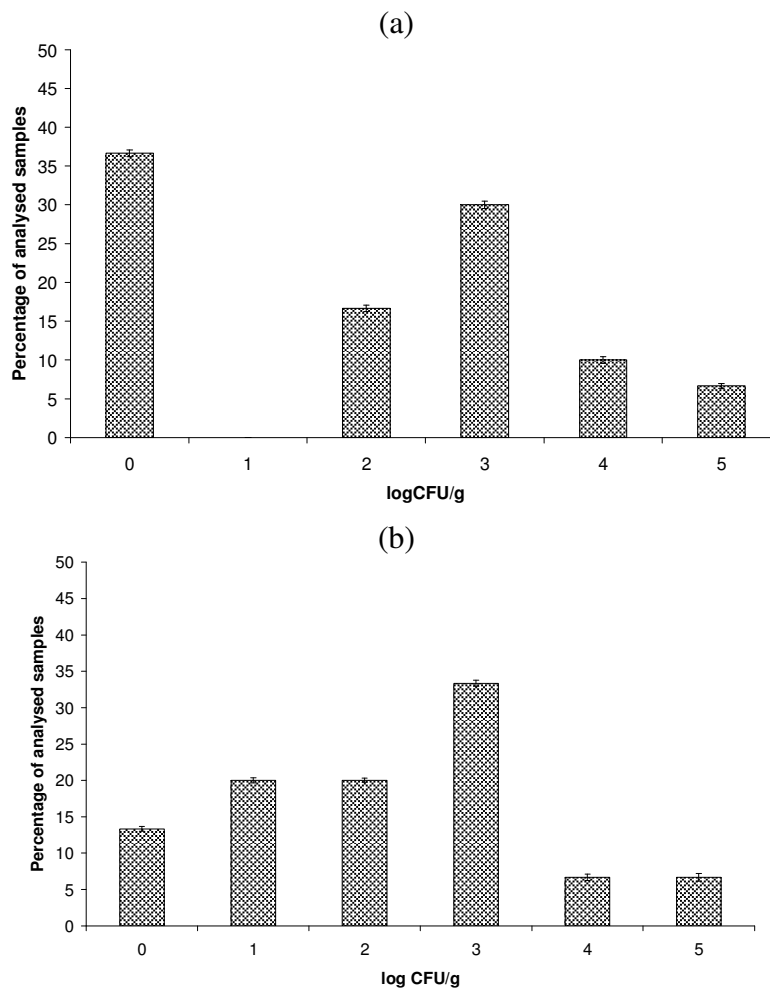


Figure 4. Contaminating microflora distribution in the total analysed samples of wheat dough; coliforms (a) and mesophilic *Bacillus* (b).

tuted by coliforms and mesophilic *Bacillus*. The level of contamination is variable from one sample to another. The content of the coliforms and mesophilic *Bacillus* va-

ries between 5×10^2 and 5×10^4 cfu/g (Figures 4a and 4b). The coliforms count is considered lower than those reported by previous studies (Faucher et al., 1999).

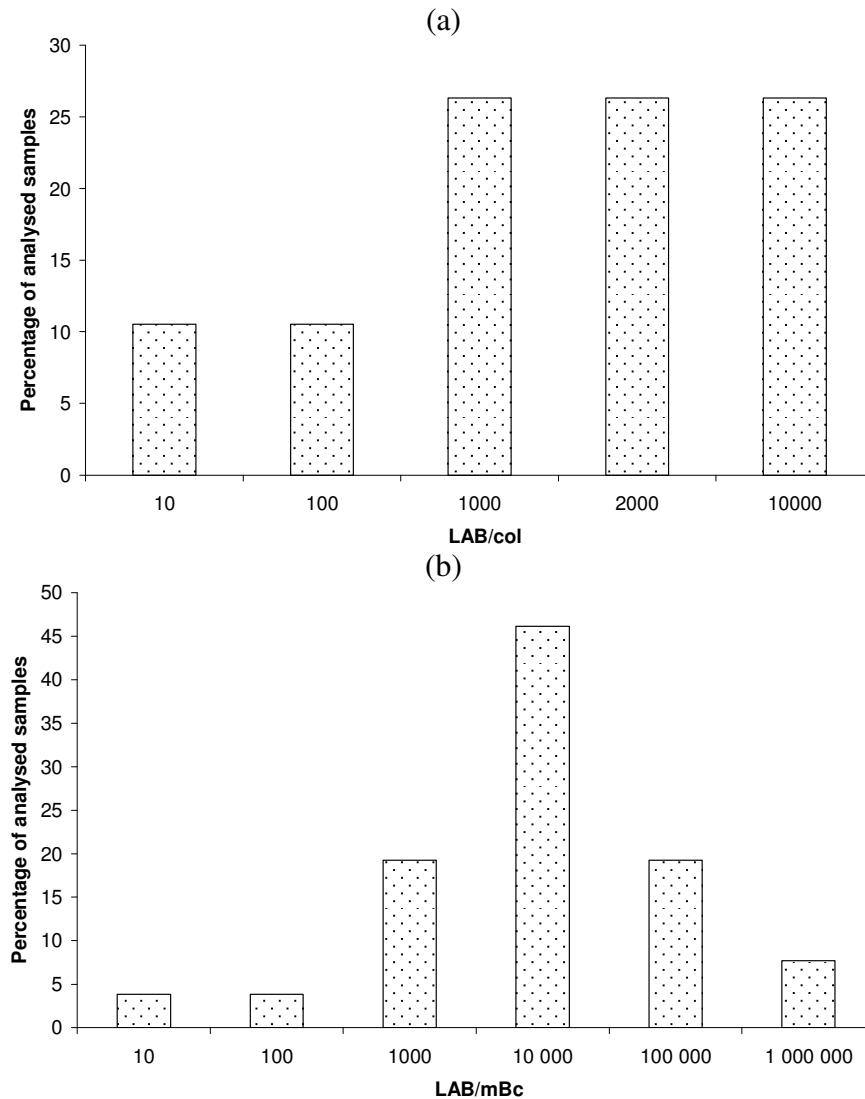


Figure 5. Lactic acid bacteria and coliforms (LAB/col) (a) and lactic acid bacteria and mesophilic *Bacillus* (LAB/mBc) (b).

Populations of coliforms were not detected in more than 35% of analysed samples. However, coliforms numbers of 30% of the samples were 10^3 cfu/g. The counts exceeded the coliforms numbers reported by Şimşek et al. (2006) and Gül (1999). It becomes evident that the hygiene practices implemented by the bakeries were not enough efficient to reduce and minimize the contamination levels by coliforms. It is therefore recommended that the bakeries revise their manufacturing processes in order to reduce contaminations and hygiene risks. In fact, contaminations have been shown to be transferred through food handlers, surfaces, equipment and environment.

The count of bacilli is higher than those showed by the study of Faucher et al. (1999). This population ranged from 10^2 to 10^3 cfu/g in our study. The *Micrococcaceae*

are absent in the different analyzed samples. The most frequent problems, occurring in baking industry, are mould contamination and rope spoilage (Jensen, 1998). Rope spoilage of bread is usually caused by *Bacillus* spp. The spoilage is initially noticed as an unpleasant odour, followed by a discoloured and sticky soft bread crumb (Rosenquist and Hansen, 1995; Mentés et al., 2005).

Kirschner and Von Holy (1989) have suggested that when the cell counts of *Bacillus subtilis* and *Bacillus licheniformis* reach over 10^5 cfu/g, they present a potential risk of food borne illness. In this study, mesophilic *Bacillus* were not detected in the samples at viable counts over 10^5 cfu/g.

Studies of the ratio of LAB/coliforms and LAB/mesophilic *Bacillus* show is presented in Figures 5a and 5b. The LAB exceeds coliforms by a value of 1000

for 26% of the analyzed samples (Figure 5a). Whereas, the LAB exceeds the mesophilic *Bacillus* in a more important way. This factor is about 10 000 for 45% of the analyzed samples (Figure 5b). Indeed, the spontaneous lactic flora has an antagonism effect on the contaminating microflora of the dough by developing acidity and producing bacteriocins (Hartnett et al., 2002; Gobbetti et al., 2005). This inhibits the growth of contaminating microflora such as the gram negative aerobic bacteria (*Pseudomonas*) and optional anaerobic (*Escherichia coli*, *Citrobacter*, etc), the cocci gram-positive (*Micrococcus*, *Streptococcus*) as well as the gram positive *Bacillus* (*Bacillus cereus*, *B. subtilis*) (Onno and Roussel, 1994; Katina et al., 2002). It was shown by De Vuyst and Neysens (2005) that during spontaneous fermentation, the LAB fast dominates the gram-negative enterobacteria. Rapid acid production by LAB is essential in sourdough bread production for technological and hygienic reasons (Şimşek et al., 2006). This microflora is able to retard bread staling and to inhibit the development of pathogens. Filamentous moulds were not detected in the fermented doughs samples, probably because of the fungistatic properties of LAB (Schnürer and Magnusson, 2005).

Yeasts and lactic acid bacteria are dominant and responsible of the spontaneous fermentation of the doughs. LAB occurs naturally in foods or are added as pure cultures to various food products. They have a GRAS (Generally Recognised As Safe) status (Stiles, 1996). They are considered to be harmless or even to improve human and animal health.

Conclusion

The difference in microbial composition observed between samples may be attributed to differences in microorganisms on the flour used in the bakery preparation and processing techniques. This study showed the dominance of LAB and yeasts associations which are often encountered in the production of fermented foods such as dough making. Bread and other leavened baked products can become contaminated with spoilage bacteria, mould or coliform originating from contamination of the raw materials or the bakery environment. The counts of coliforms exceeded the guidelines; almost 60% of samples exceeded 10 cfu/g. However, coliforms counts were frequently absent in more than 35% of analysed samples. The presence of coliforms confirms the lack of hygienic practices such as hand washing and other proper personal hygiene. This suggests the implementation of quality control measures so as to produce food of good standards and quality.

These results arising from microbial enumerations were important and helpful for understanding relationships between hygienic quality and organoleptic quality of baked products. Furthermore, the important ratio observed with LAB/coliforms and LAB/mesophilic *Bacillus* may

be attributed to the antimicrobial activity of LAB. Further knowledge regarding sourdough ecosystem could lead to the establishment of good manufacturing practice (GMP) of industrially baked dough products. Management commitment, proper personal and process hygiene appear to be the primary issues to be addressed in order to curb undesirable contamination of the bakery product.

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