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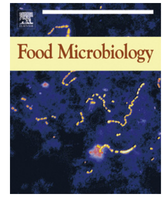

Comparison of three *Bacillus amyloliquefaciens* strains growth behaviour and evaluation of the spoilage risk during bread shelf-life

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ABSTRACT

This study aims at the characterisation of growth behaviour of three strains of *Bacillus amyloliquefaciens*, isolated from ropy bread (ATCC8473), wheat grain (ISPA-S109.3) and semolina (ISPA-N9.1) to estimate rope spoilage risk in pan bread during shelf-life using the Sym'Previus tool. Cardinal values and growth/no growth boundaries were determined in broth, while artificial spore inoculations were performed in dough for various pan bread recipes to compare experimental counts with *in silico* growth simulations. Finally, two storage scenarios were tested to determine the probability to reach a spoilage threshold during bread shelf-life. Similarly to the safety criteria fixed for *Listeria monocytogenes* contamination in foodstuff complying with EC regulation, a potential rope spoilage threshold was arbitrary fixed at 5 log CFU/g for *B. amyloliquefaciens*. This study further underlines a higher rope spoilage potential of the ISPA strains as compared to the ATCC strain, thus emphasizing the interest to characterise both wild strains and reference strain to account for biological variability. In conclusion, this study showed that available decision making tools which are largely recognized to predict behaviour of pathogenic strains, shall also be used with spoilage strains to help maintain food quality and extend shelf-life.

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1. Introduction

Bacillus amyloliquefaciens species is a spore forming rod widely used in industrial microbial cultivation for the production of enzymes (Deb et al., 2013; Schallmey et al., 2004) or secondary metabolites potentially used for biocontrol of plant pathogens (Arguelles-Arias et al., 2009; Reva et al., 2004) or various feed additives (EFSA, 2008; EFSA, 2013). *B. amyloliquefaciens* shares remarkably high level of 16S rRNA gene sequence with *Bacillus subtilis* while only showing minor differences in molecular, phenotypic and biochemical features (Welker and Campbell, 1967; Nakamura, 1987; O'Donnell et al., 1980; Gonzalez et al., 2013; Valerio et al., 2012). Even though *B. amyloliquefaciens* meets Qualified Presumption of Safety status requirements, De Jonghe et al. (2010) underlined strong proteolytic and lipolytic activities as well as the ability of *B. amyloliquefaciens* to produce both, heat-labile and heat-stable cytotoxic compounds. *B. amyloliquefaciens* is also recognized for its implication in food spoilage, which is a complex and dynamic event combining microbial and biochemical degradations that might occur during food storage. Initially present in low quantities, specific spoilage organisms constitute only a minor part of the natural microflora. But when conditions are favourable during food shelf-life, fast development of spoilage organisms will yield the synthesis of metabolites responsible for food degradation and the production of off-flavour, off-odours or slime (Huis in't Veld, 1996). *B. amyloliquefaciens* and its closely related species are particularly known to be involved in ropy bread spoilage that is characterized by an unpleasant fruity odour followed by enzymatic degradation yielding soft, sticky and stringy bread crumb making the bread inedible. The deterioration of bread texture is due to the combined effect of the proteolytic and amylolytic enzymes produced by some *Bacillus* strains that results in slime formation. Ropiness is usually occurring in the summer season when the climate is warm (25–30 °C) and humid as in the Mediterranean countries, Africa and Australia (Pepe et al., 2003; Rosenkvist and Hansen, 1995; Valerio et al., 2012; Voysey and Hammond, 1993). As previously reported, a low level ($\approx 10^2$ spores/g) of *Bacillus* spores in flour can reach up to 10^7 CFU/g in bread crumb within 2 days causing bread spoilage (Rosenkvist and Hansen, 1995). Actually, the current study was prompted by reports

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of ropy spoilage from several Italian bakeries during summer and autumn and by the assessment of a relevant contamination (more than 100 spores/g) of bacterial spores in raw materials (including semolina, grain, brewer yeast, improvers) used in bread-making (Valerio et al., 2012). In particular, authors identified *B. amyloliquefaciens* as the most frequent species associated with the rope spoilage. Moreover, in Europe bread remains a common and important food for a balanced healthy diet and the ropy spoilage could represents an industrial issue that causes economic losses for bakery industries. This phenomenon also tends to increase with the consumption of bread without preservatives and the addition of raw materials such as bran or seeds (Saranraj and Geetha, 2012). Recently, the application of LAB- derived acidic bioingredients in bread-making has been studied to improve bread quality and microbiological shelf-life (Komlenić et al., 2010; Valerio et al., 2013). In particular, the use of LAB-based bioingredients was reported to significantly influence the growth of *Bacillus subtilis* in bread by the acidification of the dough (Valerio et al., 2008).

The ability to form endospores when environmental conditions are not favourable for growth is a particular trait of *Bacillus* spoilage organisms. The extreme resistance of endospores to various treatments encountered in food industries explains the persistence of sporeforming bacteria in industrial plants and their natural prevalence in a wide range of foodstuffs. In particular dehydrated powder, spices or ingredients may constitute a major contamination source for various industries (Postollec et al., 2012). While no visible trace of spore contamination is noticed in dehydrated ingredients, the potential presence of one spore may lead to food spoilage during storage of formulated products when environmental conditions allow germination, outgrowth and vegetative growth. Therefore the type and rate of spoilage are complex and depend on spore resistance, spoilage ability and a number of intrinsic and extrinsic factors related to the food and its storage environment (Baril et al., 2012a, b). Indeed the ability of *B. amyloliquefaciens* to spoil bread is generally attributed to spore survival during bread cooking process and amylase activity of vegetative cells (Pepe et al., 2003; Valerio et al., 2012; Witthuhn et al., 2011).

Rather than describing mathematical approaches, this work mainly concerns the industrial application and transfer of validated models available for pathogenic strains (Membré et al., 2002, 2005; Leporq et al., 2005; Pinon et al., 2004; Couvert et al., 2010) to industrial relevant spoilage bacteria using the Sym'Previus tool. The objectives of this study were: i) to characterize the growth ability of three *B. amyloliquefaciens* strains, potentially responsible for ropy bread, in a wide range of temperature, pH and water activity (a_w) to determine growth/no growth boundaries; ii) to compare *B. amyloliquefaciens* counts determined in pan bread (in food) with *in silico* growth simulations for different intrinsic (pH and a_w) and environmental (storage temperature) conditions and iii) to predict the probability to overpass a spoilage threshold fixed at 5 log cfu/g in order to minimise spoilage risk as a function of industrial relevant conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacillus amyloliquefaciens ISPA-S109.3 isolated from wheat grain and *Bacillus amyloliquefaciens* ISPA-N9.1 isolated from wheat semolina, were obtained from the Culture Collection of the CNR Institute of Sciences of Food Production, Bari, Italy. These strains were selected among 13 *B. amyloliquefaciens* strains showing different Rep-PCR profiles, isolated from raw materials or bread and screened for their ability to cause the bread rope with and without heat treatments exposure as reported in Valerio et al. (2012). The strain *Bacillus amyloliquefaciens* ATCC8473 was isolated from ropy bread, obtained from the American Type Culture Collection (Rockville, MD, USA). Strains were cultured in Brain Heart Infusion broth (BHI; Difco, Becton Dickinson Co., Sparks, MD, USA) for 24 h at 37 °C. For long-term storage, stock cultures were prepared by mixing 8 ml of a culture with 2 ml of Bacto glycerol (Difco) and freezing 1 ml portions of this mixture at −80 °C. Strains were subcultured (1% vol/vol) twice in BHI for 24 h at 37 °C using an inoculation, before use in experiments.

2.2. Determination of cardinal growth parameters of Bacillus amyloliquefaciens strains

Cardinal values, *i.e.* minimal, optimal and maximal values of temperature, pH and a_w which enable bacterial growth were determined in broth. Growth kinetics were performed in 0.22 μm filtered Brain Heart Infusion broth supplemented with yeast extract (0.3% w/v) (Oxoid LTD, Basintoke Hampshire, England) and D-glucose (0.2% w/v) (Biokar Diagnostics, Beauvais, France) (BHIYG). For all strains, growth rate (μ_{max}) was determined for ten levels of temperature (from 4 °C to 55 °C), pH (from 4.5 to 9.5) and a_w (from 0.964 to 0.994). Three replicates for each condition were performed. For all a_w and pH conditions performed at 30 °C and temperatures ranging from 25 to 45 °C, the growth of *B. amyloliquefaciens* strains was automatically monitored by a Bioscreen C (Labsystems, Helsinki, Finland) using the turbidimetry method (Membré et al., 2002) according to the dilution methods reported by Cuppers and Smelt (1993). The slope of the linear relationship between detection times and logarithm of initial concentrations corresponded to the maximum growth rates (μ_{max}) (Biesta-Peters et al., 2010; Le Marc et al., 2002; Membré et al., 2005). For remaining temperatures, growth kinetics were determined manually after static incubation at tested temperatures. Sampling and bacterial counts on Plate Count Agar (Biokar Diagnostics) were performed with adequate dilution in maximum recovery diluent (MRD; Oxoid). Maximal growth rate was determined after fitting experimental kinetics by logistic model with delay and rupture (Rosso, 1995). To estimate the cardinal growth values, the experimental growth rates were plotted against each environmental factor and fitted to determine cardinal growth values (Rosso et al., 1995) yielding growth/no growth boundaries (Le Marc model, 2001, modified by Augustin et al., 2005). In order to ease the use of mathematical models and recognised approaches (see paragraph 2.6), the fitting and determination of boundaries with 10%, 50% and 90% growth probabilities were performed using Sym'Previus platform (www.symprevius.org).

2.3. Endospore stock preparation

A 0.5 ml aliquot of a 24 h pre-culture in BHI at 37 °C was used to inoculate the surface of the Modified Nutrient Agar sporulation medium (MNA) containing nutrient agar (Biokar diagnostics) supplemented with $CaCl_2$, $2H_2O$ (100 mg/L) and $MnSO_4$, H_2O (50 mg/L). Plates were incubated at 30 °C and spores were collected by scrapping when microscopic observation of the suspension indicated a sporulation of more than 80% of the cells. Spores were suspended in sterile distilled water and centrifuged at 10.000 × *g* for 15 min. After elimination of remaining vegetative cells by the addition of ethanol 1:1 (v/v) at 4 °C for 12 h, spores were washed three times in sterile distilled water and the suspension was centrifuged at 10.000 × *g* for 15 min. The final pellet was suspended in a minimal volume of sterile distilled water to obtain 10^{10} spores ml^{-1}. Spore suspensions were stored at 4 °C and kept two months before artificial inoculation in bread.

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