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ABSTRACT

Microbial spoilage of bread and the consequent waste problem causes large economic losses for both the bakery industry and the consumer. Furthermore the presence of mycotoxins due to fungal contamination in cereals and cereal products remains a significant issue. The use of conventional chemical preservatives has several drawbacks, necessitating the development of clean-label alternatives.

In this review, we describe current research aiming to extend the shelf life of bread through the use of more consumer friendly and ecologically sustainable preservation techniques as alternatives to chemical additives. Studies on the in situ-production/-expression of antifungal compounds are presented, with special attention given to recent developments over the past decade. Sourdough fermented with antifungal strains of lactic acid bacteria (LAB) is an area of increasing focus and serves as a high-potential biological ingredient to produce gluten-containing and gluten-free breads with improved nutritional value, quality and safety due to shelf-life extension, and is in-line with consumer's demands for more products containing less additives. Other alternative biopreservation techniques include the utilization of antifungal peptides, ethanol and plant extracts. These can be added to bread formulations or incorporated in antimicrobial films for active packaging (AP) of bread. This review outlines recent progress that has been made in the area of bread biopreservation and future perspectives in this important area.

Introduction

Baked goods are considered to be one of the most important products of the food industry. Bread is a staple food in many countries and consumed daily all over the world. Within the European Union (EU) the production of bread is relatively stable showing low growth in most western countries. The Germans and Austrians consume the most bread averaging at 80 kg bread per person per year, while the UK and Ireland have the lowest average annual consumption, less than 50 kg (The Federation of Bakers, 2012). In contrast to Europe, the consumption of bread products shows an increasing trend in most developing countries (Elsanhoty et al., 2013).

Over the last decade, consumer behavior in the baked goods category has changed. In particular, consumers' concerns about safety and additive contents in food have received much attention. Additionally, the number of health-conscious consumers has increased. Consequently, a high demand for "natural" and "wholesome" foods without chemical preservatives and additives exists on the market. Accordingly, bread manufacturers have increasingly moved to produce so called "clean label products" which fit this healthier lifestyle of customers. Food labels of such products have claims such as "no preservatives" or "natural." In fact, marketing the absence of additives/preservatives (21% of new products launched in Europe 2013/2014) and the inclusion of wholegrain continues to drive health-focused new product developments (Mintel, 2014). In turn, such minimally processed food without chemical preservatives

or other artificial additives should still be of high-quality and have an extended shelf life.

Spoilage of bread can be caused by bacteria, yeast and molds. However, contamination originates predominantly post baking by fungal spores being deposited from the bakery environment (Knight and Menlove, 1961). The most critical factors controlling the growth of undesirable fungi on foodstuffs are oxygen, temperature, pH, and water activity (a_w). Generally, breads have a relatively high moisture content and a_w between 0.94 and 0.97 at a pH of about 6 with sliced, prepacked and wrapped breads belong to the most susceptible bakery products for mold spoilage (Magan et al., 2003a). This is because wrapping prevents moisture loss from the bread slices allowing suitable growth conditions for fungi in a humid atmosphere. When wrapped, freshly baked breads without any added preservatives have a shelf-life of only a few days at room temperature.

Microbial deterioration of bread is of serious concern and the consequent waste problem still causes large economic losses for both the bakery industry and the consumer (Melikoglu and Webb, 2013). In 2011, Novozymes surveyed over 4000 bread consumers throughout Europe and found evidence that the main reason that bread was thrown away was because it had become moldy (van Sint Fiet, 2015). For UK households losses due to mold spoilage of bread are estimated to be about 20%; this waste represents 65,600 tonnes of bread slices and equates to £ 72 million per year (Ventour, 2008). Apart from the unpleasant sight of visible mold growth, fungi are also

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responsible for the production of mycotoxins and off-flavors, which might be produced even before fungal outgrowth is visible (Magan et al., 2003b). Thus, spoiled breads represent a hazard which can be of a high risk to consumer's health.

Physical methods like ultraviolet light, infrared radiation, microwave heating or ultra-high pressure treatments can destroy post-baking contaminants in breads. However, few studies employing these methods have been done in the recent past. In light of the continuing consumer trend towards a healthier lifestyle, studies have increasingly set targets to replace traditionally used chemical preservatives with environmental friendly, "clean-label" alternatives. Thus the use of "biopreservation," defined as the extension of "shelf life by the use of natural or controlled microbiota and/or their antimicrobial compounds" (Stiles, 1996) has become an increasingly important field of research. Furthermore, this term can also be applied to active plant ingredients or plant extracts. Additionally, recent investigations have dealt with improving protective packaging. This paper reviews the potential for biopreservation and smart packaging to insure bread safety with an emphasis on the in situ-production/-expression of antifungal compounds by lactic acid bacteria (LAB), yeast and plant derived compounds.

Cereals and their contamination with fungi and mycotoxins

Many species and varieties of cereals and pseudocereals are cultivated worldwide. Their products are an important source of nutrients for human consumption in both developed and developing countries. The major cereal crops produced worldwide are wheat, rice and maize (FAO, 2013). Most relevant for bread production in the western world is wheat followed by rye. Other relevant cereals include barley, oat, and the gluten-free crops, millet, sorghum, quinoa, amaranth and buckwheat. The latter three belong to the pseudocereals, which feature higher quality protein and the presence of abundant quantities of fiber, vitamins and minerals such as calcium and iron when compared to their cereal counterparts (Alvarez-Jubete et al., 2010).

Cereals and pseudocereal crops are susceptible to a wide spectrum of plant pathogens. During cultivation, cereal contamination by fungi can cause severe plant diseases which can cause enormous yield losses correlated with the reduction of grain quality. Common phytogenic pathogens include filamentous fungi such as *Alternaria, Aureobasidium, Cladosporium, Claviceps, Epicoccum, Fusarium* and *Helminthosporium*, with *Fusarium* infections seem to representing a major threat (Oliveira et al., 2014). Cereal grains and their products are exposed to contamination post-harvest, during storage and pre- and post-processing. The most common fungi related to losses in bakery products belong to the genera *Aspergillus, Cladosporium, Endomyces, Fusarium, Monilia, Mucor, Penicillium,* and *Rhizopus* (Dal Bello et al., 2007). Other bread ingredients, equipment or packaging material, when contaminated, could also represent a potential vehicle allowing spoilage-related and undesirable microorganisms into the baking environment (Reale et al., 2013). To guarantee food safety and quality along the food production chain, it is essential to follow guidelines like Codex Alimentarius including implementing such systems as good agricultural practices (GAP), good manufacturing practices (GMPs), good hygienic practices (GHPs) and Hazard Analysis and Critical Control Point (HACCP) systems.

In normal bakery practice molds do not survive the bread-baking process (Knight and Menlove, 1961). In contrast, mycotoxins produced by crop pathogens and food-spoilage fungi are relatively heat stable (Vidal et al., 2015; Wu and Wang, 2015) and therefore often represent a more serious problem (Osborne and Stein, 2007). The effect of processing on the mycotoxin content in grains was recently summarized by Kaushik (2015). The author concluded that the reduction in mycotoxins during baking is relatively low and varies with the toxin. An EU regulation (No. 1881/2006) defines maximum levels for the mycotoxins, aflatoxin (2 μg/kg) and ochratoxin A (OTA) (3 μg/kg) in all cereal products; for the *Fusarium* mycotoxins, deoxynivalenol (DON) (500 μg/kg) and zearalenone (50 μg/kg) specific maximum levels have been set for bread and other bakery products such as biscuits or pastries (EEC, 2006). However, for some *Fusarium* mycotoxins like T-2 and HT-2 toxins, fumonisins, enniatins and nivalenol no maximum level has yet been set. A great number of articles are available in the literature for further reading about mycotoxins. Pereira et al. (2014) give an up-to-date review about the occurrence of mycotoxins specifically in cereals and cereal-related foodstuff along with their recent methods of analysis. A review by Oliveira et al. (2014) addresses cereal fungal infections in relation with bioprotection. The authors provide an extensive overview of mycotoxins found in cereal crops, their fungal source and their possible health hazards for humans and animals. Mycotoxins may cause severe health problems with carcinogenic, nephrotoxic, neurotoxic and/or immunosuppressive effects. Wu et al. (2014) provide a more detailed description about the adverse human health impacts associated with the major groups of mycotoxins, aflatoxin, fumonisins, DON and OTA. Co-occurrence of different mycotoxins is of great concern due to possible synergic toxic effects. Of further concern are conjugated mycotoxins, which are masked by transformation, conjugation or compartmentalization. Food processing can result in conjugation. To date, no regulatory levels exist for these conjugated mycotoxins in cereals and cereal-based foods.

The presence of mycotoxins due to fungal contamination in cereals and cereal products remains a significant problem worldwide, and although monitoring occurs in most European countries, they are detected frequently in processed cereal products (Škrbić et al., 2012; Aldana et al., 2014; Błajet-Kosicka et al., 2014; Demirel and Sariozlu, 2014). Due to the higher humidity and temperature in developing countries in Africa or Asia, mold growth and mycotoxin formation in crops and food products creates a particular issue (Manjula et al., 2009; Reddy and Salleh, 2011; Adetunji et al., 2014). It is therefore important for these countries to establish broad monitoring programs to examine the occurrence and distribution of mycotoxins in food products, and train farmers about effective post-harvest management (Gnonlonfin et al., 2013; Abass et al., 2014; Shephard and Gelderblom, 2014).

Conventional food preservatives

Weak organic acids such as propionic and sorbic acid are commonly added as chemical preservatives to suppress the growth of undesired microorganisms and to lengthen the shelf life of bakery products. Generally at lower pH values, these acids are in their undissociated form and can easily penetrate the plasma membrane. Once intracellular, the acid dissociates and due to the release of charged protons, the cell cytoplasm gets acidified. Consequently, the drop in pH decreases phosphofructokinase activity, a key enzyme of glycolysis, and hence reduces the ATP yield (Krebs et al., 1983). Recent investigations have suggested that sorbic acid may act additionally as a membrane-active antimicrobial compound through the inhibition of the plasma membrane H^+-ATPase proton pump (Stratford et al., 2013a, 2009).

Within the EU, the use of chemical preservatives in bakery wares like bread and rolls is limited (EEC, 2008). Because of their higher water solubility and easier handling than their respective corrosive acids, potassium, sodium or calcium salts of propionic and sorbic acid are the forms generally used (Magan et al., 2003a). Sorbate is allowed up to 0.2% (w/w) and propionate can be added to a maximum 0.3% (EEC, 2008). To define it more precisely, these levels pertain only to prepacked sliced bread and rye bread. A maximum of only 0.1% propionate is permitted for prepacked unsliced bread. This means that for unpacked bread and in particular wheat bread made only from wheat flour, water, yeast or sourdough and salt, neither the addition of sorbate nor propionate is allowed. The addition of benzoic acid to bakery products is, although sometimes examined in studies (Suhr and Nielsen, 2004; Guynot et al., 2005), not authorized.

Suhr and Nielsen (2004) conducted an in vitro screening experiment over a wide pH and a_w range using different concentrations of weak acid preservatives in rye agar and wheat agar, against 9 fungal isolates. On wheat agar, a high propionate concentration (0.3%) generally had a strong inhibitory effect on all fungi tested at pH 4.5 and a_w 0.95, but not at pH 6, which is generally the pH of standard wheat bread. However, on rye agar (high a_w and low pH), the growth of *P. roqueforti* even occurred up to the addition of 0.3% propionate. Thus, the authors concluded that the addition of propionate to rye sourdough bread is not recommended due to the resistance of *P. roqueforti*. Furthermore, in our opinion, those results also indicate that propionate has only slight effect in mold inhibition when included in breads at pH 6.

In another study, sorbate completely inhibited fungal growth at a_w 0.90 and pH 4.5 when compared to calcium propionate assayed at the highest concentration in bread analogues at 0.3% level (Guynot et al., 2005). The current legislated maximum level of 0.2% was not tested. However, although sorbate seems to be more efficient at inhibiting spoilage, it is rarely used in bread due to its negative impact on bread volume (Lavermicocca et al., 2000). The use of 0.3% calcium propionate in vitro only partially prevented germination of conidia, in 12 tested fungal species (Lavermicocca et al., 2000). Nevertheless, some in situ experiments on the use of propionate to control bread spoilage showed shelf life prolongation and some further examples are given throughout this review.

Overall, high concentrations of sorbate or propionate are desired for antifungal activity, but this can also alter the sensory properties of the product. Prolonged usage of the same preservatives against spoilage fungi may lead to the development of fungal resistance to those chemicals (Suhr and Nielsen, 2004; Levinskaite, 2012; Stratford et al., 2013b). Moreover, concentrations below the maximum level of preservative must be chosen carefully. Sub-optimal concentrations, lower than 0.03% can result in an enhancement of fungal growth (Marin et al., 2002) and higher mycotoxin production (Arroyo et al., 2005).

Alternative preservation techniques

The disadvantages outlined regarding the use of chemical preservatives has encouraged researchers to finding alternative agents to control spoilage fungi in food products. Furthermore, strategies need to be developed to reduce mycotoxins levels ensuring food safety and consumer health.

Fermentation

Lactic acid bacteria

Microbial fermentation is one of the oldest and most economically and ecologically friendly methods of preserving foods (Zannini et al., 2012). Among bakery products the microorganisms most widely used as starter cultures, applied for example in sourdough production, are LAB. Since active compounds responsible for biopreservation are produced or released by LAB in situ, use of sourdough technology can replace chemical preservatives, guaranteeing a clean label while imparting additional positive effects, such as improved flavor, texture and nutritional properties, coupled with higher consumer acceptance (Pawlowska et al., 2012). A further benefit of LAB is that many species from the genera *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* have been referred to the European Food Safety Authority (EFSA) for safety assessment without raising safety concerns. As a result, they have been included in the Qualified Presumption of Safety (QPS) list for authorized use in the food and feed chain within the European Union (EFSA, 2012). The same applies to the US, where they enjoy the Generally Regarded as Safe (GRAS) status regulated by the U.S. Food and Drug Administration.

Recently there have been several comprehensive reviews examining LAB with respect to their antifungal activity spectrum, effective metabolites and their interactions with mycotoxins (Dalié et al., 2010; Crowley et al., 2013; Oliveira et al., 2014).

This review however, describes how specific antifungal LAB strains have been applied to extend the shelf life of bread. In general, a low preservative effect can be achieved through the use of LAB-fermented sourdough due to the pH drop and acidification associated with the production of organic acids, mainly lactic and acetic acid. However, it has been reported that chemical acidification either has no influence on mold inhibition or can only prolong shelf life to a limited extent (Ryan et al., 2011; Axel et al., 2015). Accordingly, this suggests that LAB, in addition to producing lactate and acetate, produce or release other active compounds during fermentation, which contribute to the antifungal activity when sourdough is

included in bakery products. These metabolites are usually low molecular mass compounds such as phenyl and substituted phenyl derivates (3-phenyllactic, 4-hydroxyphenyllactic, and benzoic acid), cyclic dipeptides, hydroxy fatty acids or antifungal peptides. Coupled to their relatively high minimal inhibition concentration (MIC) ranging from 0.1 to 10,000 mg/kg, they are produced at low levels in the fermentation substrate (Axel et al., 2015a). Thus, the antifungal inhibitory mechanism is believed to originate from a complex synergy effect among these low molecular mass compounds. An interesting in situ chemical application of antifungal compounds at concentration levels of about 30 mg/kg resulted in a longer shelf life (+25%) than achieved simply by acidifying the dough (Axel et al., 2015a). Moreover, the production of the antifungal compounds during fermentation is species and substrate specific (Axel et al., 2015a; Vermeulen et al., 2006). Therefore, it is necessary to measure the levels of these compounds produced both in nutrient growth media and the sourdough environment.

In the last decades, considerable effort has been directed to screen the antifungal activity of LAB in order to find suitable starter cultures for sourdough fermentation. In terms of biopreservation, these studies first rely on in vitro assays which either include dual culture plate assays and or micro titre plate assays (Magnusson and Schnürer, 2001). If a strain shows broad activity against a range of bread spoilage organisms, it is further evaluated in sourdough fermentation and bread baking to assess both its potential to extend shelf life and its effects on bread quality. Such an approach was undertaken by several research groups and findings are presented in Table 1. This table summarizes which LAB have been applied as antifungal starter culture in sourdough and bread fermentation. It also presents the mold activity spectrum and the antifungal compounds analyzed which were related to the biopreservative extension of shelf life Furthermore, it includes a few studies that assess whether the level of mycotoxins can be decreased by means of fermentation.

In an in vitro screening with 95 strains of different lactobacilli and pediococci against *A. niger* CH101, *Penicillium sp.* CH102 and *F. graminearum* CH103, *Lb. plantarum* CRL778 performed best (Gerez et al., 2009) and also in situ (Gerez et al., 2010). In a follow-up study by the same group, this same strain was applied in situ as a biopreservative culture inoculated into a slurry composed of water, wheat and quinoa flour (Gerez et al., 2014). The growth of *A. niger* 13D and levels of OTA on the resulting bread slices were decreased by 60% independent to a_w. The antifungal effect was related to the production of lactic, acetic, 3-phenyllactic acid and 4-hydroxy-phenyllactic acids. In contrast, Vidal et al. (2014) found that OTA levels were confirmed to be quite stable during the bread making process, regardless of whether sourdough was added or not. Traditional wheat sourdough type I was used for the latter experiment, where the specific microbiota were not determined. Sourdough type I, is a mixture of flour and water, which is fermented spontaneously by LAB and yeasts and continuously back-slopped in various fermentation steps (Corsetti, 2013). A Type II is obtained through a unique one-step fermentation using a portion of the mature sourdough from Type I or defined starter cultures (Corsetti, 2013). Elsanhoty et al. (2013) inoculated wheat grains with *A. flavus* to allow aflatoxin B and

G production. Fermentation of the contaminated flour with *Lb. rhamnosus* TISTR541 and yeast resulted in the lowest level of aflatoxin in the bread. A sourdough fermentation using a commercial starter mix (*Lb. plantarum* and *Lb. brevis*) also gave a significant decrease (58.6–66.5%) in DON content produced with *F. graminearum* MI113 artificially contaminated wheat flour (Banu et al., 2014). The detoxifying effect of LAB against mycotoxins is believed to be the result of binding to bacterial cell wall structures, with peptidoglycan being particularly important (Lahtinen et al., 2004). Other strategies for biological detoxification of mycotoxins using microorganism have been recently reviewed by Hathout and Aly (2014).

Lb. plantarum FST1.7 represents another antifungal LAB strain which has been applied to extend the shelf life of wheat and gluten-free (composite recipe using a mix of brown rice flour, buckwheat flour, corn starch and soya flour) sourdough breads, after it was shown to be active against spoilage molds and bacteria in vitro (Dal Bello et al., 2007; Moore et al., 2008; Ryan et al., 2008). The antifungal activity of *Lb. plantarum* FST1.7 was related to lactic acid, 3-phenyllactic acid and the two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro) production which were detected in the cell free supernatant. Subsequent analyses revealed the presence of 3-phenyl lactic acid and cyclic dipeptides also in the wheat sourdough (Ryan et al., 2009a, 2009b). Unfermented LAB growth media already contains cyclic dipeptides but their concentrations can increase upon fermentation with LAB (Axel et al., 2014). The level of cyclic dipeptides found in sourdough and broth are 1,000-fold lower than the MICs indicating a minor significance in the antifungal action, but synergistic effects with other compounds are possible (Niku-Paavola et al., 1999; Ryan et al., 2009a; Axel et al., 2014). In the gluten-free system, it must be stated however, that *Fusarium* outgrowth on the infected gluten-free bread slices started for all breads after 2 days. In comparison with the other gluten-free breads, the *Lb. plantarum* FST1.7 sourdough bread had the lowest increase in mold spoilage per day (Moore et al., 2008). The same strain failed to extend the shelf life when single types of gluten-free flour was fermented and incorporated in a gluten-free bread system (Wolter et al., 2014).

The biological preservation of wheat sourdough bread fermented with *Lb. amylovorus* DSM19280 inhibited the growth of *A. niger, F. culmorum, P. expansum* and *P. roqueforti* more effectively than calcium propionate (Ryan et al., 2011). The successful inhibitory activity in gluten-free quinoa sourdough bread (Axel et al., 2015) and salt-reduced wheat sourdough bread (Belz et al., 2012) reinforces the potential of this strain as an antifungal starter culture. HPLC-UV/DAD analysis of the *Lb. amylovorus* DSM19280 fermented quinoa sourdough extracts revealed the presence of the antifungal compounds hydroferulic acid, 4-hydroxy-phenyllactic acid, phloretic acid and 3-phenyllactic acid ranging from 13 to 86 ppm (Axel et al., 2015). In wheat sourdough the maximal in situ antifungal activity could not be related to *Lb. reuteri* R29 being the highest producer of carboxylic acids, since use of *Lb. amylovorus* DSM 19280 had a greater biopreservative effect, potentially due to the production of additional antifungal compounds, such as antifungal peptides (Axel et al., 2015a).

Table 1. Antifungal lactic acid bacteria used as starter cultures for bread biopreservation, their tested activity and related antifungal compounds.

Starter culture used	Activity spectrum	Antifungal compounds in biopreservative/sourdough	Substrate/Application	Reference
Lactobacillus amylovorus DSM19280 Lb. brevis R2Δ Lb. reuteri R29	Environmental molds[a]	Azelaic acid, caffeic acid, p-coumaric acid, ferulic acid, hydrocaffeic acid, hydroferulic acid, 2-hydroxyisocaproic acid, 4-hydroxyphenyllactic acid, 3-phenyllactic acid, phloretic acid, salicylic acid, vanillic acid	Wheat sourdough/ wheat sourdough bread	Axel et al. (2015)
Lb. amylovorus DSM19280	Aspergillus niger, Fusarium culmorum, Penicillium expansum, P. roqueforti, environmental molds[a]	3-Phenyllactic acid, 4-hydroxyphenyllactic acid, hydroferulic acid, phloretic acid, organic acids, cyclic dipeptides	Wheat sourdough/ wheat sourdough bread, Quinoa sourdough/ quinoa sourdough bread	Ryan et al. (2011); Belz et al. (2012); Axel et al. (2015b)
Lb. plantarum Lb. brevis (DI-PROX MTTX, commercial starter mix)	Deoxynivalenol	n.d.[b]	Wheat sourdough bread/ wheat sourdough bread	Banu et al. (2014)
Lb. plantarum CRL 778	A. niger ($a_w = 0.971$, 60% lower Ochratoxin A production)	Organic acids, 3-phenyllactic acid, 4-hydroxyphenyllactic acid	Wheat slurry, Quinoa slurry and mixtures/ wheat sourdough bread	Gerez et al. (2014)
Not specified	Deoxynivalenol, ochratoxin A	n.d.[b]	Traditional wheat sourdough type I/wheat sourdough bread	Vidal et al. (2014)
Lb. plantarum FST 1.7	A. niger, F. graminearum, F. culmorum, F. oxysporum (no activity against P. roqueforti), environmental molds[a]	Cyclic dipeptides, 3-phenyllactic acid	Wheat sourdough/ wheat sourdough bread, Gluten-free sourdough (mixture of brown rice, corn starch, buckwheat and soya flour)/ gluten-free bread, Gluten-free sourdough (quinoa, teff, buckwheat, oat, sorghum)/ gluten-free bread	Dal Bello et al. (2007); Moore et al. (2008); Ryan et al. (2008, 2009a, 2009b); Wolter et al. (2014)
Lactococcus BSN	A. niger, F. oxysporum, F. moniliforme	n.d.[b]	Wheat sourdough/ wheat sourdough bread	Varsha et al. (2014)
Lb. hammesii DSM16381	A. niger, P. roqueforti, environmental molds[a]	Monohydroxy octadecenoic acid	Wheat sourdough supplemented with linoleic acid/ wheat sourdough bread	Black et al. (2013)
Lb. rhamnosus TISTR 541	A. flavus/aflatoxin (B1, B2, G1, G2)	Aflatoxin binding cell wall	Whole wheat sourdough / Baladi bread	Elsanhoty et al. (2013)
Pediococcus acidilactici KTU05-7 Pc. pentosaceus KTU05-8 Pc. pentosaceus KTU05-10	A. fumigatus, A. niger, A. versicolor, F. culmorum, P. chrysogenum, P. expansum, environmental molds[a]	Bacteriocins like inhibitory substances, organic acids	Single LAB cell suspension 5×10^4 CFU/cm²) sprayed on the surface of baked wheat bread	Cizeikiene et al. (2013)
Commercial sourdough starter	Beauvericin, enniatin,	n.d.[b]	Wheat and rye sourdough/ wheat and rye sourdough bread	Hu et al. (2013)
Leuconostoc citreum C5 Weissella confusa HO24	Cladosporium sp., Neurospora sp., P. crustosum	Organic acids	Rice sourdough for steamed rice cake	Baek et al. (2012)
Leuconostoc citreum HO12 W. koreensis HO20	A. niger, P. roqueforti	Ethanol, organic acids	Whole wheat sourdough/Whole wheat sourdough bread	Choi et al. (2012)
Lb. paralimentarius PB127 Lb. rossiae LD108	A. japonicus	Antifungal peptides, organic acids, 3-phenyllactic acid	Wheat sourdough/ wheat sourdough bread, Panettone	Garofalo et al. (2012)
Lb. plantarum ATCC 20179 Lb. acidophilus ATCC 20079	Environmental molds[a]	Organic acids	Wheat sourdough/ traditional Iran flat bread (Sangak)	Najafi et al. (2012)
Lb. fermentum Te007 Lb. paracasi D5 Lb. pentosus G004 Pc. pentosaceus Te010 (Co-cultures)	A. niger, A. oryzae	Protein-like compounds	LAB cells or supernatants added to wheat bread dough/ wheat bread	Muhialdin et al. (2011)
Lb. acidophilus	Environmental molds[a]	Organic acids	Wheat sourdough/ wheat sourdough bread	Plessas et al. (2011)

Table 1. (Continued)

Starter culture used	Activity spectrum	Antifungal compounds in biopreservative/sourdough	Substrate/Application	Reference
Lb. sakei (Co-culture)				
Lb. plantarum LB1 + *Lb. rossiae* LB5 (Co-culture)	*P. roqueforti*, environmental molds[a]	Antifungal peptides, organic acids; acetic acid, formic acid, lactic acid, 3-phenyllactic acid	Sourdough fermented wheat germ (freeze dried)/ wheat bread	Rizzello et al. (2011)
Lb. buchneri FUA 3252	*A. clavatus*, *Cladosporium sp.*, *Mortierella sp.*, *P. roqueforti*, environmental molds[a]	Acetic acid, ethanol, lactic acid, propionic acid	Whole wheat sourdough/ whole wheat sourdough breads	Zhang et al. (2010)
Lb. diolivorans DSM14421 (Co-culture)				
Lb. reuteri CRL1100	*A. niger*, *Penicillium sp.*	Organic acids, 3-phenyllactic acid	Wheat flour slurry/ wheat sourdough bread	Gerez et al. (2009,2010)
Lb. plantarum CRL778				
Lb. brevis CRL772				
Lb. brevis CRL79 (single + co-cultures)				
Lb. brevis AM7	*P. roqueforti*, environmental molds[a]	Antifungal peptide and protein	Wheat sourdough + water-soluble extract (27%, vol/wt, 5 mg of protein/ml) from *Phaseolus vulgaris cv. Pinto*/ wheat sourdough bread	Coda et al. (2008)
Lb. Casei	Environmental molds[a]	n.d.[b]	Wheat sourdough/Iranian Lavash bread	Fazeli et al. (2004)
Lb. fermentum				
Lb. plantarum				
Lb. plantarum 21B	*A. niger*	n.d.[b]	Wheat sourdough/wheat sourdough bread	Lavermicocca et al. (2000)

[a]Shelf life test without fungal spore inoculum/ or exposure to bakery air; [b]not determined.

In fact, few studies have reported the use of sourdough in gluten-free products for mold inhibition (Moore et al., 2008; Baek et al., 2012; Axel et al., 2015). In contrast to wheat bread, gluten-free breads usually have higher water contents and a_w (Moore et al., 2004; Hager et al., 2012) This higher water availability makes gluten-free products more susceptible for fungal spoilage. In the absence of the gluten network, the movement of water from the gluten-free bread crumb to the crust is enhanced (Sciarini et al., 2010). Thus, combating fungal contaminations in gluten-free systems is more difficult. The same applies for salt-reduced bread. Salt acts as a preservative agent due to its ability to reduce a_w.

An interesting and so far unique approach was undertaken by Rizzello et al. (2011), exploiting the potential of sourdough fermented wheat germ to delay fungal outgrowth on wheat bread. Wheat germ is the embryo containing part of the grain and one of the main by-products of the milling process. It features high quality proteins, lipids and is rich in vitamins. Use of sourdough biotechnology stabilized and improved some nutritional characteristics of wheat germ making it suitable for food processing (Rizzello et al., 2010). The use of the freeze-dried wheat germ (4%, w/w) fermented with a co-culture of *Lb. plantarum* LB1 and *Lb. rossiae* LB5 prevented fungal outgrowth on bread slices to at least 28 days of storage, behaving similar to calcium propionate (0.3%, w/w) addition (Rizzello et al., 2011). A complex synergistic system between organic acids and peptides was suggested to be responsible for mold prevention. The sequence of an antifungal peptide was found to be encrypted in the cereal protein expansin which belongs to the plant defensin-like proteins. It was hypothesised that defensins can induce

membrane permeabilization of fungi during the defence response (Picart et al., 2012).

Sourdough with propionate improves synergistic antifungal activity (Ryan et al., 2008; Zhang et al., 2010). As a result of the consumer's aversion to chemical ingredients, some studies have examined the application of natural propionic acid fermentation. In situ-propionate production of LAB with propionic acid bacteria (PAB) in co-fermentation is possible, but rather impracticable due to the slow or even absent growth of PAB in the sourdough system (Javanainen and Linko, 1993; Suomalainen and Mäyrä-Mäkinen, 1999). Co-culture fermentation of specific LAB strains enables another option of producing propionate naturally. Lactate is first converted into 1,2-propanediol by *Lb. buchneri* (Oude Elferink et al., 2001) and 1,2-propanediol is further converted into propionate by *Lb. diolivorans* (Krooneman et al., 2002). Zhang et al. (2010) used this cooperative metabolism of *Lb. buchneri* FUA 3252 and *Lb. diolivorans* DSM14421 for bread preservation. Although the bread prepared with 20% sourdough achieved the longest shelf life, high amounts of propionate (10 mM kg^{-1}) and acetate (35 mM kg^{-1}) were unfavorable in terms of sensory properties (Zhang et al., 2010). Additionally, a long fermentation time of 14 days was required to ensure the formation of sufficient amounts of propionic acid.

More studies have reported prolonged bread shelf life using combined starter cultures for sourdough fermentation (Muhialdin et al., 2011; Plessas et al., 2011). The better antifungal effect was explained by higher acidification rates.

The conversion of linoic acid to an antifungal monohydroxy-octadecenoic acid was observed in sourdough fermentation by *Lb. hammesii* DSM16381 (Black et al., 2013). The

sourdough has potential to be used as a biopreservative since the addition of 20% in bread making resulted in an extended shelf life when challenged with *P. roqueforti* and *A. niger*, and environmental contamination without inoculation.

Most of the studies presented apply the starter culture in the traditional sourdough making process. However, a different approach was followed by Cizeikiene et al. (2013). After inoculation in MRS media, the single LAB cell suspension ($\sim 5 \times 10^4$ CFU cm^{-1}) was sprayed on the surface of the baked wheat bread. The experimental procedure did not clarify whether the LAB cell suspension was washed or remained suspended in MRS medium. We judge the latter treatment as unacceptable for food applications.

Overall antifungal LAB-fermented sourdough serves as a great potential biopreservative ingredient to produce chemical preservative-free breads with an extended shelf life, coupled with other advantages like improved flavor and texture.

Yeast

The application of yeasts other than baker's yeast (*Saccharomyces cerevisiae*) with or without the combination of LAB is also suggested as a promising alternative for bread preservation (Table 2). For instance, the yeast *Wickerhamomyces anomalus* LCF1695 (formerly known as *Pichia anomala*) was used as mixed starter, in combination with *Lb. plantarum* 1A7, for sourdough fermentation. This combination, when added to a standard bread recipe, allowed a microbial shelf life of at least 14 days during storage at room temperature after artificial inoculum (10^2 conidia ml^{-1}) with *P. roqueforti* DPPMAF1 (Coda et al., 2011). In subsequent work by the same group another yeast strain, *Meyerozyma guilliermondii* LCF1353 (previously *Pichia guilliermondii*), was found out of 146 yeast strains to harbor marked antifungal activity toward the indicator fungus *P. roqueforti* DPPMAF1. As shown under pilot plant conditions, the bread including the sourdough fermented with a combined starter culture consisting of *M. guilliermondii* LCF1353, *W. anomalus* LCF1695 and *Lb. plantarum* 1A7 strain showed the longest shelf-life (Coda et al., 2013). High ethanol and ethyl acetate concentrations were determined in the water/salt-soluble dough extract resulting, together with proteinaceous compounds, in fungal inhibition. Bread quality parameters like specific volume and crumb hardness were not significantly different to the control breads.

The fungal outgrowth of *Penicillium paneum* KACC 44834 on white pan bread leavened with *P. anomala* SKM-T was significantly decreased in comparison to normal baker's yeast (Mo and Sung, 2014). The sensory qualities of bread were also improved by the presence of the pleasing flavor compounds phenylethyl alcohol and 2-phenylethyl acetate, which were only produced by *P. anomala* SKM-T. These compounds also contributed to the shelf life extension.

The antifungal activity of *W. anomalus* and *M. guilliermondii* has previously been demonstrated under silage storage conditions (Petersson and Schnürer, 1995). Nevertheless, further research on the use of these yeasts is needed to confirm the in situ-antifungal activity for bread biopreservation. The use of *W. anomalus* seems to be safe, because this species is also included in the QPS list, although authorized only when it is used for enzyme production (EFSA, 2012). However, the safety of the yeast *M. guilliermondii* needs to be assessed for use as a biological control agent in food.

Plant derived compounds

The growing interest in the application of natural ingredients with multifunctional properties in food has also led to extensive investigation of plant extracts as biopreservatives. Recently reviewed by da Cruz Cabral et al. (2013), a comprehensive summary is given on in vitro experiments regarding the use of plant extracts and essential oils for controlling common food spoilage fungi. Additionally the mode of action and the impact on mycotoxins is discussed. However, only a few in situ tests are presented, without any application on bread. It is noteworthy that we found few studies dealing with the direct addition of plant extract in bakery products (Table 3). Recent developments also employ essential oils in edible coating films of active packaging (AP) (Table 3). These will be discussed in Section 4.3.

Using a simple storage test, without artificial inoculum, Wei et al. (2009) evaluated the antifungal activity of different raisin extracts and products in conventional bread. The breads made with the raisin paste and raisin water extract (7.5%) showed the best mold-retarding properties when compared to the control containing no preservative. However, they were not significantly different to the positive control containing 0.24% propionate. The authors related the antifungal activity to a synergistic effect between the formed Maillard products and phenolic compounds. Although bread quality parameters were not included in the study, the raisin extract seems to have

Table 2. Antifungal yeasts applied for bread biopreservation, their tested activity and related antifungal compounds.

Starter culture used	Activity spectrum	Antifungal compounds in biopreservative	Substrate/Application	Reference
Pichia anomala SKM-T	*Penicillium paneum*	2-phenylethyl acetate, phenylethyl alcohol	Wheat dough/ white pan bread	Mo and Sung (2014)
Meyerozyma guilliermondii LCF1353 + *Wickerhamomyces anomalus* LCF1695 (Co-culture with *Lb. plantarum* 1A7)	*P. roqueforti*, environmental molds[a]	Antifungal peptides, ethanol, ethyl acetate	Wheat sourdough/ wheat sourdough bread	Coda et al. (2013)
W. anomalus LCF1695 (Co-culture with *Lb. plantarum* 1A7)	*P. roqueforti*, environmental molds[a]	Antifungal peptides, ethanol, ethyl acetate	Wheat sourdough/ wheat sourdough bread	Coda et al. (2011)

[a]Shelf life test without fungal spore inoculum/ or exposure to bakery air.

Table 3. Other biopreservation techniques aimed at increasing bread shelf life, their tested activity and related antifungal compounds.

Other biopreservation techniques	Activity spectrum	Antifungal compounds in biopreservative	Application	Reference
Plant derived compounds:				
Water/salt-soluble extracts of legume flour hydrolysates (soy, lentil, pea, chick pea and faba bean)	*Penicillium roqueforti*	Antifungal peptides; in pea: pea defensins 1 and 2, a nonspecific lipid transfer protein	Wheat sourdough bread	Rizzello et al. (2015)
Raisin paste	Environmental molds[a]	Maillard products, phenolic compounds	Wheat bread	Wei et al. (2009)
Raisin water extract				
Water-soluble extract from *Amaranthus* spp. seeds	*P. roqueforti*	Antifungal peptides (agglutinin sequences)	Wheat bread, Wheat sourdough bread	Rizzello et al. (2009)
Protective Packaging:				
Cinnamaldehyde	Environmental molds[a]	Cinnamaldehyde	Cellulose-derived polymers containing 5% cinnamaldehyde for cinnamon loaf (0.1g/kg wheat flour)	Lopes et al. (2014)
Clove bud essential oil Oregano essential oil	Environmental molds[a]	Eugenol, carvacrol, thymol	Nano-emulsions of the essential oil in edible films from methylcellulose/metalized polypropylene bags/ active packaging for wheat bread	Otoni et al. (2014)
Oregano essential oil	Environmental molds[a]	γ-Terpinene, *p*-cymene	Antimicrobial sachets containing more than 5% oregano essential oil/ active packaging for wheat bread	Passarinho et al. (2014)
Allyl, benzyl, and phenyl isothiocyanate	*Fusarium moniliforme*	Allyl, benzyl, and phenyl isothiocyanate	Model active packaging system (wheat bread)	Azaiez et al. (2013)
Cinnamaldehyde	*P. expansum*	Cinnamaldehyde	Antimicrobial gliadin film treated with 5% cinnamon aldehyde/ active packaging for wheat bread	Balaguer et al. (2013)
Marjoram essential oil vapor	*Aspergillus niger, P. chrysogenum, Rhizopus* spp.	n.d.[b]	Model active packaging system (wheat, wheat-rye mixed, and rye bread slices)	Krisch et al. (2013)
Sage essential oil vapor				
Cinnamon essential oil	Environmental molds[a]	Cinnamaldehyde	Polypropylene with an organic solvent base (ethyl acetate) coated with nitrocellulose containing the cinnamon essential oil (0.0215 and 0.0374 g)	Gutiérrez et al. (2011)
Cinnamon powder Clove powder	Environmental molds[a]	Cinnamaldehyde Eugenol, Eugenyl acetate	Included in biodegradable film based on cassava starch for wheat pan bread	Kechichian et al. (2010)
Further applications:				
Ethanol	Environmental molds[a]	Ethanol	Active packaging ethanol emitting sachets for wheat Ciabatta bead	Hempel et al. (2013)
Ethanol	*Chrysonilia sitophila, Hyphopichia burtonii*	0.8% and 2.0% ethanol	Surface treatment on wheat bread	Berni and Scaramuzza (2013)
Biosurfactant produced by B. subtilis SPB1	Environmental molds[a]	Lipopeptide biosurfactant	Addition of 0.075% purified lipopeptide powder dissolved in distilled water to wheat bread formula	Mnif et al. (2012)
Ethanol	Environmental molds[a]	Synergistic effect of 0.5% ethanol with sorbate and propionate	Surface treatment on wheat bread	Katsinis et al. (2008)
1-Monocaprylin, 1-Monocaprin	A. niger, *Monascus ruber, P. chrysogenum, P. jensenii,*	1-monocaprylin destabilizes membranes by increasing membrane fluidity and the number of phase boundary defects	Microfilm (65 mg/L) spread on the surface of wheat bread	Buňková et al. (2010)

[a]Shelf life test without fungal spore inoculum/or exposure to bakery air; [b]not determined.

potential to reduce chemical preservatives in food systems and should be further investigated.

Cherry laurel (*Prunus laurocerasus L.*) leaf extracts were suggested as novel biopreservatives after showing very low MIC (μg/mL) against a range of bread spoilage fungi (Sahan, 2011). The highest total antifungal effect was observed from ethanol and acetone extracts. Neither biologically active components were analyzed nor the in situ activity were tested in bread shelf life trials.

The interest in applying antifungal proteins and peptides for bread biopreservation alone or in combination with sourdough (Coda et al., 2008; Rizzello et al., 2009, 2011, 2015), and as biocontrol agents against fungal pathogens in agriculture has increased in recent years (Yan et al., 2015). A large number of antifungal proteins have been reported from a multitude of organisms including leguminous flowering plants, non-leguminous flowering plants, gymnosperms, but also fungi, bacteria, insects and mammals (Ng, 2004). They are categorized by type and function, comprising thaumatin-like proteins, chitinases, glucanases, embryo-abundant proteins, miraculin-like proteins, cyclophilin-like proteins, allergen-like proteins, defensins, thionins, and nonspecific lipid transfer proteins (Ng, 2004). The various mechanisms of antifungal action for a range of plant antifungal proteins were recently summarized by Yan et al. (2015). Antifungal chitinases act hydrolytically on fungal cell wall chitin leading to cell lysis (Graham and Sticklen, 1994).

Defensins cause membrane permeabilization (Picart et al., 2012). A similar action was proposed for lipid transfer proteins (Selitrennikoff, 2001). It is possible to observe a combination of antifungal proteins in a single plant species (Ye et al., 2000).

Water/salt-soluble extracts of different legume flour hydrolysates (soy, lentil, pea, chick pea and faba bean) were obtained by the use of fungal proteases and tested in vitro against *P. roqueforti* DPPMAF1 (Rizzello et al., 2015). Pea (*Pisum sativum*) hydrolysate, the most active antifungal biopreservative, was freeze dried and used as ingredient for bread making at a level of 1.6% (w/w). Bread made with the combination of sourdough fermented by the antifungal strain *Lb. plantarum* 1A7 and the pea flour hydrolysate had the longest shelf life. The antifungal activity was attributed to three native proteins (pea defensins 1 and 2, a nonspecific lipid transfer protein and a mixture of peptides released during hydrolysis).

Rizzello et al. (2009) also reported the use of water-soluble extract from *Amaranthus* spp. seeds during storage of gluten-free and wheat flour breads. Novel antifungal peptides (agglutinin sequences) were identified in the extract, which showed in vitro inhibition of a large number of spoilage fungal species isolated from bakeries (5 mg/mL). Three different types of bread were produced; control bread, bread with added amaranth water-soluble extract, and sourdough bread (*Lb. sanfranciscensis* E9) with added amaranth water-soluble extract. Bread slices were inoculated with *P. roqueforti* DPPMAF1 (10^2/mL). With the addition of the water-soluble extract to the breads the appearance of fungal mycelium was delayed by at least 7 days, being more pronounced for the sourdough breads. Furthermore, the addition of the water-soluble extract improved the quality of the gluten-free breads regarding taste and specific volume. *Lb. sanfranciscensis* is the most frequently used LAB as leavening agent for sourdough production (Gänzle, 2014). The antifungal activity of this strain was not specified by the authors. Thus, we speculate further improvement of the preservative effect when amaranth water-soluble extract would have been combined with sourdough fermented with a known antifungal LAB.

Protective packaging

Packaging is an important factor in extending the microbial shelf life of bakery products. Normal packed bread provides aerobic microorganisms like fungi adequate amounts of oxygen for growth. The principle of modified atmosphere packaging (MAP) revolves around reducing oxygen by replacing the headspace of the product either with carbon dioxide alone or mixtures of it and nitrogen. Generally for bakery products, the gas mixture consists of 60% or more carbon dioxide with nitrogen acting as a filler gas. Thus, carbon dioxide is the fungistatic component slowing down the proliferation of molds. Furthermore, it may alter the permeability of the cell membrane, reduce the internal pH of the cell and influencing certain enzyme systems and changing metabolic activities (Farber, 1991). Due to the highly porous texture of bakery products complete oxygen elimination is challenging (Hempel et al., 2013). Moreover, oxygen accumulation over time can occur even if packaging films of low oxygen permeability are used. The introduction of AP provided a significant improvement

over MAP. AP techniques prevents bread spoilage through the use of oxygen absorbers, ethanol emitters and other antimicrobial agents which are either incorporated as a layer in the packaging or in edible films, or placed for example as sachets in the packaging. AP can be combined with the classical MAP approach. An extensive review of shelf life in packed bakery products was published by Galić et al. (2009). This review covers many studies dealing with MAP including active packaging and describes the types of material used for cereal based food packaging. Therefore, this section presents an update in research conducted within the last 5 years (Table 3).

As previously mentioned, plant extract or essential oils can be used in antimicrobial films for AP of bread. Balaguer et al. (2013) found good in vitro antifungal activity against *P. expansum* and *A. niger* testing gliadin films containing cinnamaldehyde which is the main component of the cinnamon essential oil. In an in situ test wheat bread slices were inoculated with *P. expansum* conidial suspension (10^6 spores/mL). After 30 days, fungal growth remained absent on bread slices which were packed into mono cast polypropylene bags with a piece (13 cm × 8 cm) of the antimicrobial gliadin film treated with 5% cinnamon aldehyde at 23°C. Cellulose-derived polymers also containing 5% cinnamaldehyde (Lopes et al., 2014) and polypropylene with an organic solvent base (ethyl acetate) coated with nitrocellulose containing the cinnamon essential oil (Gutiérrez et al., 2011), present further materials which were used successfully for gluten-free and wheat bread shelf life extension. The shelf life of wheat pan bread was not increased using biodegradable films based on cassava starch with cinnamon and clove powder (Kechichian et al., 2010). The authors observed an increase in a_w of the film due to product instability. Hence, the material which supports the antimicrobial-active substance seems to be of great importance. The commission regulation (EU) No. 10/2011, also known as plastic implementation measure (PIM), controls which materials can be used for food packaging and sets specific migration limits to ensure food safety (EEC, 2011).

Otoni et al. (2014) included nano-emulsions of oregano and clove bud essential oil in edible films from methylcellulose/metalized polypropylene bags. Their antifungal efficacy was attributed to the phenolic compounds eugenol, carvacrol and thymol. Those compounds have shown antimicrobial activity against a range of bacteria due to reduction of the proton gradient through the cell membrane. The reduced proton gradient, along with the resulting depletion of the ATP pool, kills microbial cells (Lambert et al., 2001; Ben Arfa et al., 2006). In vitro tests with the essential oils inhibited spore germination of *A. niger* ATCC 16404 and *Penicillium sp.* ATCC 2147 at a concentration of 20 mg/mL (Otoni et al., 2014).

The overall sensory and physicochemical properties of the bread can be influenced by aromatic compounds like cinnamon aldehyde or thymol, although present at very low levels in the final product. Although the cinnamon aroma was noticeable to a panellist at the highest concentration level (0.374 g per self−adhesive active label of 13 × 10 cm), no significant differences were found in cinnamon taste between control (no cinnamon aldehyde) and positive samples (Gutiérrez et al., 2011). In contrast, although antimicrobial sachets containing more than 5% oregano essential oil reduced the growth rate of

molds on sliced bread, the content of γ-terpinene and p-cymene on treated bread increased throughout storage and their acceptance was reduced (Passarinho et al., 2014). Marjoram and sage essentials oil vapor treatment also showed inhibition of mold on bread slices in a model AP system (Krisch et al., 2013). However, the taste and odor was rated from strange to unacceptable.

Isothiocyanates are another group of potenial antimicrobial substances, these are hydrolysed products of sulphur containing compounds called glucosinolates, which occur naturally in cruciferous vegetables, such as broccoli, cabbage, cauliflower, kale, turnip, radish, canola, rapeseed and various mustards (Isshiki et al., 1992). Azaiez et al. (2013) demonstrated their ability in their gaseous state to inhibit the fungal growth of *Fusarium moniliforme* CECT 2987 and to reduce the levels of fumonisins produced by this strain in a bread AP model system. Allyl and phenyl isothiocyanates showed stronger activity than benzyl isothiocyanates. Allyl isothiocyanate at 500 μg/L vapor phase concentration presented the highest reduction of about 96% fumonisin B_2 in comparison to the un-treated control. Indeed, chromatograms presented in the publication evidenced that the high peak of fumonisin B_2 present in the control sample almost disappeared in the isothiocyanate treated samples. The strongly electrophilic carbon in the isothiocyanate group ($-N=C=S$) easily binds to thiol and free amino groups of amino acids, peptides and proteins (Luciano and Holley, 2009). It was suggested that isothiocyanates bind the free amino group of the fumonisin. Further investigation will examine any toxicity of the products generated by the reaction of fumonisin B_2 and isothiocyanates (Azaiez et al., 2013).

To overcome problems arising from the strong aroma of highly volatile compounds, further experiments are needed to minimize the impact on product aroma, flavor and sensory attributes, while maintaining high efficacy when used in combination with other technologies. AP has a great potential in extending the shelf life of bread. Nevertheless, further improvements are needed using better biodegradable materials, to fulfil the green approach.

Further applications

The addition of ethanol is a traditional preservative method. Hence, from the consumer point of view, ethanol might be a more preferable preservative agent over some other chemical substances (Table 3). Ethanol can be considered as a natural product. The commercial production of ethanol is mainly based on the yeast fermentation of sucrose-containing feedstocks (e.g., sugar cane, sugar beet), starchy material (e.g., corn, wheat, potatoes) and hydrolysed lignocellulosic biomass (e.g., wood, straw, grasses). A recently published review by Dao and Dantigny (2011) summarizes the control of food spoilage fungi by ethanol with a detailed explanation of its mode of action. In studies ethanol was used to control post-harvest fruit decay. According to an in vitro data based model against 12 common food spoilage molds, the inhibition concentration of ethanol was estimated in a range between 3% and 5% (Dantigny et al., 2005). All experiments were performed at high a_w values of

0.99 at intermediate pH of 5.8. However, for increasing the shelf life of bread concentrations between 0.2 and 12% are reported (Dao and Dantigny, 2011).

Katsinis et al. (2008) found an improved effect of sorbate and propionate on naturally contaminated products, when ethanol was added to the bread surface (0.5% w/w). In a challenge test against *Chrysonilia sitophila* ("the red bread mould") and *Hyphopichia burtonii* ("the chalky mould") on packed and sliced bread, growth was inhibited at very low (0.8%) and medium (2.0%) ethanol concentrations, respectively (Berni and Scaramuzza, 2013).

Due to its low toxicity, ethanol enjoys GRAS status in the USA. In the European Union, ethanol is not included in the regulation on food additives (EEC, 2008). Thus, there is no restriction about the use of ethanol as a preservative. If added to a product, it has to be listed as "ethyl alcohol" or "ethanol" on the ingredients. The results of studies presented above suggest that ethanol could act as an effective additional barrier to inhibit fungal growth in bakery products. Thus it represents an interesting alternative to the use of chemical preservatives and merits further research. A further application showing promise includes the addition of ethanol emitter in active packaging (Hempel et al., 2013).

One study tested the influence of 1-monoglyceride of medium chain fatty acids (MAG), 1-monocaprylin and 1-monocaprin on spoilage inhibition (*P. chrysogenum, P. jensenii, Monascus ruber, A. niger*) on bread (Buňková et al., 2010). The microfilm of MAG (65 mg/L) spread on the bread surface showed fungal inhibition up to 14 days storage (Table 3). MAG belong to food additives in accordance with the principle of "quantum satis" addition for good manufacturing practice and commonly acts as emulsifier in breads (EEC, 2008). A mode of action was hypothesized that 1-monocaprylin destabilizes membranes by increasing membrane fluidity and the number of phase boundary defects (Hyldgaard et al., 2012).

The use of biosurfactants, in particular lipopeptides produced by microorganisms has attracted the food industry due to their wide range of functionalities including their antimicrobial activity (Campos et al., 2013). Antibacterial and antifungal lipopeptides are mostly synthesized by strains of *Bacillus subtilis* and are classified into 3 main classes; surfactin, iturin, and fengycin (Muthusamy et al., 2008). *B. subtilis* belong to the QPS *Bacillus* species, with no safety concerns being reported from their usage in food products (EFSA, 2012). Mnif et al. (2012) evaluated the influence of biosurfactant produced by *B. subtilis* SPB1 on bread quality at concentrations of 0.025, 0.05, 0.075, and 0.1% (flour weight basis). Bread prepared with 0.075% SPB1 biosurfactant showed higher specific volume when compared to a control containing the same amount of soya lecithin. Texture profile analysis of bread prepared with and without biosurfactant showed also decreased firmness. Microbiological analysis of the 0.075% SPB1 biosurfactant containing bread had fungal and bacterial counts of 2×10^3 and 4×10^2 cfu/g, respectively; these counts were 1 log less than the control after 8 days of storage which was attributed to the significantly lower a_w values of bread with added SPB1 biosurfactant. However, the a_w was in general low, with values below 0.8 for all the breads produced. Microbial counts do not provide sufficient data if not compared to a specific reference level. The

reference values of microbial counts of 10^2 and 10^5 cfu/g for molds and bacteria, respectively, were suggested for frozen, ready to eat, bakery wares (i.e., rolls or croissants) from the German Society for Hygiene and Microbiology (DGHM, 2006). These reference values are based on which micro-flora can be expected and are tolerated in terms of good hygiene practice. Furthermore, the sample size needs to be sufficient and appropriate representing at least a small sale unit of 50 g (DGHM, 2006). There are other antimicrobial agents derived from microorganisms showing antifungal activity. Natamycin produced by *Streptomyces natalensis* is listed as preservative E235 in the EU regulation on food additives (EEC, 2008). In Europe, it is permitted only for surface treatment for the protection of cheeses and sausages. The GRAS status in the USA allows its addition also to yoghurt (FDA, 2014).

Further studies would be needed in order to confirm the in situ antifungal activity of MAG, and the other mentioned antimicrobial agents on bakery products, including assessing product safety.

Conclusion

In order to meet the needs and to fulfil the high consumer demand for additive-free bakery products, current research has focused on biopreservation to extend the shelf life of bread. Furthermore, the ineffective usage of chemical preservatives due to the development of resistance by several fungi has increased the pressure to find alternative agents.

The application of biopreservatives derived from plants or biosurfactants seem interesting alternatives to chemical preservatives. More reports are expected about the use of antifungal proteins. Antimicrobial essential oils need to be further evaluated, where progress could also lead to a better sensory profile. Protective packaging material should focus on biodegradable materials ensuring customer satisfaction.

Obviously, all data presented in Section 4 were obtained from different baking technologies and methodologies defining the mold-free shelf life of the resulting product. Furthermore, varying contamination levels or inoculum sizes per slice of bread, and storage conditions make an overall comparison of shelf life-extension potential between different methodologies difficult. In our opinion, moldiness should be assessed with the first visible appearance of fungal colonies on bread and bread slices. Nevertheless, in some studies, a bread slice was considered moldy if more than 1% of the total surface area was covered with fungi (Ryan et al., 2008, 2011) or all the inoculated spots started to show fungal growth (Zhang et al., 2010). Thus, a standardized method for defining bread moldiness is vital to allow meaningful comparisons to be made.

Sourdough fermented with antifungal strains serves as a high-potential biological ingredient to produce gluten-containing and gluten-free breads that meet presently consumer needs with improved nutritional value, bread quality and safety due to an extended shelf life. Despite the relatively high abundance of LAB, the antifungal potential and binding properties for mycotoxins is highly strain specific, which should be considered as a criterion for the selection of LAB for use as starter cultures in food and feed. The application of antifungal starter strains is still

limited and more strains need to be investigated in situ. Further research is required to improve the shelf life of gluten-free bread. Research into the removal of mycotoxins in food matrices is still in its infancy. Many novel methods have been introduced with high potential for biopreservation of bakery products. The next step will be to up-scale the use of such biopreservatives in the marketplace, potentially in combination with other technologies such as intelligent packaging, which will necessitate production of such compounds at an industrial scale.

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