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Modelling of the antifungal activity of propionic and sorbic acid against spoilage molds and validation in bread and cake

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Abstract

Propionic and sorbic acids are weak acids widely used as antifungals in baked products. Their use increases shelf life and reduces food waste and mycotoxins production. This study, conducted at Ghent University (Belgium), investigates the relationship between weak acids, pH and six molds in bakery products. More specifically, the susceptibility of *Aspergillus niger*, *Cladosporium ramotenellum*, *Aspergillus montevidensis*, *Aspergillus ruber*, *Penicillium brevicompactum*, and *Penicillium roqueforti* to the antifungal action of propionic acid and sorbic acid was studied, both in-vitro, on MEA (Malt Extract Agar) plates with established conditions of water activity (0.93, 0.95, and 0.97), pH (5.0, 5.5, 6.0), and preservative concentration (0, 500, 1000, 1500, and 2000 mg acid/L MEA), and by validation carried out on bread (0, 497, 990, 1967, and 2901 mg propionic acid/kg), cake pH 7.2 (0, 493, 1004, 1488, and 1988 mg sorbic acid/kg), and lemon cake pH 5.2 (0, 475, 951, 1429, and 1897 mg sorbic acid/kg). The undissociated component (C_{HA}) was estimated through three different Henderson-Hasselbalch equations: the standard one, model 1 (proposed in 2000 by Wilson et al.), and model 2 (Wemmenhove et al., 2018). The chemical and physical characteristics of the matrix (W/O, pH, aw, moisture) were studied to evaluate their relationship with the anti-mold effect of acids. *In-vitro* growth/non-growth patterns were correlated with shelf-life studies on bread and cakes. On bread, due to the strong propionic acid resistance of *Penicillium brevicompactum* and *Penicillium roqueforti*, the minimum inhibitory concentration (MIC) was above 2901 mg propionic acid/kg. In the cake at pH 7.2, the antifungal action of sorbic acid was unsatisfactory, and the maximum amount of sorbic acid (1988 mg sorbic acid/kg) was not enough to achieve 30 days of shelf-life. In lemon cake at pH 5.2, the minimum inhibitory concentration (MIC) was 1429 mg sorbic acid/kg; the shelf life reached thirty days. The standard Henderson-Hasselbalch equation was adequate to calculate C_{HA} in low-fat matrices (MEA and bread), and model 2 effectively calculated C_{HA} in high-fat matrices (cakes).

Riassunto

L'acido propionico e sorbico sono acidi deboli ampiamente utilizzati come antimicotici nei prodotti da forno. Il loro uso aumenta la shelf-life e riduce gli sprechi alimentari e la produzione di micotossine. Questo studio, svolto presso Ghent University (Belgium), analizza la relazione tra acidi deboli, pH e sei muffe nei prodotti da forno. In particolare, è stata studiata la sensibilità di *Aspergillus niger*, *Cladosporium ramotenellum*, *Aspergillus montevidensis*, *Aspergillus ruber*, *Penicillium brevicompactum* e *Penicillium roqueforti* all'azione antifungina dell'acido propionico e dell'acido sorbico, sia in vitro, su piastre MEA (Malt Extract Agar) con condizioni stabilite di attività dell'acqua (0,93, 0,95 e 0,95), pH (5,0, 5,5 e 6,0) e concentrazione di acidi (0, 500, 1000, 1500 e 2000 mg di acido/L MEA), sia con la validazione effettuata su pane (0, 497, 990, 1967 e 2901 mg di acido propionico/kg), torta a pH 7.2 (0, 493, 1004, 1488 e 1988 mg di acido sorbico/kg) e torta al limone a pH 5,2 (0, 475, 951, 1429 e 1897 mg di acido sorbico/kg). La componente di acido non dissociata (C_{HA}) è stata stimata attraverso tre diverse equazioni di Henderson-Hasselbalch: standard, modello 1 (proposto nel 2000 da Wilson et al.) e modello 2 (Wemmenhove et al., 2018). Le caratteristiche chimiche e fisiche della matrice (W/O, pH, aw, umidità) sono state studiate per valutare la loro relazione con l'effetto antimuffa degli acidi. I modelli di crescita/non crescita in-vitro sono stati correlati con studi di shelf-life su pane e dolci. Nel pane, a causa della forte resistenza all'acido propionico di *Penicillium brevicompactum* e *Penicillium roqueforti*, la concentrazione minima inibitoria (MIC) è stata superiore a 2901 mg di acido propionico/kg. Nella torta a pH 7,2, l'azione antimicotica dell'acido sorbico è risultata insoddisfacente e la quantità massima di acido sorbico (1988 mg di acido sorbico/kg) è stata insufficiente per arrivare a 30 giorni di shelf-life. Nella torta di limone a pH 5,2, la concentrazione minima inibitoria (MIC) è stata di 1429 mg di acido sorbico/kg; la durata di conservazione ha raggiunto i trenta giorni. L'equazione standard di Henderson-Hasselbalch è risultata adeguata nel calcolo di C_{HA} su matrici a basso contenuto di grassi (MEA e pane), mentre il modello 2 è risultato più adeguato per calcolare C_{HA} in matrici ad alto contenuto di grassi (torte).

Lists of abbreviations

[A-]: concentration of the acid's conjugated base

[HA]: concentration of the undissociated acid

A-: conjugate base of the acid

AACC: American Association of Cereal Chemists

ADI: acceptable daily intake

AM: *Aspergillus montevidensis*

AN: *Aspergillus niger*

ANOVA: Analysis of variance

AR: *Aspergillus ruber*

aw: water activity

BA: benzoic acid

BAL: bronchoalveolar lavage

Bw: body weight

CA: citric acid (citric acid-1 hydrate)

CAGR: Compound Annual Growth Rate

$C_{HA,model\ 1}$: concentration of the undissociated acid calculated with Henderson-Hasselbach equation model 1

$C_{HA,model\ 2}$: concentration of the undissociated acid calculated with Henderson-Hasselbach equation model 2

$C_{HA,standard}$: concentration of the undissociated acid calculated with standard Henderson-Hasselbach equation

C_{HA}: concentration of the undissociated acid

CR: *Cladosporium ramotenellum*

C_{TOT}: total concentration of the acid

DG18: Thermo Scientific™ Dichloran-Glycerol 18%

DTO: Department of Applied and Industrial Mycology

EC: European Community

EFSA: European Food Safety Authority

EPA: Environmental Protection Agency

EU: European Union

FAO: Food and Agriculture Organization

FLD: Farmer's lung disease

GHG: Greenhouses Gas

H+: hydrogen ions

HA: undissociated acid

HP: hypersensitivity pneumonitis

IMPDH: inosine 5′-monophosphate dehydrogenase

ISO: International Standard Organization

IUPAC: International Union of Pure and Applied Chemistry

Ka: acid dissociation constant

Kp: partitioning coefficient of the acid

MAP: Modified Atmosphere Packaging

maq: g water/100g system

MEA: Malt Extract Agar

MIC: Minimal inhibitory concentration

minert: g inert fraction/100 g

moil: g oil/100g system

mTOT: total weight of the system

MUCL: Mycotheque de l'Université catholique de Louvain

PA: propionic acid

PB: *Penicillium brevicompactum*

pH: potential of hydrogen

pKa: negative log of Ka

PML: progressive multifocal leukoencephalopathy

ppm: parts per million

PR: *Penicillium roqueforti*

QPS: Qualified presumption of safety

r: mass fraction of the lipid phase in the W/O emulsion

SA: sorbic acid

SDG: Sustainable Development Goals

SL: Shelf life

spp: *species plures*

SPSS: Statistical Package for the Social Sciences

SV: Specific Volume

triNaC: tri-sodium citrate dehydrate

UN: United Nations

UNGA: United Nations General Assembly

WFP: World Food Programme

ρaq: water density

ρoil: oil density (g/L)

φ(vaq): water volume fraction in the system

φ(voil): oil volume fraction in the system

1. Introduction

1.1. Food waste

"Food waste" is food and the associated inedible parts removed from the human food supply chain in retail, food service, and households.

Food Waste includes edible parts that are the parts of food intended for human consumption and inedible parts that are components associated with food that is not intended to be consumed by humans. Examples of inedible parts are bones, rinds, and pits/stones.

One-third of the globally produced food is lost or wasted. This amount of food is equivalent to about 1.3 billion tons per year, worth about $ 1 trillion. Moreover, this waste is not the same all over the world. For example, 40% of losses in developing countries occur at the post-harvest and processing levels. On the contrary, in industrialized countries, over 40% of losses occur at the retail and consumer level (WFP, 2020) (Gustavsson, J.,2011).

Figure 1 SDG goals 12.3, UN SDG report, 2022

In 2020, 13.3% of the world's food was lost after harvesting and before reaching retail markets. These losses occur during on-farm activities, transport, storage, processing, and wholesaling (Figure 1).

Total food waste produced worldwide in 2019, by sector, from retail (13%) to the household (61%), also including food service (26%) was 931 million metric tons (UN *SDG report*, 2022) (Figure 2)

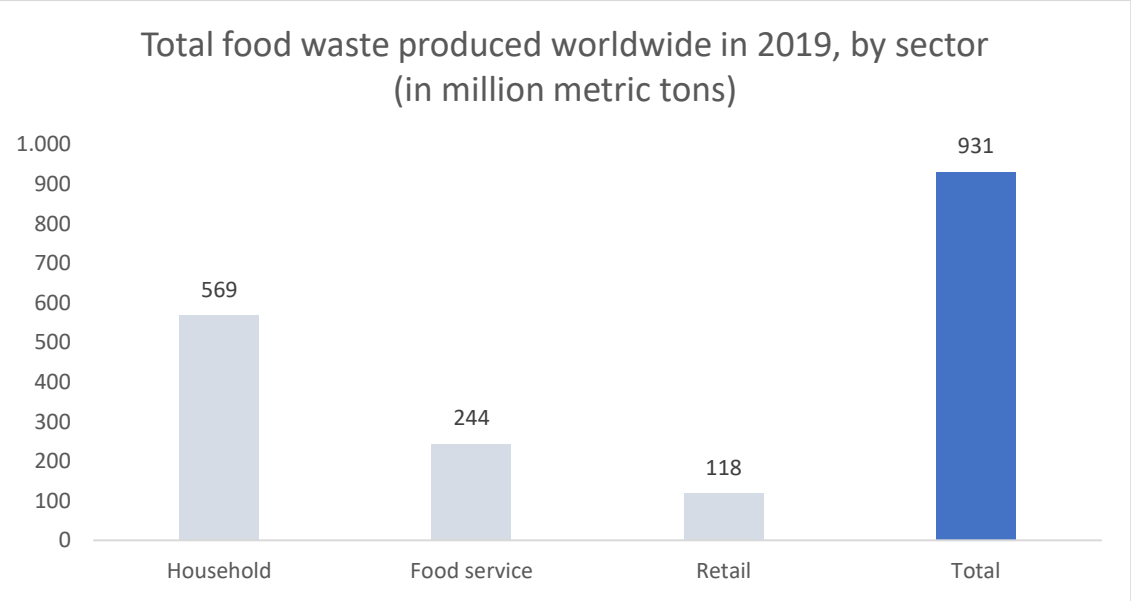

Figure 2 Total food waste produced worldwide in 2019, by sector (UN SDG report, 2022)

Household *per capita* food waste generation is generally found to be similar between country income groups, suggesting that action on food waste is relevant in the same way in high, upper-middle, and lower-middle-income countries (Table 1).

Income group	Average food waste (kg/capita/year)		
	Household	Food service	Retail
High-income countries	79	26	13
Upper middle-income countries	76	Insufficient data	
Lower middle-income countries	91	Insufficient data	
Low-income countries	Insufficient data		

Table 1 Average food waste (kg/capita/year) for high, upper-middle, lower-middle, and low-income countries in the household, food service, and retail. Research by World Bank classification (FOOD WASTE INDEX REPORT 2021)

While data do not permit a solid comparison across time, food wastes in households and food services appear to be higher than the previous FAO estimate (FAO SOFA, 2019).

1.1.1. Food waste, Greenhouses Gas (GHG), and politics (UN SDG goals)

Food waste is a significant worldwide issue that continues to attract attention in the scientific community and politics (Iakovlieva and Eriksson, 2021).

The value of food produced in Europe is around 344 billion euros, and the food waste is approximately 103 billion euros (Iakovlieva and Eriksson, 2021).

In the USA, Environmental Protection Agency (EPA) estimated that food loss produced 170 million metric tons of CO_2eq/year (but not include the methane emissions from food waste rotting in landfills). This uneaten food also contains enough calories to feed more than 150 million people yearly (Epa, U., and Office, I., 2021).

Food waste contributes to the production of Greenhouses Gas (GHG) with around 1.6 kg of CO_2eq for every kg of food waste (Iakovlieva and Eriksson, 2021), about 186 Mt CO_2 eq/year only in Europe (Scherhaufer et al., 2018) with a food waste of 88 Mt /year, equivalent to 173 kg per person (European Parliament, 2017). Estimates suggest that 8-10% of global GHG emissions are associated with food that is not consumed (UNEP Food Waste Index Report 2021) (SDG 12.3).

Reducing waste is a difficult challenge. Cutting global food waste by half by 2030 is one of the UN's top priorities. Food waste is one of the organization's 17 Sustainable Development Goals SDG (Sustainable Development Goals) (WFP, 2020), (Iakovlieva and Eriksson, 2021), FAO, target 12.3 says, "*By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses*". The Sustainable Development Goals (SDGs) are 17 goals developed by the United Nations General Assembly (UNGA), which aim by 2030 to improve the condition of peace and prosperity for people and the planet (SDG, Department of Economic and Social Affairs).

1.2. Bakery food production and lost

Bread and bakery products are one of the most consumed foods. The bakery sector increased from 29,641 million in 2010 to 30,328 million kilograms by 2017.

Although a slight reduction (from 20.4 to 18.8 million kg) in bread consumption has been recorded from 2010 to 2021, preserving pastry goods and cakes has been documented (Figure 3).

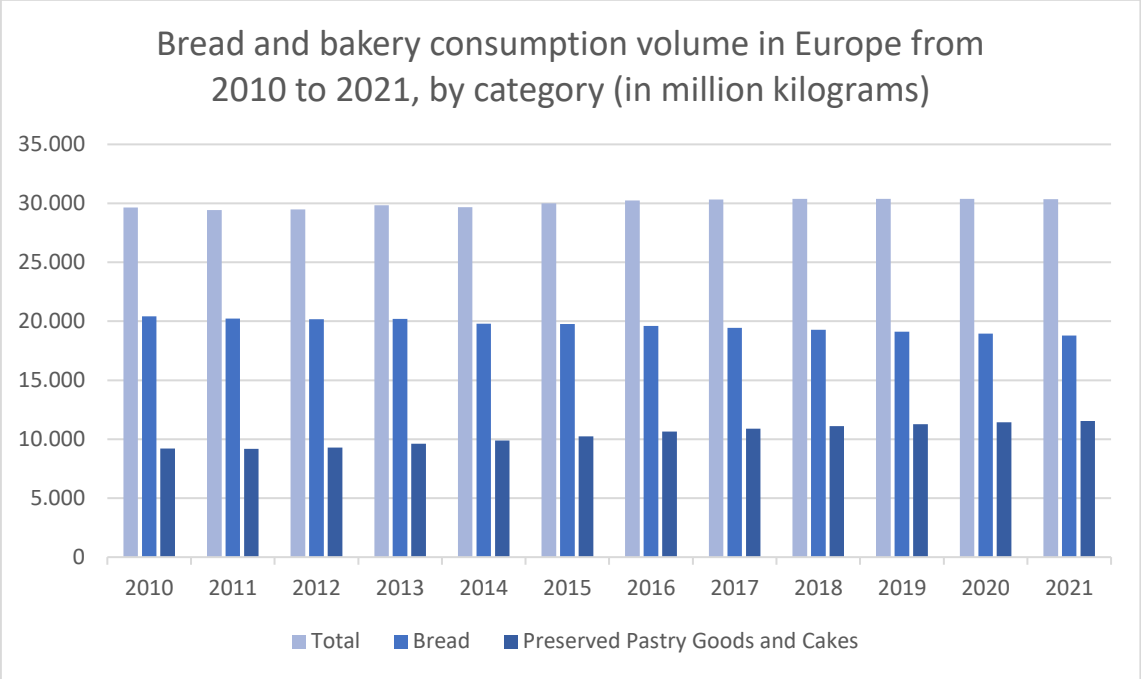

Figure 3 Bread and bakery consumption volume in Europe from 2010 to 2021, by category (in million kilograms), Statista. (December 1, 2017).

Unfortunately, a large quantity of bakery products is lost/wasted, and it is not consumed.

Compared with other products such as meat and dairy, bread loss has a lower GHG impact: around 0.5kg CO_2eq/kg finished bread, but if we consider the volume of bread loss, the total emissions are significant.

1.2.1. Causes behind bakery loss

About 5-10% of the world's food supply is lost every year due to the mere presence of fungi and mycotoxins. For example, the total annual cost in the United States is estimated to be in the hundreds of millions and billions of dollars (Cook., 2009).

Bakery products account for 4-5% losses due to fungi and mycotoxins. To limit this loss, many stores offer this perishable good at a discount, resulting in an overall loss of economic revenue (Cook., 2009).

Mold spoilage is the leading cause of loss in the baking industry; mold spoilage causes an economic loss and can also determine serious health diseases by producing mycotoxins (Gerez et al., 2010). Mold spoilage is usually characterized by a mycelium, sometimes visible before reaching the end of the shelf-life (SL), thus causing product rejection by the consumer (Dagnas and Membré, 2013; Garcia et al., 2019).

The most problematic bakery products from the microbiological point of view are those intended to be stored for an extended period (more than the time the label recommends) at room temperature as packaged sliced toast bread and par-baked bread (Debonne Els, 2020).

In Europe, the economic losses for bread related to fungi spoilage are estimated to be around 200 million/year, from 1% to 5% (variability depends on the season). In tropical regions, the percentage increases to 11% (Garcia et al., 2019).

Regarding bakery wastes, it is essential to determine in which part of the chain these products are lost: Scherhaufer et al. (2018) estimated that consumers cause around 68% (17 Mt/year) of the waste). The main reasons for bread and pastries disposal are reported in figure 4.

Research by Voedselverlies en consumentengedrag Bij Vlaamse huishoudens on Flemish families in Belgium (Figure 4) showed that 38% of the consumers stated that the most crucial factor behind bakery products is "*The product had gone off/did not taste good anymore*". It is clear that the quality of the products when they are eaten is an essential factor, and lowering the food quality can make the products unsuitable for human consumption.

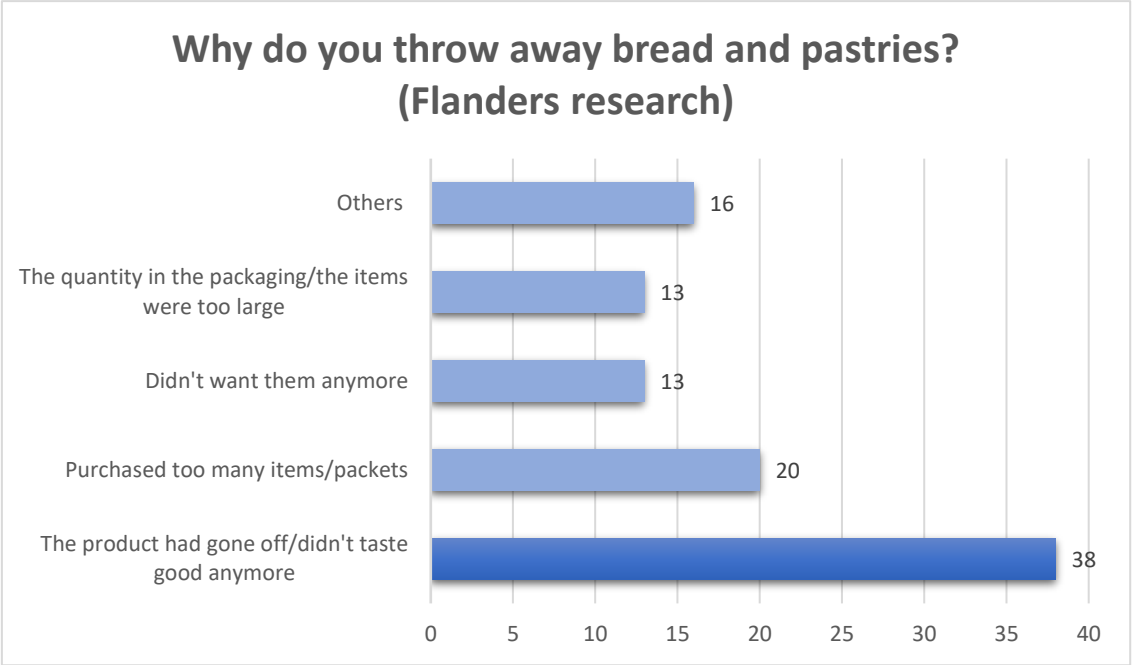

Figure 4 Causes behind food waste, Flanders research, Voedselverlies en consumentengedrag bij Vlaamse huishoudens (2019).

The lower quality of the baked products is likely due to their hardening after contact with air. However, another equally important reason (and indeed more dangerous) is the spoiling caused by the development of microorganisms.

1.3. Microbiological spoilage in bread and bakery products

Worldwide, bread is the most consumed bakery product and one of the most consumed foods. Bread is a staple food obtained from fermentation, forming followed by leavening and subsequent baking of a dough made of flour and water; other ingredients that differ according to local traditions are often added, for example, some different cereals. Indigenous microorganisms can raise bread (e.g., mother yeast), chemicals (e.g., baking soda), industrially produced yeast, or high-pressure aeration. All the cases mentioned aim to deliver gas bubbles that fluff up the dough. Natural sourdough or mother is a mixture of flour and water naturally contaminated by microorganisms (mainly lactic bacteria and yeasts in raw materials). The development of microorganisms creates a native microflora (bacterial and yeast) inside the mass in which the culture of lactic bacteria predominates.

The water activity of bakery products is between 0.75 and 0.98. The bread's water activity is usually higher than 0.90 (generally 0.96-0.98). The pH is typically around 5.5 and 6. It is mainly stored at room temperature. Thus, bread is primarily sensitive to mold growth.

Extending shelf life in bread and bakery products is mandatory to reduce waste and concur in a globalized world. Strategies for extending shelf-life include using food-grade preservatives, modified atmosphere packaging, refrigeration, and humectants (Garcia et al., 2021). Nowadays, there is also an increase in interest in clean-label food products and, consequently, more studies regarding natural and friendly antimicrobial preservation strategies (Samapundo et al., 2016; Debonne, 2020; Garcia et al., 2021).

Bread and other bakery products are subjected to various spoilage problems, i.e., physical, chemical, and microbial.

Microorganisms are the leading cause of spoilage problems because they can also cause health damage to human beings (e.g., toxins, mycotoxins) (Saranraj Sivasakthivelan, 2015). The most common microbial spoilage agents are represented by bacteria and fungi (yeast and molds) (Marriott, 2006). Table 2 reports the primary causative agents of bread microbial spoilage.

Table 2 Major causative agents of bread microbial spoilage (Melini, and Melini, 2018).

Spoilage Agents		Properties of Colony
Moulds	*Penicillium* spp.	Blue/green, flat, spread rather slowly
	Aspergillus niger	Black, fluffy, spreading with sporeheads often clearly visible
	Aspergillus flavus	Olive green
	Aspergillus candidus	Cream
	Aspergillus glaucus	Pale green
	Cladosporium spp.	Dark olive green, flat, spread slowly
	Neurospora stophila	Salmon pink, fluffy and fast spreading
	Rhizopus nigricans	Grey/black, very fluffy and fast spreading
	Mucor spp.	Grey
Bacteria	*Bacillus subtilis* or *Bacillus licheniformis*	Irregular shape, white and dull colour
Yeasts	*Hyphopichia burtonii*	Slow growth on bread surface, low, white, spreading colonies
	Pichia anomala	
	Scopsisfi buligera	
	Pichia burtonii	Very fast growth on bread
	Zygosaccharomyces bailii	Smooth, round, convex and white to cream coloured
	Torulaspora delbrueckii	
	Pichia membranifaciens	
	Candida parapsilosis	

1.3.1. Bacterial growth

Bacillus genera are the leading cause of bacterial spoilage. Bacterial growth is usually possible (excluding osmophilic bacteria) if water activity is higher than 0.9. For example, *Bacillus subtilis* is a *Bacillus* that can commonly cause spoilage in raw bakery materials.

Other *Bacillus* species, such as *Bacillus megaterium,* and *Bacillus cereus*, have been associated with the formation of ropy bread. Ropy bread has a fruity unpleasant odor. Subsequently, the crumb becomes soft and filamentous due to enzymatic degradation and bacteria's production of extracellular polysaccharides (Pepe. et al., 2003) (Figure 5).

Figure 5 Rope production in bread started with *Saccharomyces cerevisiae* T22 after 1 day of storage at 23°C, Pepe et al., 2003.

1.3.2. Fungi

Fungi include yeasts (single cells fungi) and molds. The best conditions for fungal growth are moist habitats. Intermediate pH is optimum for their growth, but many can survive and grow at extreme pH from 2 to 9. They also have high osmotic resistance, and thus they can stay at high salt or sugar concentrations. Moreover, they usually survive at a low temperature.

1.3.2.1. Yeast spoilage

The optimum pH for yeast is around pH 4-6, but they can easily grow in a broader range.

The minimal water activity for yeast is around 0.88. Typically, water activity is higher in bread. For this, yeast spoilage could be a problem. However, for some cakes and bakery products where water activity is lower than 0.88 (less or no water and sugar), water activity could be humble enough to defeat some molds and all the yeasts (Debonne, 2020).

Yeasts reproduce by gemmation with a generation time of around 2-3 hours (higher than bacterial). For this reason, 40-60 hours could be necessary before food appears spoiled (Marriott et al., 2006).

Deterioration of bread can be due to chalk yeasts (chalk yeasts are also called chalk molds). Figure 6 reports an example of chalk yeast spoilage in MAP (Modified Atmosphere Packaging).

Figure 6 Colonies of *Saccharomycopsis fibuligera* grown on Modified Atmosphere Packaging packed durum wheat bread after 7 days of storage (Giannone et al., 2016).

There are 24 known chalk molds. *Saccharomycopsis fibuligera, Hyphopichia burtonii, Zygosaccharomyces bailli,* and *Saccharomyces cerevisiae* have been reported as dominant species, but only *Saccharomycopsis fibuligera* and *Hyphopichia burtonii* are considered as true chalk yeasts (Giannone et al., 2016)

Wickerhamomyces anomalus (formerly *Pichia anomala*) and *Saccharomycopsis fibuligera* were responsible for the early spoilage of commercial Modified Atmosphere Packaging par-baked bread (Debonne, 2020, Debonne et al., 2021, Giannone, et al., 2016).

1.3.2.2. Molds spoilage

Molds tolerate more significant variations in pH than bacteria and yeasts. Molds are typically acid tolerant (pH <4) and can usually grow in a pH range from 1.5 to 11. For this reason, pH is not the primary factor in controlling molds (Marriott et al., 2006), and water activity variations are generally used for controlling mold growth. Optimal water activity is around 0.9, but minimal water activity for many molds is 0.8, and at water activity <0.83, no mycotoxin production is recorded (Debonne, 2020). Xerophilic molds such as *Eurotium amstelodami* and *Eurotium chevalier* can grow a lower water activity (0.75) (Abellanaet al. 1999). Other xerophilic molds grow at water activity as low as 0.65 (Vytřasová et al., 2002).

Mold spoilage is by far the most critical loss cause for bakery products.

Penicillium spp. is the leading cause of mold spoilage in bread, with around 90% cases, with a minimal water activity for growing of 0.85-0.90.

Not only spoilage and mycotoxins production is an essential factors to consider. *Aspergillus spp., Penicillium spp., Eurotium spp.,* and *Fusarium spp.* are present in agricultural products and can produce mycotoxins in bakery products (Debonne, 2020).

1.4. Mycotoxins

As reported by WHO (World Health Organization), mycotoxins are "*Toxic compounds that are naturally produced by certain types of molds (fungi). Molds that can produce mycotoxins grow on numerous foodstuffs such as cereals, dried fruits, nuts, and spices*" (WHO, 2018). Mycotoxins are thus toxic secondary metabolites (Battilani et al., 2020).

The presence of mycotoxins in food and feed can cause adverse effects on human and animal health as gastrointestinal disorders, kidney disorders, immune deficiency, and cancer. The effects vary from person to person, based on the individual's sensitivity. Some personal factors increase individual sensitivity to mycotoxins, such as vitamin deficiency, low-calorie intake, alcohol abuse, and the presence of infectious diseases (Omotayo et al., 2019).

The most relevant mycotoxins are produced by molds of the genera *Aspergillus spp., Penicillium spp., Eurotium spp.,* and *Fusarium spp*., and with a lower frequency from *Alternaria spp*. and *Claviceps spp*. The most common mycotoxins (aflatoxins, ochratoxin A, and *Fusarium* toxins such as deoxynivalenol) are a problem for human and animal health (Battilani et al., 2020).

Mycotoxins have a ubiquitous presence: they are widely present in cereals, fruits, and vegetables.

Mycotoxigenic fungi are commonly not host-specific. They are mainly associated with a specific crop depending on its growing area and the weather conditions of the place of growth. A single fungus can produce multiple types of mycotoxins. Mycotoxins may change due to fungal-host plant interaction or during raw product processing. As a result, humans, and animals are frequently exposed to more than one mycotoxin simultaneously. Temperature, relative humidity, rainfall, and water activity are the most critical factors influencing the fungal colonization of the substrate (Table 3).

Table 3 Some fungal species with corresponding mycotoxins and their primary crop hosts (Battilani et al., 2020).

Fungi source	Mycotoxins[(a)(b)]	Crops of primary concern
***Aspergillus* spp.**		
A. carbonarius	OTA	grapes, pistachio nuts
A. flavus	AFB1, AFB2, STC	maize, rice, grapes, sorghum, nuts (mainly peanuts, pistachio and almond nuts)
A. niger	OTA, FB2	grapes, sorghum, nuts (mainly peanuts, pistachio and almond nuts)
A. ochraceus	OTA	rice, grapes, nuts
A. parasiticus	AFB1, AFB2, AFG1, AFG2, STC	maize, nuts (mainly peanuts, pistachio and almond nuts)
A. tubingensis	OTA	sorghum
A. versicolor	STC	rice
Fusarium spp.		
F. armeniacum	T-2, HT-2	rice
F. avenaceum	ENNs, MON	small grains (mainly wheat, barley, oat), grapes
F. culmorum	DON, AcDONs, NIV, ZEN	maize, small grains (mainly wheat, barley, oat)
F. equiseti	ZEN	maize, small grains (mainly wheat, barley, oat)
F. fujikuroi	FB1, MON, GA	rice
F. graminearum	DON, AcDONs, NIV, ZEN	maize, small grains (mainly wheat, barley, oat), rice
F. incarnatum	ZEN	sorghum
F. langhsethiae	T-2, HT-2	maize, small grains (mainly wheat, barley, oat)
F. nivale	NIV	small grains (mainly wheat, barley, oat)
F. oxysporum	ENNs, MON, BEA	grapes
F. poae	NIV, T2- HT2, DON, AcDONs, DAS, ENNs	small grains (mainly wheat, barley, oat)
F. proliferatum	FBs, BEA, FUS, MON	maize, small grains (mainly wheat, barley, oat), rice, grapes, sorghum
F. pseudograminearum	DON	small grains (mainly wheat, barley, oat)
F. pseudonygamai	FBs	sorghum
F. sporotrichioides	T-2, HT-2	maize, small grains (mainly wheat, barley, oat), grapes
F. subglutinans	BEA, FUS, MON	maize, rice
F. temperatum	FBs, BEA, FUS, MON, ENNs	maize
F. thapsinum	ZEN	sorghum
F. verticillioides	FBs	maize, grapes, sorghum
***Penicillium* spp.**		
P. aurantiogriseum	PAC	rice
P. citreonigrum	CTN	rice
P. citrinum	CIT	rice
P. commune	CPA	rice
P. expansum	PAT, CIT	grapes
P. islandicum	CC, uteoskyrin	rice
P. rugulosum	Rugulosin	rice
P. verrucosum	CIT, OTA	wheat, rice, grapes
Alternaria spp.		
A. alternata	ATs	grapes
A. infectoria	ATs	rice
A. tenuissima	ATs	rice
***Claviceps* spp.**		
C. africana	EAs	sorghum
C. purpurea	EAs	small grains (mainly wheat, barley, oat)
C. sorghi	EAs	sorghum
C. sorghicola	EAs	sorghum

AFs: aflatoxins; AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2; ATs: Alternaria toxins; BEA: beauvericin; CC: cyclochlorotin; CIT: Citrinin; CPA: cyclopiazonic acid; CTN: citreoviridin; DON: deoxynivalenol; AcDONs: acetyldeoxynivalenols; EAs: ergot alkaloids; ENNs: enniatins; FBs: fumonisins; FB1: fumonisin B1; FB2: fumonisin B2; FUS: fusaproliferin; DAS: diacetoxyscirpenol; GA: gibberellic acid; HT-2: HT-2 toxin; MON: moniliformin; NIV: nivalenol; OTA: ochratoxin A; PAC: penicillic acid; PAT: patulin; STC: sterigmatocystin; T-2: T-2 toxin; ZEN: zearalenone

Molds infection and the subsequent possible synthesis of mycotoxins start in the field and could continue during harvesting, storage, and processing if conditions permit. Mycotoxins are resistant and stable compounds, and their final amount in the food or feed is a sum of mycotoxins produced during the entire food or feed chain (Battilani et al., 2020).

1.4.1. Mycotoxins production

Molds growth can occur over a wide range of water activity and temperature. The optimal conditions for fungal growth and mycotoxin biosynthesis do not always match.

Figure 7 compares the temperature needed for the growth and the production of mycotoxins by different molds.

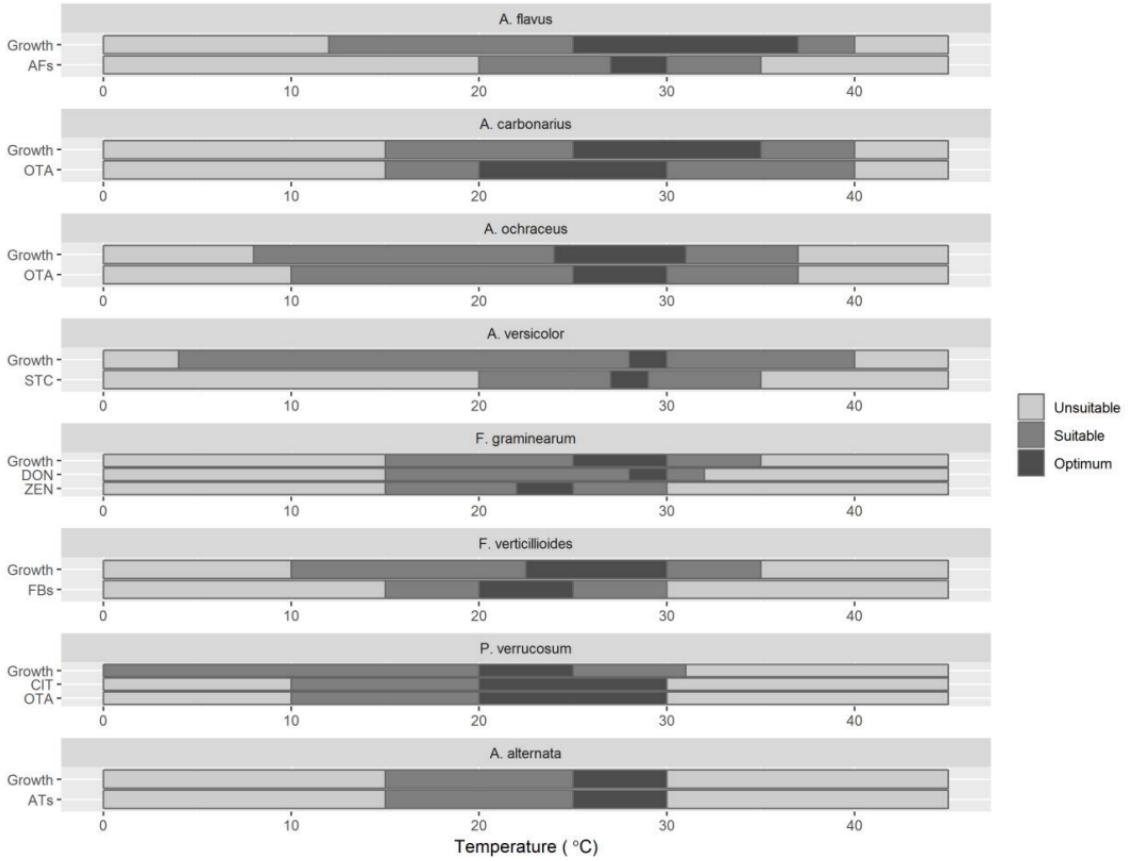

Figure 7 Comparison between the temperatures necessary for the growth of some molds and the production of mycotoxins (Battilani et al., 2020).

Battilani et al. 's 2020 study report that mold growth and mycotoxin production temperatures do not match. Often, the growth temperatures of molds have a more

comprehensive range than the production temperatures of mycotoxins. For example, *Aspergillus flavus* has a wide range of suitable growth (12°C -40°C), while the suitable range of production of Afs (aflatoxins) is narrower (20°C -35°C)

Water activity is a critical parameter. Not only about temperature but also concerning water activity, the ranges of mycotoxin production and mold growth vary. Figure 8 compares unsuitable, suitable, and optimum water activity for mold growth and mycotoxins production. Suitable water activity for growth typically presents a broader range than suitable mycotoxin production (e.g., *Aspergillus flavous*, *Aspergillus ochraceus,* and *Fusarium verticillioides*)

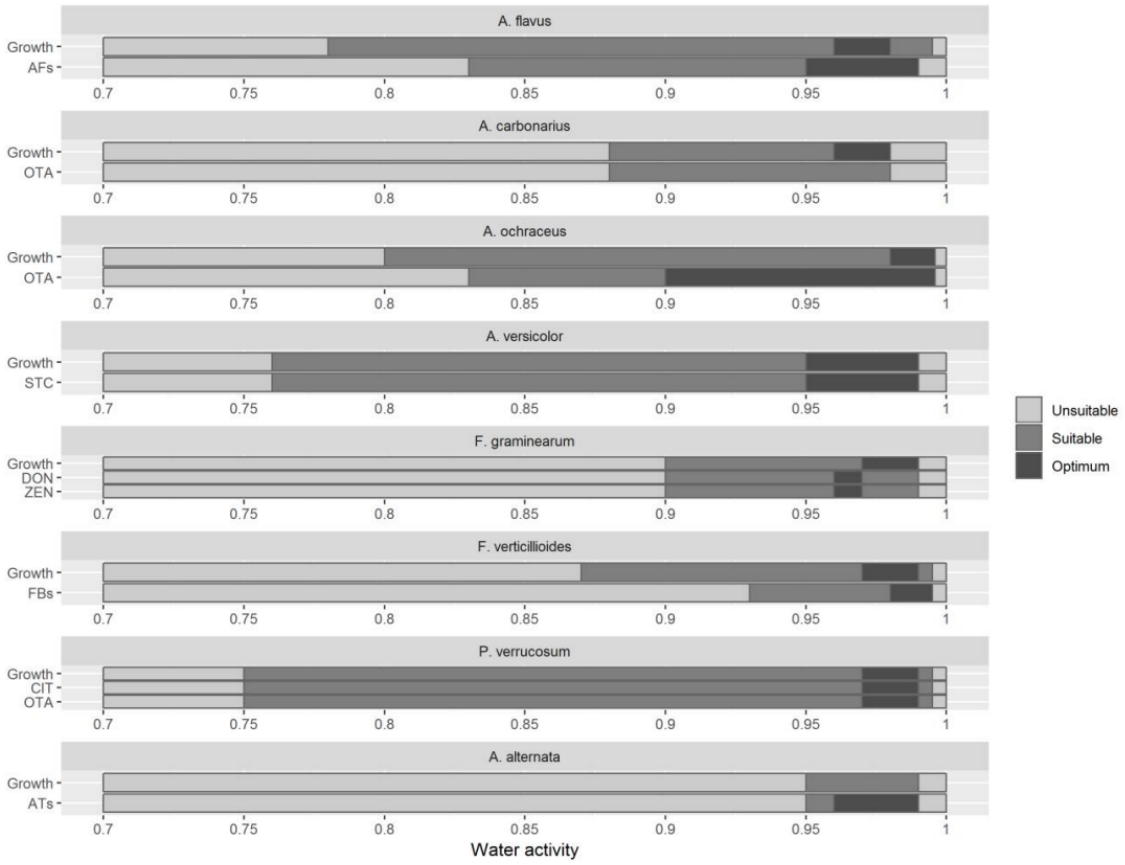

Figure 8 Comparison between the water activity necessary for the growth of some molds and the production of mycotoxins, Battilani et al., 2020.

1.5. Preservatives

In bread and other bakery products, organic acids such as sorbic and propionic acids are typically used as preservatives at a concentration of around 0.1% (Debonne, 2020).

Organic acids act lowering the pH of food products. At lower pH, the equilibrium between dissociated and undissociated acid is more shifted in favor of the undissociated one.

Replacing chemical preservatives such as propionates and sorbates is of particular interest to the food industry (Samapundo et al., 2016). Recently, more and more consumers have tried to avoid chemical preservatives in food products (Figure 9).

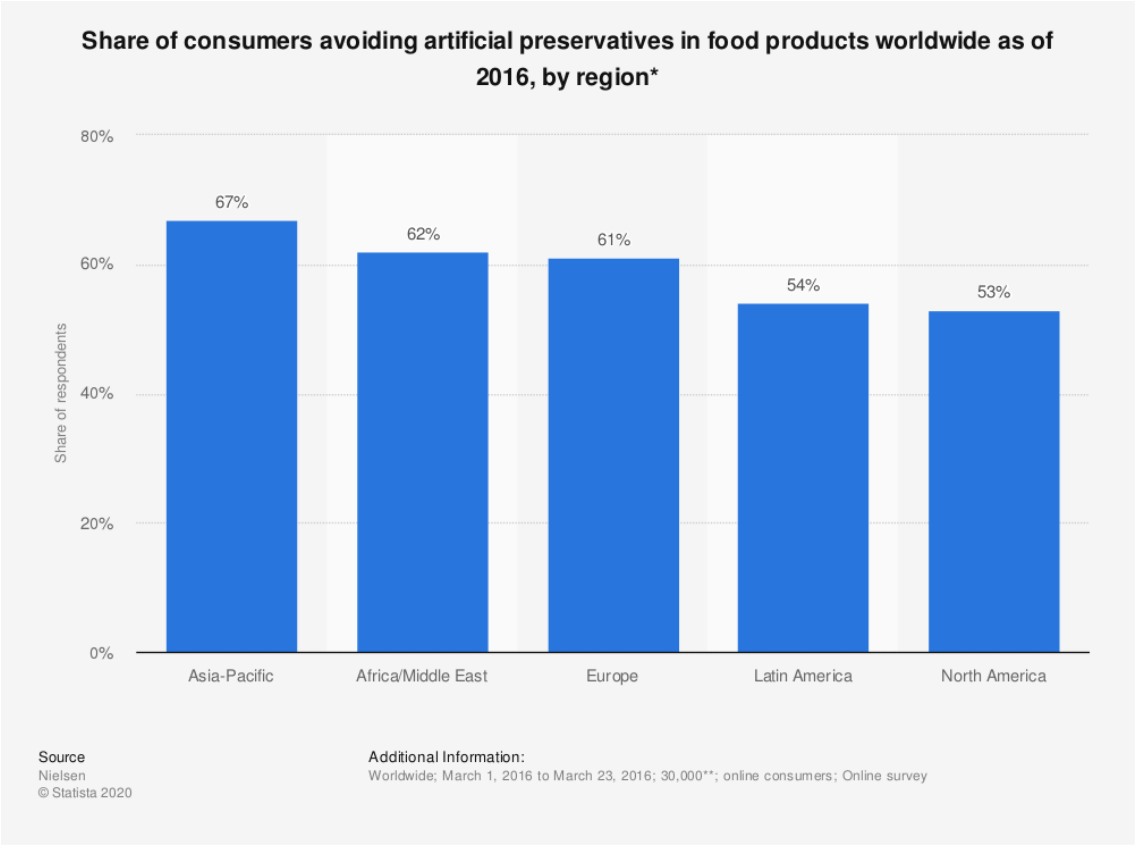

Figure 9 Share of consumers avoiding artificial preservatives in food products worldwide as of 2016, by region. (Statista 2016).

At the same time, there is a crucial request for chemical preservatives from Europe and America, and every year there is a positive CAGR (Compound Annual Growth Rate). In the Asia-Pacific continent, the demand is even higher.

Propionic, sorbic, and benzoic acids are the most used food preservatives, especially in bakery conservations.

Propionic acid inhibits *Bacillus,* molds, and fungal spores, but not yeasts to the same extent, and has therefore been the traditional choice for bread storage.

Sorbic acid is considered to be more effective than propionic acid. It inhibits molds and yeasts and is used in various food products, including refined baked goods, pastry products, and bread (Gioia, 2017).

1.5.1. Sorbic acid

Sorbic acid ($CH_3(CH)_4CO_2H$), IUPAC name (2E,4E)-Hexa-2,4-dienoic acid (Figure 10) is an organic compound used as a food preservative and was first isolated in 1859 from the rowan tree (*Sorbus aucuparia*) (Wen and Marth, 1985).

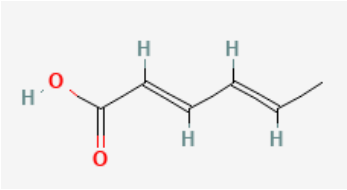

Figure 10 Sorbic acid, 2D structure (PubChem, sorbic acid)

Sorbic acid is a white crystalline solid or powder, and it is used in the food sector as a food preservative and antimicrobial agent. The molecular weight is 112.13, relatively odorless; it has a slightly acidic taste, a boiling point of 228°C, and a melting point of 134°C. It is slightly soluble in water and soluble in ethanol. It is used as salts (sodium sorbate, potassium sorbate, and calcium sorbate) for foods application (National Center for Biotechnology Information, 2022). It has a pKa of 4.76 at 20°C. The optimal pH for the antimicrobial effect is below pH 6.5. It is a good inhibitor of molds, yeast, and fungi (Debonne, 2020; Wen and Marth, 1985; Shibamoto and F.Bjeldanes, 1993; Zeece, 2020). The spectrum of activity of sorbic acid for what concern molds and yeast is wider than propionic acid. Unfortunately, sorbic acid also affects yeasts used in the doughs as

Saccharomyces cerevisiae, although it is possible to use an encapsulated sorbic acid after doughs are risen (Debonne, 2020).

There is no evidence of the toxicity of sorbic acid. It is absorbed from the body and mainly excreted as expired carbon dioxide. Another National Center for Biotechnology Information research concluded that "*Application of 150 mg of sorbic acid to human skin for 1 hr produced severe irritation*". Regarding the oral intake, the same research concluded that no adverse effects were observed in rats fed with sorbic acid at dietary levels of 1, 2, 4, and 8% for 90 days; mice fed with a diet containing 15% sorbic acid for 88 weeks showed a high incidence of hepatoma (EFSA, 2015).

Sorbic acid (E200), Sodium sorbate (E201), Potassium sorbate (E202), and Calcium sorbate (E203) have an acceptable daily intake (ADI) of 25 mg/kg body weight (EFSA, 2015).

The sorbic acid market is predicted to grow from $3.5 billion in 2019 to $5.2 billion in 2030, with a 4.7% CAGR (Compound Annual Growth Rate) from 2020 to 2030.

1.5.2. Propionic acid

Propionic Acid (CH_3CH_2COOH) (IUPAC propanoic acid) (Figure 11). Johann Gottlieb found it for the first time among sugar degradation products in 1844 (Johann Gottlieb, 1844).

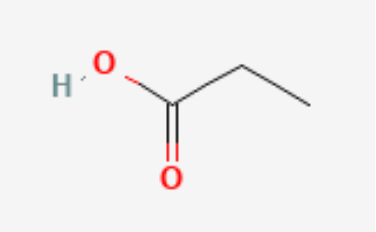

Figure 11 Propionic acid, 2D structure (PubChem, propionic acid)

Propionic acid is a colorless liquid, and it is used in the food sector as a food preservative and antimicrobial agent. The molecular weight is 74.08; it has a pungent/rancid odor, a boiling point of 141.1°C, and a melting point of -21°C. It has a pKa of 4.87 (at 20°C), and the optimum pH is 5.0-5.55. At pH 6, only 7% of the propionic acid is undissociated,

compared to 71% at pH 4.5 (Gioia, 2017). It is miscible with water, alcohol, and organic solvents (National Center for Biotechnology Information, 2022).

Propionic acid can be used as a preservative against molds and some bacteria in food and feed. The primary use is mainly in baked goods. It is allowed both in the EU (EFSA, 2014) and in the USA (in 1984, it was recognized as GRAS (Generally Recognized as Safe)) with no limitations (*Propionic Acid Handling/Processing*, 2008).

Propionic acid (E280), sodium propionate (E 281), calcium propionate (E 282), and potassium propionate (E 283) are thus considered safe. Moreover, propionates are naturally occurring substances in the regular diet.

The acceptable daily intake (ADI) from EFSA for propionic acid and propionates is 0.7 to 21.1 mg/kg Body-Weight/day and 3.6-40.8 mg/kg Body-Weight/day (EFSA, 2014). Till today there is no evidence that propionates are hazardous to public health if used within these levels (Debonne, 2020).

In 2021, the market for propionic acid was valued at approximately 1.7 billion dollars worldwide. This market is estimated to increase to 2.5 billion dollars by 2029 with an approximately CAGR (Compound Annual Growth Rate) of 4.6% from 2022 to 2029 (AgileIntel Research, 2022) (Figure 12).

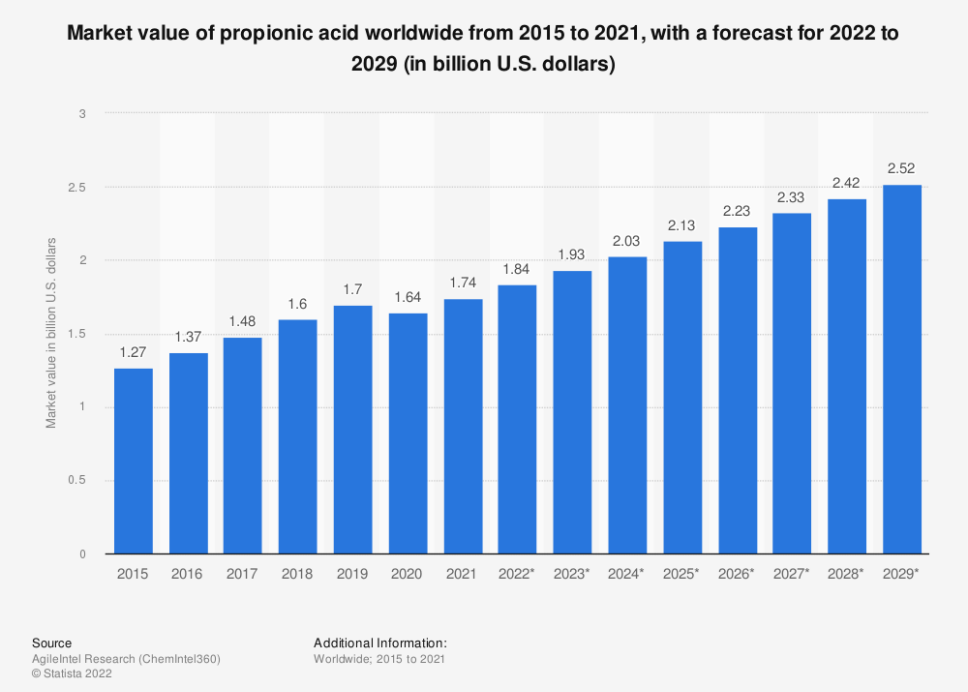

Figure 12 Market value of propionic acid worldwide from 2015 to 2021, with a forecast for 2022 to 2029 (in billion U.S. dollars) (AgileIntel Research (ChemIntel360), 2022).

Europe is the largest market for propionic acid (44% of total propionic acid consumption), but APAC (Asia-Pacific) is the fastest-growing propionic acid market. The "food and beverage" sector is the highest contributor to the global propionic acid market.

1.5.3. Benzoic acid

Benzoic acid (C6H5COOH) (Figure 13) is the simplest aromatic carboxylic acid. It has a molecular weight of 122.1, it looks like a white crystalline powder, slightly soluble in water, with no or a slight benzaldehyde odor, almost tasteless, a bit bitter, boiling point 249.2 °C, melting point 122.4 °C, and a pKa of Benzoic acid is 4.19 at 20°C.

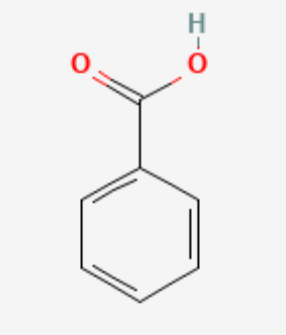

Figure 13 Benzoic acid, 2D structure (PubChem, benzoic acid)

Many plants naturally produce benzoic acid. Benzoic acid is used to make other chemicals in perfume and flavoring, and the salts of benzoic acid are generally used as preservatives in many foods.

Benzoic acid (E 210), sodium benzoate (E 211), potassium benzoate (E 212), and calcium benzoate (E 213) have been studied, and the EFSA panel derived an acceptable daily intake (ADI) of 5 mg/kg of body weight a day.

The market value of benzoic acid was around 1.1 billion $ worldwide in 2021. The global market value of benzoic acid by 2029 is expected to increase slightly to around 1.46 billion US dollars (Statista, 2022) (Figure 14).

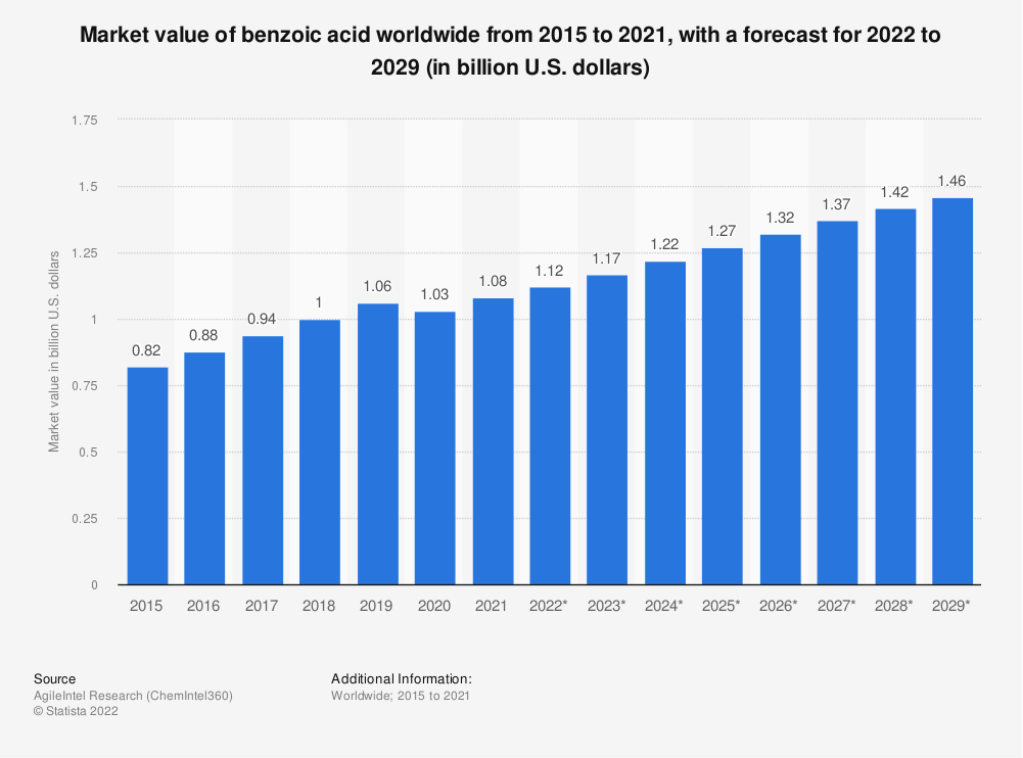

Figure 14 Market value of Benzoic Acid worldwide from 2015 to 2029 (in billion U.S. dollars), (Statista 2022).

1.6. How weak organic acids exert their antimicrobial effect

Organic acids are considered weak acids because the acid's dissociation depends on the environment's pH. The most important antimicrobial component is the undissociated one.

In solution, there is an equilibrium between the acid (HA) and dissociation products:

$$HA \rightleftarrows (H^+ + A^-)$$

The active part is the undissociated acid in the aqueous phase of the medium. The undissociated part of the acid can easily penetrate inside microorganism cells; for this reason, weak acids have an effect as preservatives, unlike strong acids, which dissociate entirely and cannot cross the plasma membrane of microorganisms.

The undissociated part (HA) crosses the membrane until the external and internal concentrations are equal. The undissociated part (HA) is lipide soluble and, thus, can move through microorganisms' membranes (Hirshfield et al., 2003; Debonne et al., 2019; Wade, Fondamenti di Chimica Organica, 2014) (Figure 15).

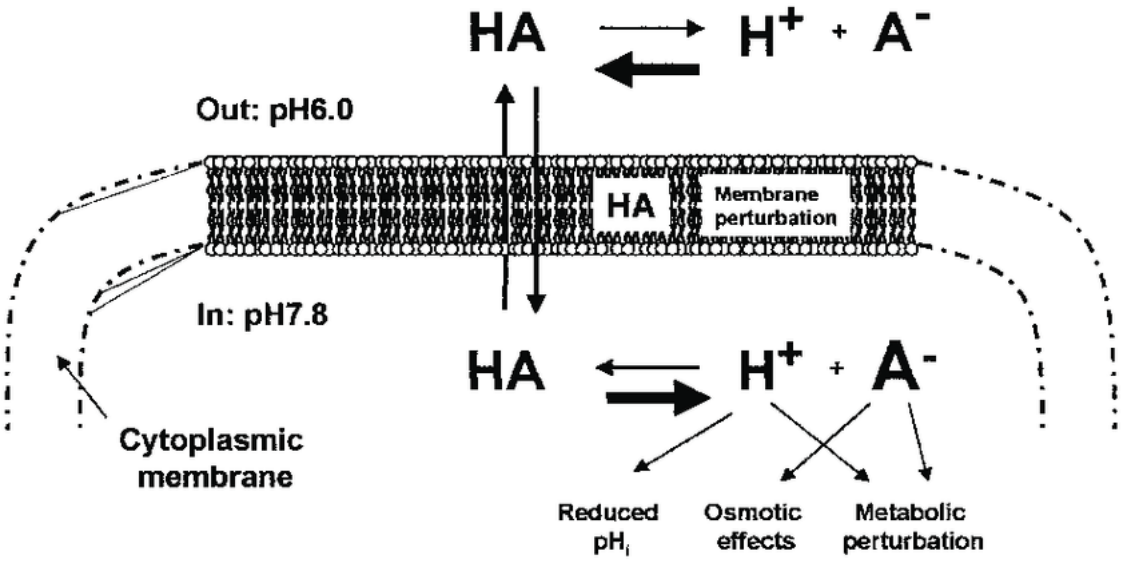

Figure 15 Generalised antimicrobial mode of action of weak organic acids (Hirshfield et al., 2004).

The leading cause of growth inhibition is the perturbation of the membrane. The undissociated part inside the cell partially dissociates to give H + and A-. This reaction depends on the pKa of the acid and the pH of the manner.

Undissociated weak acids enter the plasmatic membrane of microorganisms by crossing the phospholipid bilayer. The concentration with undissociated weak acids enter the plasmatic membrane depends on their lipophilicity.

There is a good correlation between the antimycotic effect of weak organic acids and their lipophilicity. A further possibility is that the growth inhibition caused by weak acids may result from the accumulation of inhibitory levels of A- (anion) within the cell's cytoplasm. The high concentration of A- in the cytoplasm can cause various problems for the cell:

- Significant increase in the osmolarity of the cytoplasm;
- If left unchecked, the anion would cause a potentially lethal increase in the pressure of the cell turgor due to the high amount of water drawn from outside the cell. The cell would compensate for this high osmotic pressure by releasing cytoplasmatic solutes of the cytoplasm (e.g., glutamate pool) to the outside;
- the high levels of weak acid anions that accumulate in the cytoplasm affect cellular metabolism, influencing the activity of enzymatic reactions within the cytoplasm (Hirshfield et al., 2003).

1.6.1. Standard Henderson-Hasselbalch equation

Organic acids are considered weak when they do not dissociate completely in water. Their level of dissociation changes according to the pH of the medium. The active part of the acid is the undissociated one.

The acid dissociation constant, Ka, is the equilibrium constant of the reaction and a quantitative measure of the strength of an acid in solution. The negative log of K_a is pKa and is typically used for communicating the acidity of different acids.

$$K_a = \frac{[A^-][H_3O^+]}{[HA]}$$

From this equation, we obtain the standard Henderson-Hesselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

- [A-] is the molar concentration of the acid's conjugated base;
- [HA] is the molar concentration of the undissociated acid.

Standard Henderson-Hesselbalch equation describes the relationship between pH and acid concentration using its pK_a.

From the Henderson-Hesselbalch equation, the percentage of dissociation ([H+]/[HA]) can be derived and used for determining the concentration of the undissociated acid (C_{HA}) and the total concentration of the acid (C_{TOT}).

$$C_{HA} = C_{tot}\left(1 - \frac{\%_{dissociation}}{100}\right)$$

Where:

- C_{HA}: concentration of undissociated acid
- C_{TOT}: total concentration of the acid

C_{HA} is expressed as mmole undissociated acid/L aqueous phase.

For the in-vitro trial, the final concentration of active undissociated acid (C_{HA}) was expressed in mmole/L MEA. For the in-vivo trial (bread and cake), for the recalculation of the concentrations, it was necessary information about product moisture (Soljic, 2019).

1.6.2. The effect of pH on the concentration of undissociated acids

The dissociation equation for sorbic acid is:

$$C_6H_8O_2 \rightleftarrows C_6H_7O_2^- + H^+$$

$$K_a = \frac{[C_6H_7O_2^-][H^+]}{[C_6H_8O_2]}$$

When the pH of the emulsion and pKa of the acid are equal, the concentration of undissociated and dissociated acids are identical. With a decrease in the pH of the emulsion (bakery product), there is consequentially an increase in undissociated acid and an increase in the antimicrobial effect of the acid (Soljic, 2019).

The effect of pH on the concentration of undissociated sorbic acid (pKa=4.76) is explained in Table 4:

Table The effect of pH on the concentration of undissociated sorbic acid.

pH	3.5	4	4.5	5	5.5	6	7
[HA]%	94.8	85.2	64.5	36.5	15.4	5.4	0.5

For propionic acid (pKa=4.87) (Table 5), the effect of pH on the concentration of the undissociated part is:

Table 4 The effect of pH on the concentration of undissociated propionic acid.

pH	3.5	4	4.5	5	5.5	6	7
[HA]%	95.9	88.1	70.1	42.6	19	6.9	0.7

In food products, microorganisms grow in the aqueous phase, and the chemical and physical structure of the food influences microbial development.

The lipid phase of foods retains part of the undissociated component of weak acids, making them less concentrated in the aqueous phase (and therefore less effective from an antimicrobial point of view) because the undissociated compound is lipophilic (Figure 16).

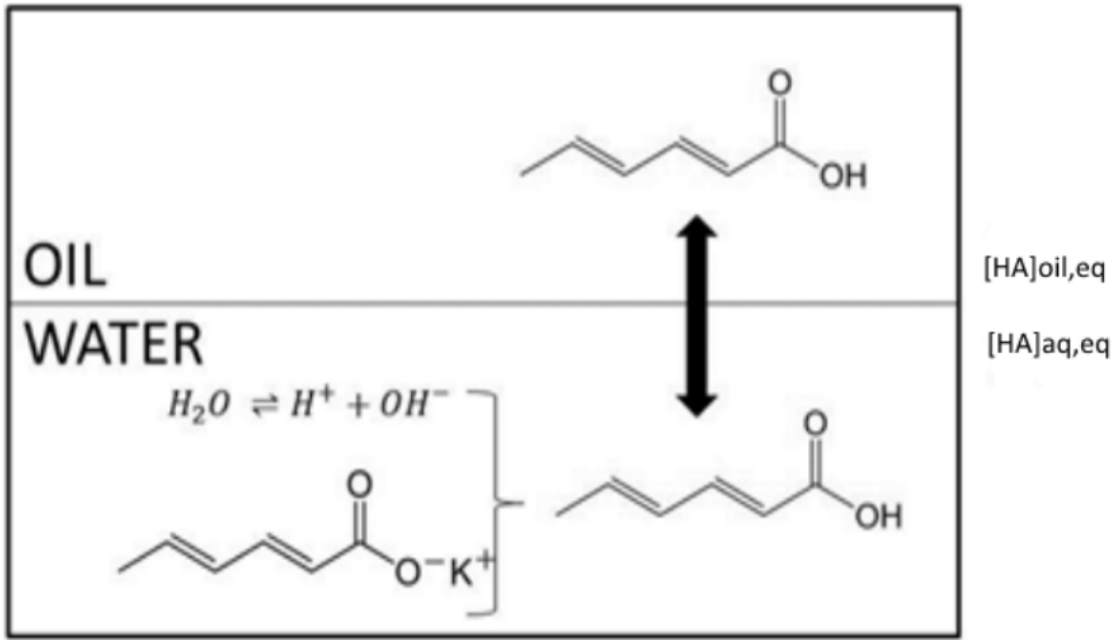

Figure 16 Sorbic acid partitioning behavior in a W/O emulsion. Šoljić 2019.

The equation shows the calculation of Kp (partitioning coefficient of the acid) between the oil and the aqueous phase where $[HA]_{oil,eq}$ and $[HA]_{aq,eq}$ (mol/L) (Soljic, 2019).

$$K_p = \frac{[HA]_{oil,eq}}{[HA]_{aq,eq}}$$

In general, in the industry, the quantity of weak acids used as preservatives depends on the pH of the food, the amount of fat (oil phase), ingredients, interferents, or salts.

Sorbic acid has a favorable oil/water partition coefficient, higher than benzoic acid. In a water/oil emulsion, a relatively high quantity of sorbic acid remains in the water phase. For these characteristics, the salts of sorbic acid (and propionic acid, too) are added to the liquid phase (water for the bread and eggs for cake). Adding salt and sugar to increase the microbiological stability of the foods also increase the repartition of the undissociated organic acid in the oil phase (Soljic, 2019).

1.6.3. Modified Henderson-Hasselbalch equation (Model 1)

To correctly represent the distribution of propionic and sorbic acid in a W/O emulsion, it is necessary to consider both the volumetric mass (kg/L) and the percentage weight (%) of the lipid and aqueous phase.

The new modified form of the Henderson-Hasselbalch equation, proposed by Wilson et al. (2000) and studied by Soljic (2019), is the following:

$$C_{HA.} = \frac{C_{TOT}}{m_{TOT} x \left(K_p \, x \, \frac{r}{\rho_{oil}} + (1 + 10^{pH - pK_a}) \, x \, \frac{1 - r}{\rho_{aq}} \right)}$$

Where:

- m_{TOT}: total weight of the system=100g
- m_{aq}: g water/100g of:
    - bread system: 45.5 g
    - cake system: 29.8 g
    - lemon cake system: 34.4 g
- m_{oil}: g oil/100g of:
    - bread system: 0.46 g
    - cake system:17.5 g
    - lemon cake system: 16.3 g
- m_{inert}: g inert fraction/100 g of:
    - bread:54.0 g
    - cake: 52.7 g
    - lemon cake: 49.3 g
- Kp: partitioning coefficient of sorbic acid and propionic acid.
    - Kp_{sorbic_acid}: 3.5
    - $Kp_{propionic\_acid}$: 2.14 (log Kow = 0.33)
- r: mass fraction of the lipid phase in the W/O emulsion: moil/m_{TOT};
    - molar weight of sorbic acid: 112.128 g/mole
    - molar weight of propionic acid: 74.07854 g/mole;
- ρ_{oil}: oil density (g/L)

- ρ_{oil} in bread g/cm^3
- ρ_{oil} cake/lemon cake: 0.92 g/cm^3

- ρ_{aq}: water density: 0.997 g/cm^3

Preservatives in emulsion are expressed in mg/kg or ppm. Converting ppm to mg/kg is easy in the aqueous phase because water could be considered 1kg/L, while in the oil phase, the concentration of preservatives expressed in mg/kg or mg/L is not the same.

In the modified form of the Henderson-Hasselbalch equation, C_{HA} is expressed in mmole/L aqueous phase; it is also possible to express C_{HA} in mg/kg or ppm. To convert C_{HA} mmole/L aqueous phase to mg/kg is essential to consider the acid's molecular weight.

1.6.5. Henderson-Hasselbalch equation (Model 2)

In 2018 Wemmenhove et al. proposed a new Henderson-Hasselbalch equation. The purpose was validation in cheese. Cheese is a high-fat food, so a different distribution of the acid component occurs. Wemmenhove et al., introduce two new parameters ($\varphi_{v_{aq}}$) and ($\varphi_{v_{oil}}$), which are respectively water and oil volume fraction in the system. Wemmenhove et al., 2018 equation is:

$$C_{HA} = \frac{C_{TOT}}{\varphi_{v_{aq}} + 10^{pH-pK_a} \times \varphi_{v_{aq}} + K_p \times \varphi_{v_{oil}}}$$

1.7. Preservative regulamentation

Food additives (EFSA definition): "*are substances added intentionally to foodstuffs to perform certain technological functions, for example to color, to sweeten or to help preserve foods.*"

The selection of the acids to be tested was made based on the legal limits set by Regulation No. 1333/2008 of the European Commission on approved food additives, including preservatives, and the terms of their use.

Certain conditions must be respected for using a food additive. They must be safe for the consumer, technologically necessary, they must provide an advantage for the consumer, and they must not be misleading to the consumer (Debonne, 2020).

Regulation (EC.) No 1333/2008 of the European Parliament and of the Council of December 16, 2008, on food additives: "*preservatives are substances that prolong the shelf-life of foods by protecting them against deterioration caused by micro-organisms and/or which protect against the growth of pathogenic micro-organisms.*".

The maximum dosage levels of organic acids in bread products and prepacked fine bakery wares (with a water activity >0.65) are listed in the revised Annex of EU Reg. 1333/2008, Reg. No. 1129/2011. Table 6 shows bakery products' maximum dosage level (in ppm) of sorbates and propionates.

Table 5 Max dosage level (ppm) for sorbates and propionates in bakery products

Preservative	**Max. dosage level (mg/kg)**	**Bakery product**
E 200, E 202, E 203	2000	Pre-packaged sliced bread and rye bread; partially baked, pre-packaged bakery products intended for retail and bread with lowered energetic value
E 280, 281, E282, E283	3000	Pre-packaged slices of bread and rye bread
E 280, 281, E282, E283	2000	Bread with lowered energetic value; partially baked, pre-packaged bread, bread rolls, and pita
E 280, 281, E282, E283	1000	Pre-packaged bread

E 200: sorbic acid, E202: potassium sorbate, E203: calcium sorbate, E280: propionic acid, E281: sodium propionate, E282: calcium propionate, E283: potassium propionate

In prepacked sliced and rye bread, the maximum level of propionic acid is 3000 mg/kg. In fine bakery ware, the limit of propionic acid is 2000 mg/kg product (EU, 2008, 2011).

According to EU Reg. No. 1129/2011, sorbic acid is allowed at a maximum level of 2000 mg/kg product, including prepacked sliced bread and rye bread, par-baked, prepacked bakery ware, and fine bakery ware (with a water activity > 0.65) (EU, 2011).

According to the Directive of the European Parliament and the Council, number 95/2 / EC, propionic and sorbic acid can be added to baked goods in concentrations up to 3000 and 2000 ppm, respectively (European Union, 1995).

Benzoic acid, allowed in concentrations up to 1500 ppm, is used in many acidic food products, although it is mainly associated with fruit preservation. It is also used in combination with sorbic acid for sweets and other products.

1.8. Most important molds in the bread and bakery products' spoilage

Among the most important species in the deterioration of bakery products, there are, *Aspergillus niger, Cladosporium ramotenellum*, *Aspergillus montevidensis* (formerly known as *Eurotium amstelodami*), *Aspergillus ruber* (formerly known as *Eurotium rubrum*), *Penicillium brevicompactum*, *Penicillium roqueforti* (Debonne, 2020) (Garcia et al., 2019).

1.8.1. *Aspergillus spp.*

The *Aspergillus* genus includes hundreds of species, important in natural ecosystems and the human economy, and has a biotechnological potentiality. Some strains can cause disease. Aspergillosis is the name given to all animal diseases caused by the growth of any member of the genus *Aspergillus* on a living host (Bennet, 2010).

Aspergillus was first cataloged in 1729 by the Italian botanist Pier Antonio Micheli, the founder of modern mycology. After microscopical observations of molds, Micheli decided to give it the name *Aspergillus* because of its shape, similar to an aspergillum (holy water sprinkler). One of the major aspergilli is *Aspergillus niger* (Figure 17). *Aspergillus niger* in nature can be found in soil or decaying plant material (Schuster et al., 2002; Vijayanandraj et al., 2006). Moreover, *Aspergillus niger* is one of the most important molds in biotechnology. *Aspergillus niger* is widely used in biotechnology to produce extracellular organic acids and enzymes in the food industry and has obtained GRAS status (Cabañes and Bragulat, 2018; Schuster et al., 2002). The first practical application of *Aspergillus niger* dates to 1919, and it was industrially used for its ability to produce citric acid in the 1960s; *Aspergillus niger* is used for producing enzymes for food processing, baking, starch, and others (Schuster et al., 2002). It is also used for bio-transformations and waste treatment (Varshney, 2016).

Unfortunately, some strains can produce ochratoxin A (OTA), a mycotoxin with nephrotoxic and carcinogenic activities. Hence, no filamentous fungi have the QPS (Qualified presumption of safety) (Cabañes and Bragulat, 2018; Gil-Serna et al., 2019; Schuster et al., 2002). *Aspergillus niger* is generally considered a non-pathogenic mold. Humans are daily exposed to its spores without diseases, and only rarely *Aspergillus niger* creates a systemic infection in humans (Vijayanandraj et al., 2006), although it is one of

the most notably responsible for otomycosis (fungal ear infections) (Munguia and Daniel, 2008).

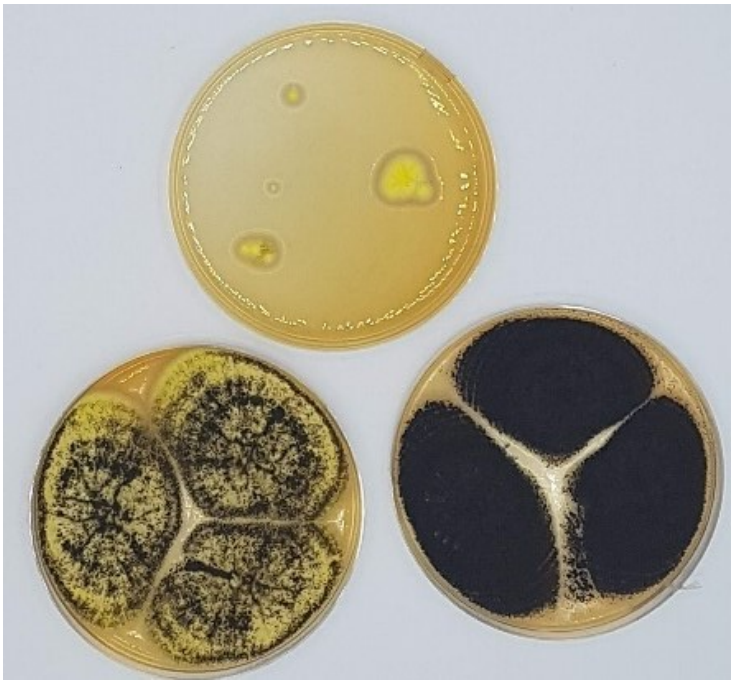

Figure 17 Different stages of development of *Aspergillus niger* in Petri dishes

1.8.2. *Cladosporium spp.*

Cladosporium is one of the most important molds, with 759 genera (Dugan et al., 2004). *Cladosporium* is a ubiquitous organism, and its spores can be found in air, soil, water, and food products. Some studies found that *Cladosporium* spores dominate 80% of all the spores in various regions of Europe (D'amato and Spieksma, 1995).

The main problems caused by *Cladosporium* are related to plant infection. For example, in cereal or the leaves of the tomato, it causes dark dots, and *Cladosporium cucumerinum* is known as an important cucumber pathogen causing damage to leaves, petioles, and fruits (Ogórek et al., 2012)

The best environmental conditions for *Cladosporium* growth are low temperatures and high humidity.

Cladosporium spp. can cause allergic reactions in humans, especially in people affected by asthma; in people without immunity deficits, rarely, it could evolve into opportunistic infections. *Cladosporium ramotenellum* (Figure 18) is one of the most important. *Cladosporium ramotenellum* is a quite common saprobic species (Bensch et al., 2015; Schubert et al., 2007). Before 2015 *Cladosporium ramotenellum* was isolated only by Schubert et al. 2007 from hypersaline water and an air conditioning system. Now we know that *Cladosporium ramotenellum* has a wider geographic distribution. Lee et al. 2011 discover that it causes sapwood discoloration and is also known to be common in indoor environments.

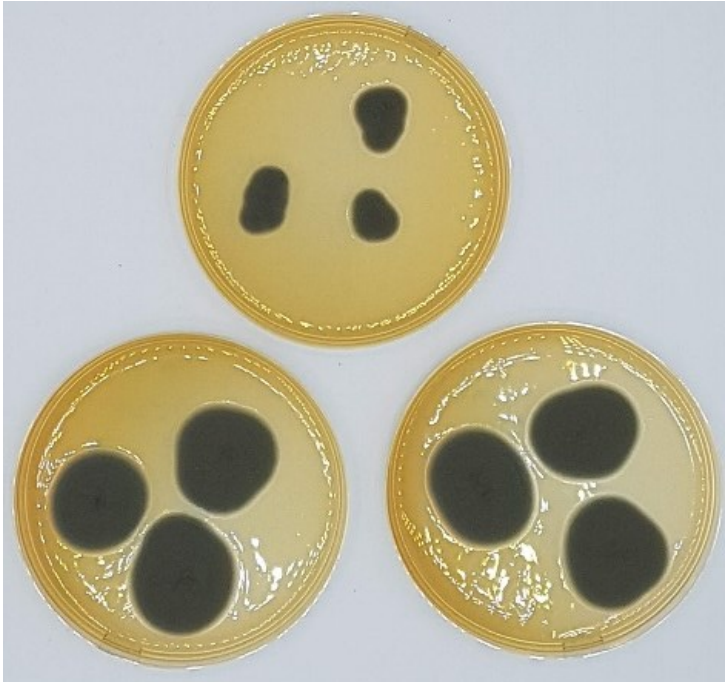

Figure 18 Different stages of development of *Cladosporium ramotenellum* in Petri dishes

1.8.3. *Eurotium spp.*

Eurotium species are the epitome of xerophilic fungi. They can see growth in high saline or sugary foods (water activity 0.7-0.72); (Azeem et al., 2016). *Eurotium* species have no real preference for substrate in stored commodities, but *Aspergillus* and *Eurotium* are more important than *Penicillium* as myco-flora in tropical conditions of storage (Hocking,

2014). Unfortunately, *Eurotium* species are great mycotoxins producers (Butinar et al., 2005).

Farmer's lung disease (FLD) is a type of hypersensitivity pneumonitis. It is common in agricultural areas and is mainly caused by the *Eurotium* species. One of the most important *Eurotium* is *Eurotium amstelodami*, now named *Aspergillus montevidensis* (Figure 19). It is known for causing hypersensitivity pneumonitis (HP), such as farmer's lung disease (FLD). Hypersensitivity pneumonitis diagnoses are based on interstitial markings on chest radiographs, serum antibodies, and lymphocytic alveolitis on bronchoalveolar lavage (BAL). It is possible to find *Aspergillus montevidensis* in sexual, asexual, or vegetative forms, but it is not known which of these is the predominant form in the agricultural environment. *Aspergillus montevidensis* is a significant mold responsible for the deterioration of baked goods. *Aspergillus montevidensis* can survive in MAPs containing up to 50% CO_2 (Abellana et al., 2001).

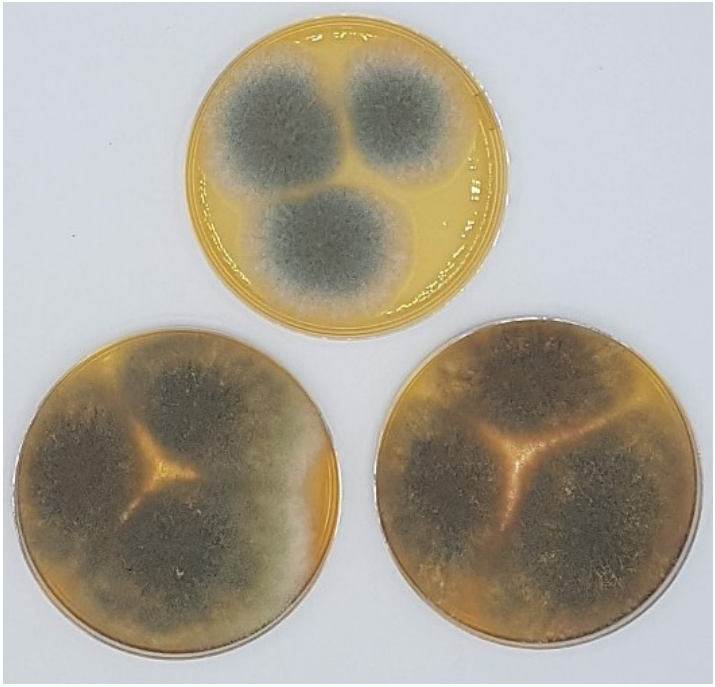

Figure 19 Different stages of development of *Aspergillus montevidensis* in Petri dishes

Aspergillus ruber (halophile) is one of the few species able to survive in conditions of hyper-salinity (water activity <0.669), *Aspergillus ruber*'s mycelial and spore stages can survive incubation in concentrated brine (Kis-Papo et al., 2014). For this ability,

Aspergillus ruber was found in the water of the Death Sea (Kis-Papo et al., 2014). *Aspergillus ruber* is not known for mycotoxin production (Frisvad, 2014).

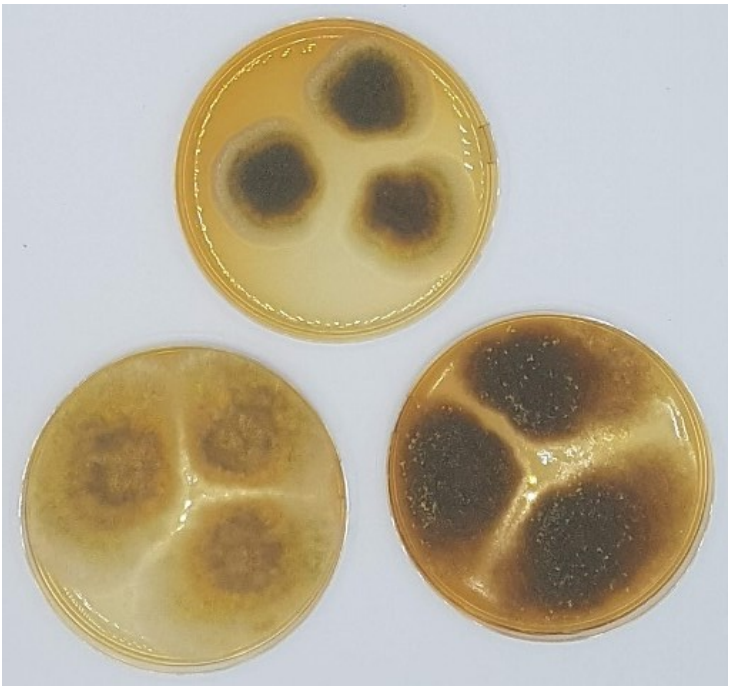

Figure 20 Different stages of development of *Aspergillus ruber* in Petri dishes

1.8.4. *Penicillium spp.*

Penicillium is an ascomycete critical in food spoilage but essential for the food industry. *Penicillium roqueforti* is needed for Roquefort or Gorgonzola production and industrial production of substances (probably the most famous one is penicillin).

The fungus was described for the first time by the German naturalist and botanist Johann Heinrich Friedrich Link in his work of 1809 called "*Observationes in ordines plantarum naturales*". *Penicillium* is derived from Latin, meaning "painter's brush" (Haubrich, 2003).

Penicillium brevicompactum (Figure 21) synthesizes mycophenolate mycotoxin acid (MPA). Mycophenolate mycotoxin acid inhibits inosine 5′-monophosphate dehydrogenase (IMPDH) and the proliferation of some cancer cells. It is used in medicine to prevent rejection of lung allografts, manage Chron's Disease, and manage systemic sclerosis. At the same time, this molecule has some side effects, like nausea and diarrhea. It can increase cancer risk, anemia, gastrointestinal bleeding, and progressive multifocal

leukoencephalopathy (PML) (Mycophenolate Monograph for Professionals, ASHP 2019).

House dust and other cellulose-rich internal materials are primary habitats for *Penicillium brevicompactum,* known to degrade cellulose fibers (Environmental Group). *Penicillium brevicompactum* has been isolated from a wide range of foods, but in conditions of optimal moisture is commonly encountered in the indoor air (Ndagijimana, 2008).

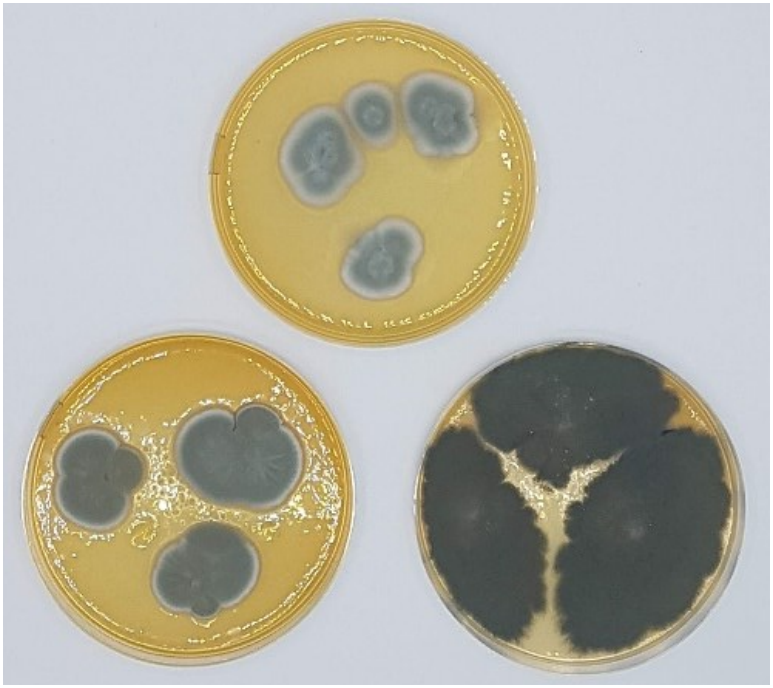

Figure 21Different stages of development of *Penicillium brevicompactum* in Petri dishes

Penicillium roqueforti (Figure 22) is a saprotrophic fungus that can be isolated in live and decaying plants.

Penicillium roqueforti is a common spoilage organism in various foods but is also used as a starter crop for many blue kinds of cheese, such as Roquefort and Gorgonzola. It is responsible for these cheeses' specific flavor, color, and characteristics.

The characteristics of these cheeses may be due to the different manufacturing methods and the specific strains of *Penicillium roqueforti* used.

In the dairy industry, it is common to call different strains of *Penicillium roqueforti* by technological names (e.g., *Penicillium gorgonzolae*, *Penicillium stilton*).

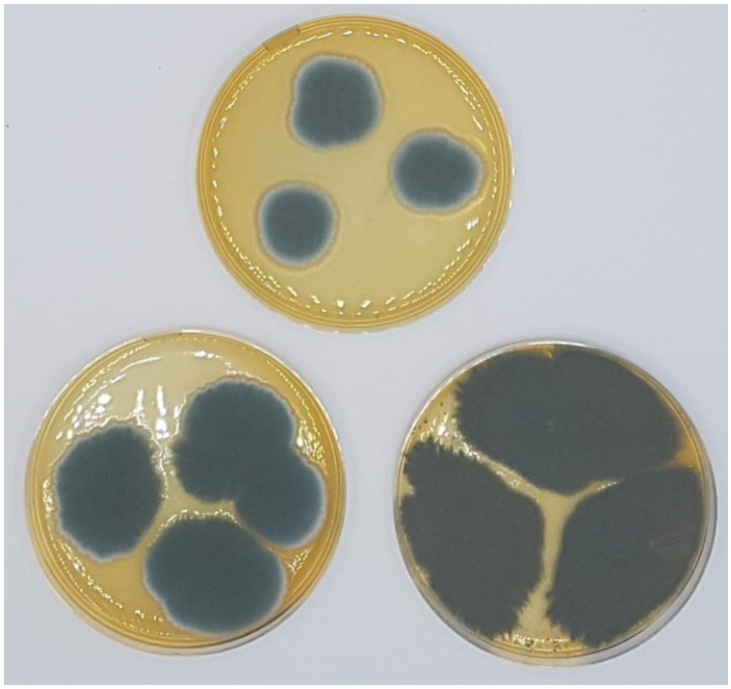

Figure 22Different stages of development of *Penicillium roqueforti* in Petri dishes

2. Aim of the thesis

The present work, carried out at Ghent University (Belgium), aimed to verify the relationship between mold development in baked products and the antimycotic action carried out by propionic and sorbic acid (used within legal limits).
In particular, the work focused on understanding, through the study of the Henderson-Hasselbalch equation, how the dissociation of weak organic acids, due to the change in pH of the medium considered, interacts with the antimycobacterial capabilities of the food preservative.

3. Materials and Methods

3.1. Fungal strains

This study used six molds based on their importance in bread and bakery product spoilage. Strains are reported in Table 7.

Table 6 The six molds used in this study and their code.

Mold's name	Strain number
Cladosporium ramotenellum	DTO 364-E4
Aspergillus niger	DTO 359-C5
Aspergillus montevidensis (formerly known as *Eurotium amstelodami*)	MUCL 15640
Aspergillus ruber (formerly known as *Eurotium rubrum*)	MUCL 29386
Penicillium brevicompactum	DTO 357-E3
Penicillium roqueforti	MUCL 046746

Original codes of purchase/origin: MUCL: Mycotheque de l'Université catholique de Louvain (UCLouvain). DTO: Department of Applied and Industrial Mycology; CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Fungal strains were maintained in the culture collection of the Laboratory of Applied Mycology MYCOLAB, Department of Food Technology, Safety and Health, Ghent University (Ghent, Belgium).

The six molds were kept active on malt extract agar (MEA, Thermo Scientific™ Malt Extract Agar (Dehydrated)) or dichloran-glycerol agar (DG18, Thermo Scientific™ Dichloran-Glycerol 18% (DG18) (ISO) Agar Base (Dehydrated)); for *Eurotium spp*.)

3.2. Refreshing fungal culture

The fungal culture was refreshed in Petri dishes containing MEA/DG18 media. The media were sterilized in an autoclave (121°C and 15 min.). Petri dishes were stored in the refrigerator for up to 1 week. Cultures were maintained in Petri dishes containing specific growth media and transferred to new fresh media when necessary. For this purpose, the fungal colonies in the old Petri dishes were scraped with a sterile eyelet and transferred to a new Petri dish. As a caution, the transfer process was repeated three times. Petri dishes were then incubated for one week at 26 ° C to allow the Petri dishes containing grown and sporified cultures were poured with 5 mL of a solution of Tween 80 (1 g Tween in 80/Lin water) and gently scraped to collect fungal sporesween in 80/Lin water) and gently scraped. The spore suspension was collected and filtered through sterile cotton to remove impurities and recover spores. The entire procedure was conducted under a laminar flow hood in sterile conditions.

The spore suspension was then centrifuged for 15 minutes at 8000 rpm at 4°C, the supernatant was removed, the pellet dissolved in 25mL of Tween-PBS, and the spores were resuspended by Vortexing. The operation was repeated three times.

As described below, the concentration of spores in the suspension was then determined by a microscope evaluation and Thoma counting chamber.

3.4. *In vitro* experiments

In the *in-vitro* experiments, the effect of organic acids on fungal spores' germination in solid media (MEA) at different water activity and pH was evaluated.

The media's water activity (aw) was corrected using 99.5% glycerol (VWR Chemicals). The media were prepared in 3 Glass Schott bottles of 2 liters each, containing 1800 g of water + glycerol as reported in table 8.

Table 7 Glycerol and water amounts to obtain water activity of 0.93-0.95-0.97 in the three bottles used for the experiments

water activity	**% glycerol (g/g)**	**water (g)**	**glycerol (g)**	**Total (glycerol + water) (g)**
0,93	23,4%	1379	421	1800
0,95	14,3%	1543	257	1800
0,97	5,2%	1707	93	1800

The pH of the solutions of glycerol and water were modified as described in table 9, using 0.1 M citric acid (CA) (citric acid-1 hydrate) and tri-sodium citrate dehydrate (Merck). MEA powder was added, and the media was sterilized in an autoclave.

Table 8 Citric acid (CA) and tri-sodium citrate dehydrate (triNaC) amounts were used to obtain different MEA pH (5, 5.5, and 6)

pH	**CA (g)**	**triNaC (g)**	**MEA (g)**
5	6,92	16,84	90
5,5	4,15	20,75	90
6	1,38	24,66	90

CA: citric acid-1-hydrate; triNaC: tri-sodium citrate dehydrate.

- pH 5 and water activity 0.93 bottle
- pH 5.5 and water activity 0.95 bottle
- pH 6 and water activity 0.97 bottle

Calcium propionate (Sigma-Aldrich, Merck; 186.22 g/mole) (for propionic acid) and potassium sorbate (Sigma-Aldrich, Merck; 150.22 g/mole) (for sorbic acid) were used as preservatives. A solution (10 w/w%) of propionic acid and sorbic acid was prepared, filter sterilized (using a Thermo Scientific Nalgene Rapid-Flow Sterile Single Use with 0.2 µm size pore), and then added to the previously autoclaved sterilized MEA with different pH and aw as reported in table 10. The media, with a different combination of pH and aw, were poured into Petri dishes and let solidify.

Table 9 Amounts of Propionic acid and Sorbic acid added to the MEA media with different water activity, and pH

Concentration (mg/L)	Propionic acid (mL)	Sorbic acid (mL)
0	0.00	0.00
500	4.52	2.41
1000	9.05	4.82
1500	13.57	7.23
2000	18.10	9.65

The water activity of the media was checked with a Lab Master-water activity (Novasina), and the pH was matched with a portable pH meter (model HI 83141, Hanna Instruments).

In total, for propionic acid, were prepared forty five different combinations of pH (5.0-5.5-6.0), water activity (0.93-0.95-0.97) and total concentration of acid (mg acid/L MEA): 0, 500, 1000, 1500, 2000. For sorbic acid, only thirty different combinations were prepared because pH 6.0 was excluded. Three replications were conducted for each condition.

For propionic acid, the concentrations in mmole acid/L MEA were 0, 6.7, 13.5, 20.2, 26.9 mmole/L MEA; for sorbic acid the concentrations were 0, 4.5, 8.9, 13.4, 17.8 mmole/L MEA (in both cases for 0, 500, 1000, 1500, 2000 ppm).

Considering pH 5 and pH 6, at water activity 0.93, water concentration ranged from 71.7% to 72.6%, respectively. At water activity 0.95, aqueous concentrations ranged from 80.3% to 81.3% respectively, and at water activity 0.97, aqueous concentrations ranged from 88.8% to 89.9% respectively.

The different water concentrations were used to calculate the concentrations of undissociated acid in MEA, calculated by the Henderson-Hesselbalch equation.

The concentration range for propionic acid was 0-18.1 mmole propionic acid/L waterin MEA. For sorbic acid the range was 0 to 9.1 mmole sorbic acid/L waterin MEA.

After solidifying the agar into the Petri dishes, three spots (20 µL and 10^2 spores each) of the mold spore solution (containing $5*10^3$ spores/ml) were applied to each Petri dish.

A total of twelve replications (each Petri dish contained three spots) were conducted for each condition. Plates containing propionic acid and sorbic acid were incubated at 25°C. Each day the plates were checked for the appearance of molds. The checking process lasted 30 days for the propionic acid and 60 days for the sorbic acid.

3.5. *In vivo* experiment

3.5.1. Evaluation of the effect of organic acids on fungal spores' germination in bread and cake

The flour used for each experiment was Epi B type 55 (Brabomills NV (Merksem, Belgium)), with a dry weight of 85%.

The flour composition was as follows: 100 grams of dry matter contained10.6 g protein, 1.2 g fat, and 0.5 g ash. The following ingredients were added to the flour: 59.5 g water/100 g flour, 1.5 g table salt/100 g flour, 1 g instant dry baker's yeast (Algist Bruggeman, Belgium)/100 g flour, 0.3 g malt flour/100 g flour, and 5 mg ascorbic acid/100 g flour.

The following concentrations of propionic acid were added to kg of bread:0, 500, 1000, 2000, 3000 mg propionic acid. As propionic acid was added as calcium propionate, the correct amounts of propionic acid were recalculated as follows: 0, 497, 990, 1967, 2901 mg propionic acid/kg bread. Calcium propionate was dissolved in water before adding it to the flour.

A spiral mixer (De Danieli Verhoest Machinery) was used to mix the ingredients for8 minutes. After mixing, the dough was taken to a leavening chamber for ten minutes at 30°C and relative humidity of about 80%. After the first leavening, the dough was divided into four loaves of 400g each. Each loaf was rolled through a Brabender Rounder. The rolled buns were placed in the leavening chamber for the second leavening for 30 min at 30°C and 80% relative humidity.

After the second rising, the buns were flattened through a rolling pin. After flattening, the loaves were folded. The folded buns were placed in metal baking pans greased with oil and placed again into the fermentation chamber to carry out the third fermentation (for 65 minutes, at 30°C and 80% relative humidity).

After the third fermentation, the pans containing the fermented buns were placed in the oven (MIWE aeromat FB12 -4.64). The baking operation took thirty minutes: 14 minutes at 230°C, 13 minutes at 200°C, and 3 minutes at 190°C). For the cooking operations, 240 ml of steam was used.

Five different loaves of bread were prepared for each acid concentration. The procedure was repeated, and ten loaves per acid were made at the end.

3.5.2. Production of cake with sorbic acid

The cakes contained the following ingredients for 100 g of dough: 25 g wheat flour (Epi B), 25 g extra-fine white sugar/, 25 g eggs/, 25 g margarine (70% oil), 3.4 g of baking powder (75% disodium diphosphate + sodium bicarbonate and 25% wheat starch). To make the lemon cake, 6.2g of lemon juice (strained through a strainer)/100 g of dough was added.

As propionic acid, also sorbic acid was added as salt (potassium sorbate). The new concentrations of sorbic acid in the cake were 0, 493, 1004, 1488, and 1988 mg sorbic acid/kg of cake. The concentrations of sorbic acid in the lemon cake were 0, 475, 951, 1429, 1897 mg sorbic acid/kg lemon cake.

The ingredients were blended by a planetary mixer (Hobart). The margarine was mixed in the planetary mixer for thirty seconds (softening process). The margarine was scraped from the mixer surfaces, and the sugar was added. The margarine and sugar were mixed for two minutes. The process of scraping the surfaces was repeated. After scraping the surfaces, the egg and flour (into which the baking powder had previously been mixed) were added. The mixture was stirred for five minutes. After the stirring operation, the last scraping process was carried out so that the mixture came off the walls (especially the margarine). After scraping, the mixer was used for thirty seconds. The mixture was poured into five aluminium baking pans. A dough quantity of 250 g was weighed for each pan. The pans with the dough were placed in the oven (MIWE aerobat FB12-4.64) for forty minutes at 175°C.

The baking operation of the cakes was carried out for each sorbic acid concentration (0-500-1000-1500-2000 ppm). Each operation was carried out twice on different days.

3.6. Challenge test (inoculation of bread and cake with fungal spores)

Before the inoculation, cakes and loaves of bread were made to cool down. Loaves of bread and cakes were weighed, volume was measured, and a small amount was used to carry out pH and water activity measurements (Table11 and chapter "Product characterization").

Then the loaves and cakes were cut into slices (using a sanitized knife). To have as uniform samples as possible, end slices of bread and cakes containing more crust were excluded from the experiments.

The bread and cake slices were inoculated (20 µL) with 10^2 spores. Three inoculations were made for each slice, and the slices were packed in plastic bags. The bags were heat-sealed. The composition of the bags was polyamide 20µm, polyethylene 70µm).

The bags were stored in a room at standard temperature and humidity. Temperature and relative humidity were checked using Testo 174 H loggers (for more information, see chapter "Temperature and relative humidity of conservation").

A total of 12 replicates were obtained for each condition studied.

Each day the integrity of the bags was checked. Overall, the bread slices and cake slices were checked for thirty days. Any fungal growths on bread and cake slices were reported.

In parallel, negative controls (5-6 bread-cake slices for each studied condition in which no spores were inoculated) were carried out. The to verify the shelf life of the non-inoculated product. During storage, some slices from the negative controls were used to examine the product's changes in pH, water activity, and moisture.

3.7. Product characterization

One hour after baking, weight, volume, pH, water activity, and moisture were determined.

The bread and cakes' volume were measured by a Volscan Profiler 600 (Stable Micro Systems), and the bread and cakes were weighed using a KERN balance (± 0.01 g). The measure of specific volume was obtained with volume /weight (mL/g). The water activity of bread was determined by a LabMaster- water activity (Novasina) (samples for water activity were taken from the crumb), and pH was determined by pH meter (model HI

83141, Hanna Instruments) (samples for pH were taken from the crumb). Moisture was measured by the weight loss of the samples after 105°C x 8 hours (AACC Method 44-15.02 method). pH, water activity, and moisture content for bread were also monitored on days 6 and 12 of storage. The cake's pH, water activity, and moisture content for the cakes were tested on days 10 and 20 of storage.

3.8. Data processing

3.8.1. Development of growth/ no-growth models (G/NG)

In-vitro inoculation envisaged the inoculation of three spots of the mold spore solution after solidifying the agar into the Petri dishes. Each spot was 10^2 spores (20µL and $5*10^3$ spores/ml). Twelve replicate spots per condition were carried out to set up the model. The plates containing propionic acid and sorbic acid were incubated at 25 °C for 30 days and 60 days, respectively.

As described above, the bread and cakes were sliced and challenged with three spots of 10^2 spores (20 µL) after cooling. Per bread/cake slice, three spots were applied on the crumb.

After inoculating the samples (both *in-vitro* and *in-vivo*) with the spores, the samples were observed, and changes were noted daily.

Based on the binary data of growth / non-growth, models were developed to evaluate the influence of C_{TOT} (total acid mmole / L MEA or total acid mmole / L aqueous phase in bread or cake), C_{HA} (mmole HA / L aqueous phase), pH, water activity and time (days) to the growth/no growth of the molds.

For each mold (*Aspergillus niger*, *Eurotium amstelodami*, *Eurotium rubrum*, *Cladosporium ramotenellum*, *Penicillium brevicompactum,* and *Penicillium roqueforti*) were built different models. C_{TOT} (mmole acid/L H_2O or MEA), C_{HA} (mmole undissociated acid/L H_2O or MEA), pH, water activity (because it is always less than 1, was normalized with values from 0 to 100), and time were used for modeling.

Debonne et al. (2019) and Vermeulen et al. (2012) describe the data used in an ordinary logistic regression model.

The equation of the models is a polynomial and logit(p) = log10(p/(1-p)) with p as the probability of growth.

The predicted growth/no-growth interfaces were plotted in Matlab 9.3 (The Mathworks, Inc., Natick, MA, USA). The growth/no growth plots presented in this study show the probability of growth (90%, 50% and 10%, respectively p = 0.9, 0.5 and 0.1). Minimal inhibitory concentrations (MIC) are based on the probability of 90% of no growth (p=0.1) during shelf life. Shelf life is respectively 30 days for MEA, bread + propionic acid, cake, and lemon cake sorbic acid, and 60 days for MEA and sorbic acid.

3.8.2. Statistical analysis

The statistical software SPSS was used to perform a multiple comparison analysis to evaluate the significant difference between the samples.

Based on the distribution of the results, several tests were carried out:

- If the results were normally distributed, a Tukey or Dunnett T3 test was used to describe the means with 95% confidence (p = 0.05).
- On the other hand, if the data did not appear to be distributed normally, a Dunn's test for multiple comparisons was performed. A Kruskal-Wallis ANOVA preceded Dunn's test

4. Results and discussion

4.1. Influence of propionic and sorbic acid on the characteristics of MEA

As described in the paragraph "Material and Methods, *In vitro* experiments," before inoculation experiments, the pH and water activity of MEA were analyzed. In pH measurements (Figure 23), a weak acid's dissociation increases as the pH value decrease. Therefore, the maximum values of C_{HA} (undissociated acid) were found at pH values close to pKa.

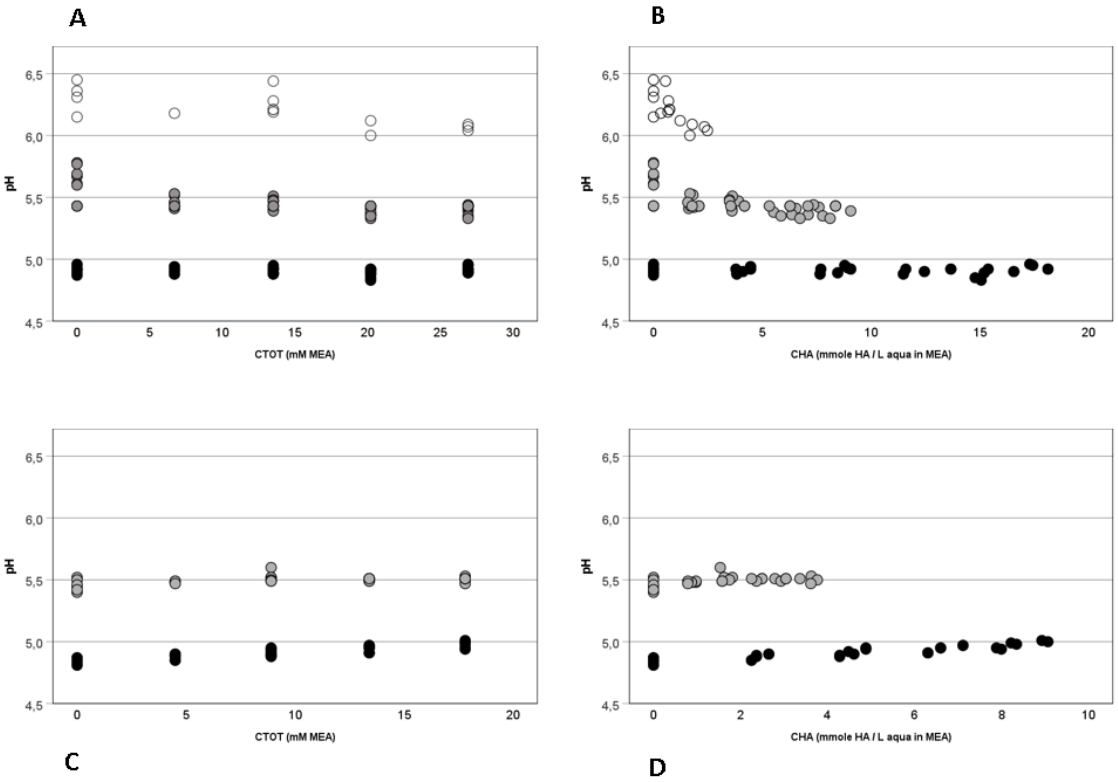

Figure 23 In MEA, the link between pH and C_{TOT} (mmole total acid /L MEA), and pH and C_{HA} (mmole HA/L H_2O in MEA) for propionic acid (A, B) and sorbic acid (C, D). The colors of the dots stand for the theoretical pH (pH = 5.0 (black), 5.5 (grey) and 6.0 (white).

The results of pH obtained for the MEA (Figure 23) ranged from a minimum of 4.8 to a maximum of 6.5 for propionic acid and from a minimum of 4.8 to a maximum of 5.6 for sorbic acid.

The concentration in the aqueous phase was calculated and it ranged from 71.7 (pH 5.0) to 72.6% (pH 6.0) for water activity 0.93, 80.3–81.3% for water activity 0.95 and 88.8–89.9% for water activity 0.97. These aqueous concentrations were used to calculate the

correct concentration concentrations of undissociated acid (C_{HA}) (mmole/L aqueous phase of MEA medium) using the standard Henderson-Hesselbalch equation (no oil phase was assumed to be in MEA). The concentration range of undissociated acid for propionic acid was 0– 18.1 mmole HA/L aqua in MEA. The concentration range of undissociated acid for sorbic acid was 0–9.1 mmole HA/L aqua in MEA.

As regards the water activity, the values obtained slightly differed from the theoretical values; for the water activity values of the propionic acid, the variation ranged from 0.901 to 0.971, while the water activity values of the sorbic acid ranged from 0.909 to 0.961. The theoretical values of water activity were 0.93, 0.95, and 0.97.

4.2. Influence of propionic acid on the physical and chemical characteristics of bread

Table 11 shows the values of pH, water activity, specific volume (SV), and moisture in the bread, cake, and lemon cake in the different experimental conditions.

Table 10 Influence of propionic acid on bread's physical and chemical characteristics (pH, water activity, specific volume (SV), and moisture).

Propionic acid (mg/kg)	**pH**	**Water activity**	**Specific Volume SV (mL/g)**	**Moisture (g/100 g)**
Bread + propionic acid				
0	5.7 ± 0.0	0.953 ± 0.005	3.9 ± 0.2	45.6 ± 0.0
500	5.5 ± 0.0	0.955 ± 0.006	3.8 ± 0.1	45.5 ± 0.4
1000	5.1 ± 0.1	0.955 ± 0.004	3.4 ± 0.1	45.6 ± 0.1
2000	5.1 ± 0.1	0.955 ± 0.004	3.1 ± 0.0	45.5 ± 0.1
3000	5.0 ± 0.2	0.951 ± 0.003	2.7 ± 0.1	45.2 ± 0.1
Mean day 0	NO	0.954 ± 0.004	NO	45.5 ± 0.2
Mean day 6	5.4 ± 0.3	0.952 ± 0.004	/	43.6 ± 2.2

NO: there are significant differences observed for different C_{TOT} values/: missing data

The quantities of propionic acid in bread were 0, 500, 1000, 2000, and 3000 ppm. The different amounts of acids caused the pH of the bread to drop from 5.7 $\pm$ 0.0 (0 ppm) to

5.0 ± 0.0 (3000ppm). Water activity decreased during conservation: on day 0, the aw of bread was 0.954 ± 0.004; on day six, aw was 0.952 ± 0.004.

Increasing concentration of propionic acid strongly affects the bread's volume. From 0 ppm of propionic acid to 3000 ppm specific volume decrease was 3.9 ± 0.2 mL/g and 2.7 ± 0.1 mL/g, respectively. The reduction of specific volume was 30.77%.

The moisture of bread did not change significantly with the increased propionic acid concentration. During storage, moisture changes significantly. In bread, moisture on day 0 was 45.5 ± 0.2 g/100 and on day 6 was 43.6 ± 2.2 g/100. The reduction was 4.18%. Figure 24 shows five different bread with different propionic acid concentrations.

Figure 24 Bread with propionic acid. From the left to the right different concentrations of propionic acid were used (0-500-1000-2000-3000 ppm)

4.3. Influence of sorbic acid on the physical and chemical characteristics of cake and lemon cake

The quantities of sorbic acid in cake and lemon cake were 0, 500, 1000, 1500, and 2000 ppm. The different amounts of acids have not caused variation in pH measurement (Table 12).

On day 0, the water activity of the cake was 0.891 $\pm$ 0.005, and the water activity of the lemon cake was 0.899 $\pm$ 0.006. On day 10, for both cakes, water activity was respectively 0.870 $\pm$ 0.027 and 0.881 $\pm$ 0.021.

The specific volume of the cakes (the normal one and lemon cake) remained constant with the increase in sorbic acid concentration. Still, at the same time, the specific volume of lemon cake (pH 5.2) was lower than traditional cake (pH 7.2) (2.0 $\pm$ 0.1 compared with 1.4 $\pm$ 0.0) (30% different). Lemon cake has a lower volume than normal cake (pH7.2) because of the interaction between the baking powder (75% disodium diphosphate + baking soda and 25% wheat starch) and the acid provided by the lemon. As the lemon reacts with the diphosphate and bicarbonate, CO_2 is produced, which escapes before baking.

The increased sorbic acid concentration did not significantly influence the moisture of cake and lemon cake. Storage deeply influenced moisture changes. Moisture reduction during conservation of cakes (ten days) changed from 34.4 $\pm$ 0.9 g/ 100 g to 30.3 $\pm$ 4.3 g/ 100g (reduction of 11.9%). Moisture reduction also changes the concentration of undissociated acids.

Table 11 Influence of sorbic acid on the physical and chemical characteristics (pH, water activity, specific volume (SV), and moisture) of cake and lemon cake.

Sorbic acid (mg/kg)	**pH**	**water activity**	**Specific Volume SV (mL/g)**	**Moisture (g/100 g)**
Cake + sorbic acid				
0	6.9 ± 0.2	0.890 ± 0.004	2.0 ± 0.0	28.9 ± 0.3
500	7.2 ± 0.1	0.893 ± 0.003	2.0 ± 0.2	29.8 ± 0.1
1000	7.2 ± 0.1	0.900 ± 0.004	2.0 ± 0.1	30.5 ± 0.1
1500	7.4 ± 0.1	0.892 ± 0.004	2.0 ± 0.0	30.3 ± 0.1
2000	7.4 ± 0.2	0.885 ± 0.003	2.0 ± 0.1	29.4 ± 0.5
MEAN day 0	7.2 ± 0.2	0.891 ± 0.005	2.0 ± 0.1	29.8 ± 0.7
MEAN day 10	7.2 ± 0.2	0.870 ± 0.027	/	25.9 ± 4.3
Lemon cake + sorbic acid				
0	5.2 ± 0.0	0.909 ± 0.010	1.4 ± 0.0	34.5 ± 0.2
500	5.2 ± 0.0	0.904 ± 0.002	1.4 ± 0.0	34.3 ± 0.4
1000	5.1 ± 0.0	0.902 ± 0.004	1.4 ± 0.1	35.5 ± 0.1
1500	5.2 ± 0.0	0.894 ± 0.001	1.4 ± 0.0	34.0 ± 1.6
2000	5.2 ± 0.0	0.895 ± 0.001	1.5 ± 0.0	33.9 ± 0.9
MEAN day 0	5.2 ± 0.0	0.899 ± 0.006	1.4 ± 0.0	34.4 ± 0.9
MEAN day 10	5.2 ± 0.1	0.881 ± 0.021	/	30.3 ± 4.3

/: missing data

Figure 25 and Figure 26 show cake and lemon cake cuts respectively, with different sorbic acid concentrations.

Figure 25Cut cake with sorbic acid. From the left to the right different concentrations of sorbic acid were (0-500-1000-1500-2000 ppm)

Figure 26 Cut lemon cake with sorbic acid. From the left to the right different concentrations of sorbic acid were used (0-500-1000-1500-2000 ppm)

4.4. Temperature and relative humidity of conservation

The bread and cake samples were stored in a climate room, and temperature and relative humidity were monitored and recorded throughout the storage period. The data were retrieved using a Testo Interface 174T/174H and Software ComSoft Basic (Testo SE and Co. KGaA, Germany). The temperature was set at 19.7 ± 0.7 °C (Figure 27) and the relative humidity at 59.6 ± 5.3 % (Figure 28). The frequency of analysis (for both temperature and humidity) taken by the two loggers was ten minutes for logger 1 (blue line) and six hours for logger 2 (orange line).

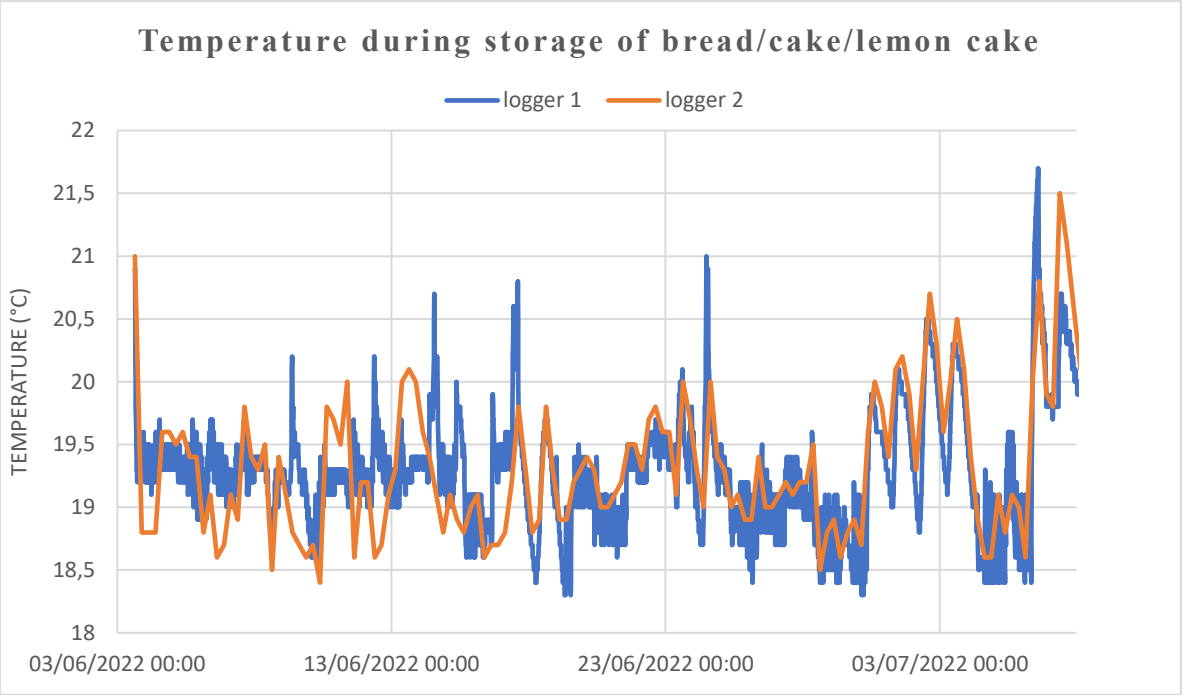

Figure 27 Storage temperatures (°C) reported by data logger Testo Interface 174T/174H. Logger 1 (blue lines), with a frequency of analysis of 10 minutes. Logger 2 with a frequency of analysis of 6 hours.

Temperatures, between day and night, changed slightly. The result was most noticeable through logger 2 (orange), which had a six-hour analysis frequency. Temperature differences settled at a maximum of 1.5 °C. Until the end of June, temperatures did not exceed 20.1 °C (June 13, 2022), while the lowest temperature recorded was 18.4 °C (June 10, 2022). Since the beginning of July, temperatures have risen more, reaching 22.5°C on July 8. Fortunately, the results of the experiments were unaffected as the last helpful measurement stopped on June 23 on a slice of cake inoculated with *Aspergillus ruber*.

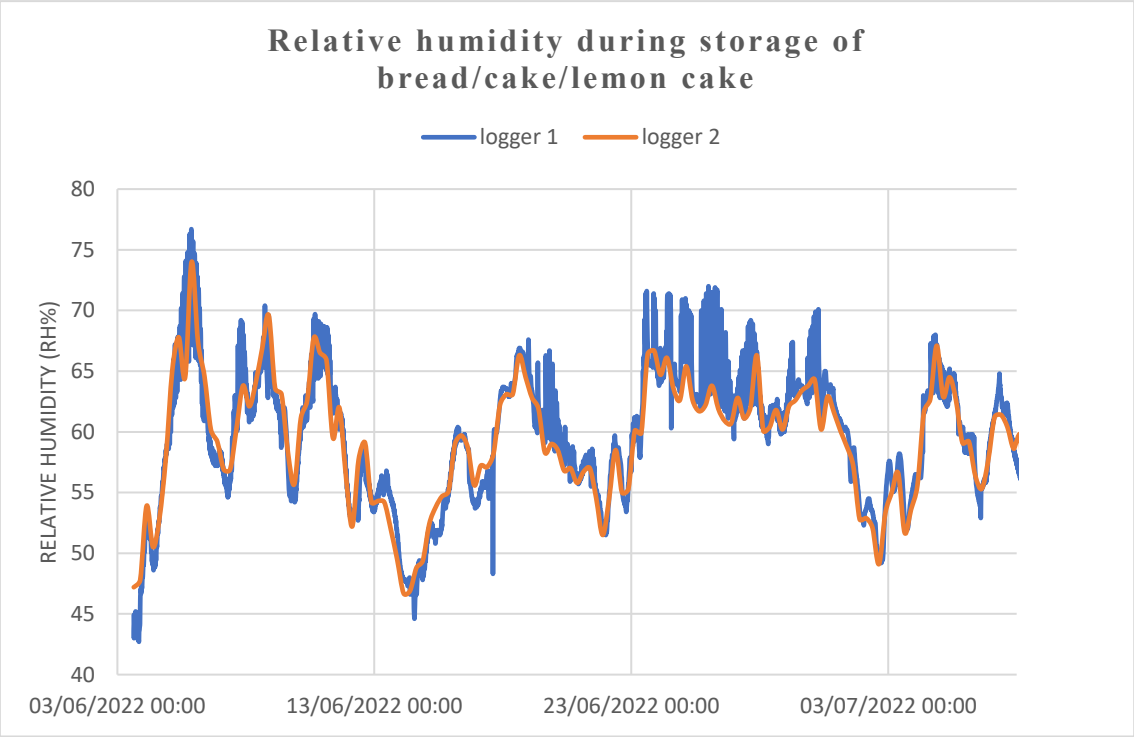

Figure 28 Storage relative humidity (RH%) reported by data logger Testo Interface 174T/174H. Logger 1 (blue lines), with a frequency of analysis of 10 minutes. Logger 2 with a frequency of analysis of 6 hours.

The measured relative humidity did not change significantly. However, it turns out to be a less critical parameter because slices of bread or cakes appeared to be sealed inside plastic bags.

4.5. Undissociated acid (C_{HA})

The values of C_{HA} (mmole undissociated acid/L H_2O) were estimated using the Henderson-Hasselbalch equation (standard, model 1 and model 2) thanks to the values of C_{TOT} (mmole total acid/L H_2O), pH, and humidity at day 0.

Figures 23 A and B represent the calculated propionic acid, and C and D are sorbic acid concentrations. The color of the dots represents theoretical pH (white is pH 6, grey pH 5.5, and black pH 5). In the case of MEA, a correlation between pH - C_{TOT} values (A and C) and pH - C_{HA} is shown in figure 23.

For the calculation of the concentration of undissociated acid, the standard Henderson-Hasselbalch equation was used because it was assumed that no oil was present in the MEA. The estimation of C_{HA} is almost the same between the standard and modified

Henderson-Hasselbalch equation (model 1). At the maximum acid concentration (2000 mg total acid/L MEA), there were 18.1 mmoles of undissociated propionic acid/ L and 9.1 mmoles of undissociated sorbic acid/L.

Table 13 shows propionic and sorbic acid's theoretical concentrations in *in-vitro*, bread, cake, and lemon cake. It is possible to see a lack in the modified Henderson-Hasselbalch equation (model 1); in bread-propionic acid, the theoretical concentration $C_{HA,\ model\ 1,}$ is higher than $C_{TOT,}$ which is impossible. Using the modified Henderson-Hasselbalch equation (model 2), the theoretical result fits the practical one.

In lemon cake, the standard Henderson-Hasselbalch equation and the Henderson-Hasselbalch equation model 2 show the C_{HA} relatively in line with each other (13.1 and 9.1 at 2000ppm, respectively). Henderson-Hasselbach equation model 1, at the pH value close to pKa, estimated a protonation of almost 100%, and the C_{HA} and C_{TOT} values were similar (respectively, 45.1 and 49.1 at 2000ppm).

We should expect that as partitioning coefficient of the acid Kp (oil/water) increases, the undissociated acid C_{HA} present in the aqueous phase decreases. In model 1, the C_{HA} and C_{TOT} values are similar. In contrast, the C_{HA} value remains within certain limits in model two.

Model 2 was proposed for the first time by Wemmenhove et al. in 2018, studying the dissociation of lactic acid on a very fat matrix (cheese). When referring to lean matrices (e.g., bread or MEA), which therefore have little / no amount of oil, the standard model and model 2 are very similar and can be used to estimate C_{HA} values. If, on the other hand, we are referring to a matrix rich in fat (oil), such as lemon cake, model 2 is more suitable than the standard model, as it also considers the slight loss of C_{HA} from the aqueous phase because the oily phase should absorb the C_{HA}. It is possible to conclude that model 2 of Wemmenhove et al., (2018) can be used not only for cheeses but also for baked goods such as cakes or bread.

Table 12 Calculated propionic and sorbic acid concentrations in the in-vitro experiment, bread, cake, and lemon cake. Concentrations range: from 0 to 2000 mg total acid per kg product for in-vitro, cake, and lemon cake, and from 0 to 3000 mg total propionic acid per kg product for the bread. C_{TOT} (mmole acid/L MEA), $C_{HA,standard}$ (mmole HA/L H_2O in bread/cake, determined by the standard Henderson-Hasselbalch equation), $C_{HA,model\ 1}$ (mmole HA/L H_2O in bread/cake by model 1) and $C_{HA,model}$ 2 (mmole/L H_2O).

	Theoretical concentration: mg acid/kg				
	500	**1000**	**1500**	**2000**	**3000**
***In vitro*-propionic acid C_{TOT}** (mmole/L MEA)	6.7	13.5	20.2	26.9	/
***In vitro*-sorbic acid C_{TOT}** (mmole/L MEA)	4.5	8.9	13.4	17.8	/
Bread, propionic acid					
C_{TOT} (mg/kg)	497	990	/	1967	2901
C_{TOT} (mmole/kg)	6.7	13.4		26.5	39.2
C_{TOT} (mmole/L H_2O)	14.7	29.4		58.3	86.1
$C_{HA,standard}$ (mmole/L H_2O)	3.5	11.2		22.2	37.6
$C_{HA,model\ 1}$ (mmole/L H_2O)	15.3	49.4		98.2	144.8
$C_{HA,model\ 2}$ (mmole/L H_2O)	3.3	10.8		21.5	31.6
Cake (pH 7.2), sorbic acid					
C_{TOT} (mg/kg)	493	1004	1488	1988	/
C_{TOT} (mmole/kg)	4.4	9.0	13.3	17.7	
C_{TOT} (mmole/L H_2O)	15.2	30.9	45.8	61.2	
$C_{HA,standard}$ (mmole/L H_2O)	0.1	0.1	0.1	0.1	
$C_{HA,model\ 1}$ (mmole/L H_2O)	0.2	0.4	0.4	0.5	
$C_{HA,model\ 2}$ (mmole/L H_2O)	0.1	0.1	0.1	0.1	
Lemon cake (pH 5.2), sorbic acid					
C_{TOT} (mg/kg)	475	951	1429	1897	/
C_{TOT} (mmole/kg)	4.2	8.5	12.7	16.9	
C_{TOT} (mmole/L H_2O)	12.3	24.6	37.0	49.1	
$C_{HA,standard}$ (mmole/L H_2O)	3.3	7.7	9.9	13.1	
$C_{HA,model\ 1}$ (mmole/L H_2O)	11.3	25.9	34.0	45.1	
$C_{HA,model\ 2}$ (mmole/L H_2O)	2.3	5.1	6.8	9.1	

/: missing data. C_{TOT} (mmole /L MEA), $C_{HA,standard}$ (mmole HA/L H_2O in bread/cake, determined by the standard Henderson-Hasselbalch equation); $C_{HA,model\ 1}$ (mmole /L H_2O) in bread/cake, determined by the modified Henderson-Hasselbalch equation model 1); $C_{HA,model\ 2}$ (mmole/L H_2O) in bread/cake, determined by the modified Henderson-Hasselbalch equation model 2).

4.6. *In-vitro* predictive Growth/No-Growth models

The main goal of this research was to evaluate the relationship between propionic acid and sorbic acid and six different bread molds. Other growth conditions were tested, and different results of Growth/No-Growth were obtained.

The following Growth/No-Growth models were fitted in SPSS Statistics 27 (SPSS, Inc., Chicago, IL, USA) and plotted by Matlab 9.3 (The Mathworks, Inc., Natick, MA, USA).

In the *in-vitro* Growth/No-Growth tests, propionic acid and sorbic acid were tested as preservatives in the cultured medium MEA.

The concentrations tested for propionic and sorbic acid in MEA were 0, 500, 1000, 1500, and 2000 mg/kg MEA.

Table 13 Calculated concentrations of propionic and sorbic acid in the in-vitro experiment. Concentration ranges of 0-2000 mg/kg MEA were used, and the C_{TOT} (mmole/L MEA) was calculated.

Theoretical concentration (C_{TOT}: mg/kg MEA)	**0**	**500**	**1000**	**1500**	**2000**
***In vitro*-propionic acid** C_{TOT} (mmole/L MEA)	0	6.7	13.5	20.2	26.9
***In vitro*-sorbic acid** C_{TOT} (mmole/L MEA)	0	4.5	8.9	13.4	17.8

4.7. Minimal inhibitory concentration (MIC) of propionic acid

Minimal inhibitory concentration is the minimum concentration of a component that prevents the visible growth of a microorganism. In our case, minimal inhibitory concentration is the lowest concentration of propionic acid or sorbic acid that prevents the visible growth of molds on MEA, bread, or cakes. In table 15, minimal inhibitory concentration for propionic acid in MEA, in function of water activity and pH is shown against six molds: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR).

Table 14 Minimal inhibitory concentration in in-vitro (MIC_{TOT} (mmole/L MEA) of propionic acid in function of water activity and pH. Six molds were considered: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Cladosporium ramotenellum* (CR), *Aspergillus ruber* (AR), *Penicillium brevicompactum* (PB), *Penicillium* roqueforti (PR)

		***In vitro* (MEA)**					
		AN	**AM**	**AR**	**CR**	**PB**	**PR**
Water activity	**pH**	**Propionic acid**					
0.93	**5.0**	26.9	18	20	> 26.9	18	26
	5.5	> 26.9	> 26.9	> 26.9	> 26.9	24	> 26.9
	6.0	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9
0.95	**5.0**	> 26.9	4	22	> 26.9	26	> 26.9
	5.5	> 26.9	26.9	> 26.9	> 26.9	> 26.9	> 26.9
	6.0	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9
0.97	**5.0**	> 26.9	NG	24	> 26.9	> 26.9	> 26.9
	5.5	> 26.9	NG	> 26.9	> 26.9	> 26.9	> 26.9
	6.0	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9	> 26.9

NG: No Growth observed

The growth/no growth plots presented in this study show the probability of growth (90%, 50% and 10%, respectively p = 0.9, 0.5 and 0.1). Minimal inhibitory concentrations (MIC) are based on the probability of 90% of no growth (p=0.1) during shelf life. Results of table 15 are shown in Figures 29, 30, 31, 32, 33, and 34.

4.7.1. The impact of water activity, pH, and propionic acid in in-vitro Growth/No-Growth predicted models

In this study, not only the pH was considered, but also water activity. Growth/no-growth curves on MEA of six molds (*Aspergillus niger* (AN), *Cladosporium ramotenellum* (CR), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR)) are described subsequently on Figure 29, 30, 31, 32, 33, 34.

Figure 29 shows that for *Aspergillus niger* at water activity 0.93, pH 5, and C_{TOT} 26.9 mmol/L, shelf life could be more than thirty days. In all other combinations of water activity and pH, MIC_{TOT} of *Aspergillus niger* is higher than 26.9 mmol/L (Table 15).

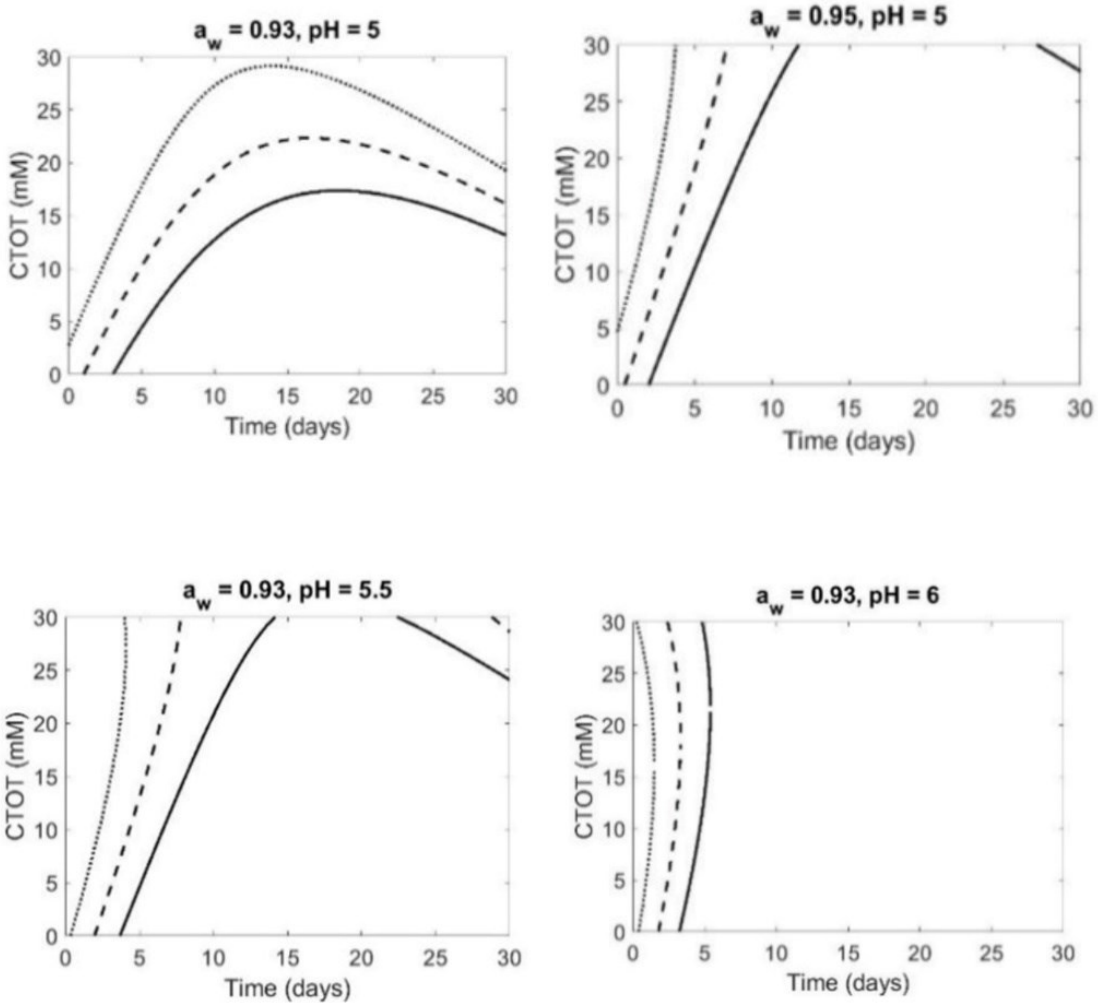

Figure 29 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid on *Aspergillus niger*. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line).

Aspergillus montevidensis (Figure 30) appears to be particularly sensitive to changes in water activity in conjunction with the antifungal action of propionic acid. At aw 0.97, no growth of *Aspergillus montevidensis* was observed. At aw 0.95, the growth of *Aspergillus montevidensis* was significantly reduced. As is shown in Table 15, the MIC_{TOT} at water activity 0.93 is 18 mmole/L, while the MIC_{TOT} at water activity 0.95 and pH 5 is 4 mmole/L, but at pH 5.5 and water activity, 0.95 MIC_{TOT} results are 26.9 mmole/L. It is known how some molds (such as *Eurotium*) can change the pH of the substrate; thus, this pH change can result in a different effect of the preservative.

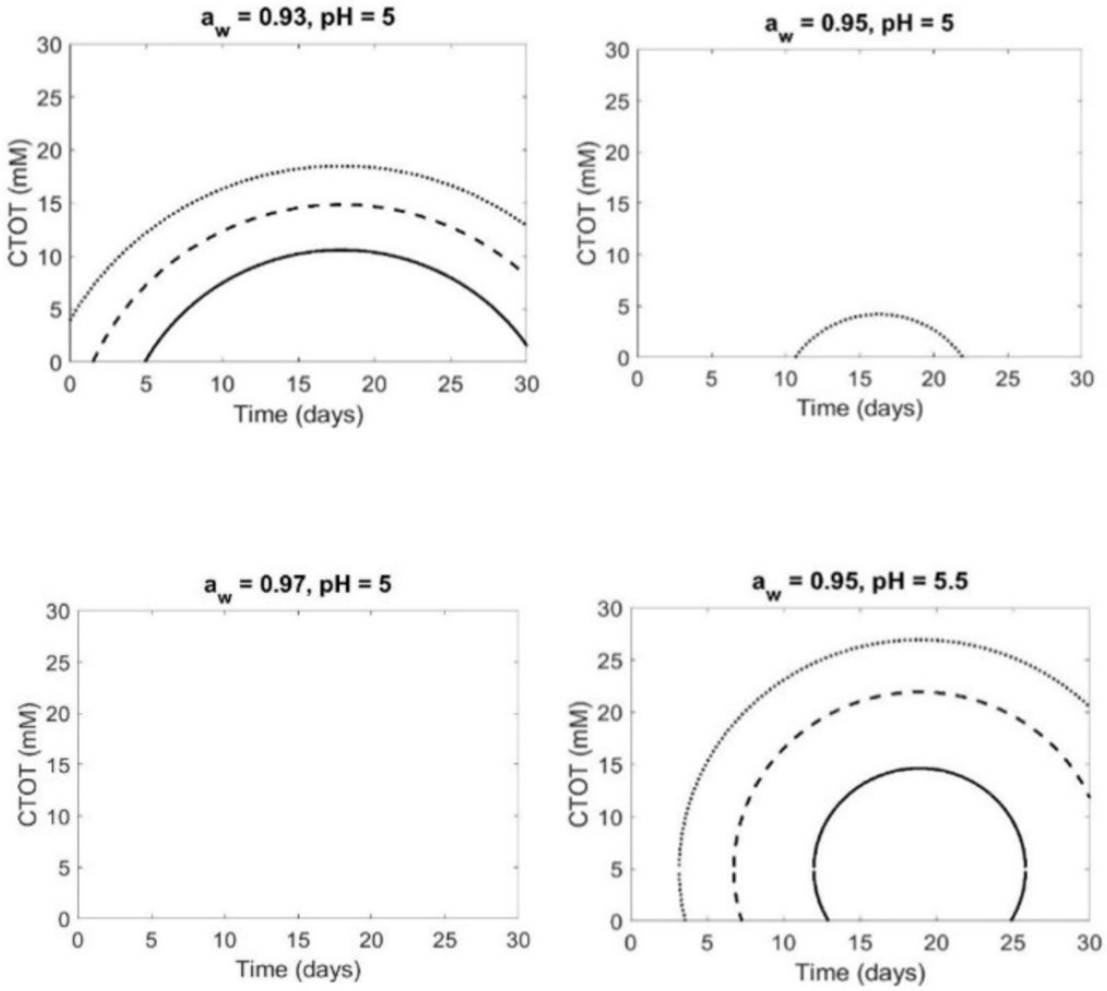

Figure 30 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line). *Aspergillus montevidensis* is considered.

Aspergillus ruber (Figure 31) showed almost no sensibility to variation of pH and water activity. At pH 5.5 and pH 6 (for all three water activities), MIC_{TOT} exceeded the maximum of 26.9 mmole/L in MEA (2000 mg/kg MEA), but at pH 5, for all three water activities, MIC_{TOT} was lower than 26.9 mmole/L in MEA (Table 15). With values of 20, 22, and 24 mmole/L in MEA, respectively.

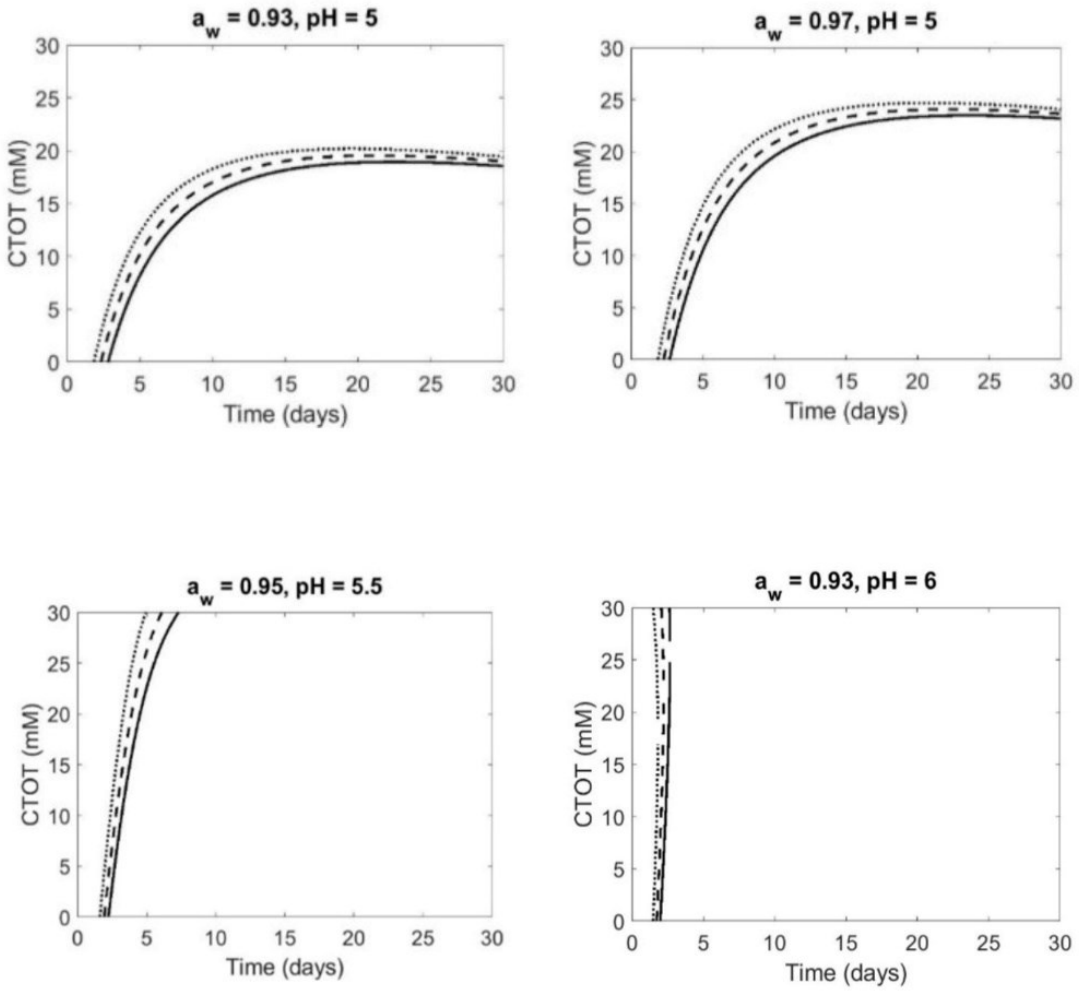

Figure 31 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line). *Aspergillus ruber* is considered.

Cladosporium ramotenellum (Figure 32) showed no difference in growth by changing water activity from 0.93 to 0.95. This result is almost the same with water activity at 0.97. Variations in pH show no changes in growth/non-growth ratios. In any case, MIC_{TOT} (Table 15) is higher than C_{TOT} of 26.9 mmole/L of MEA.

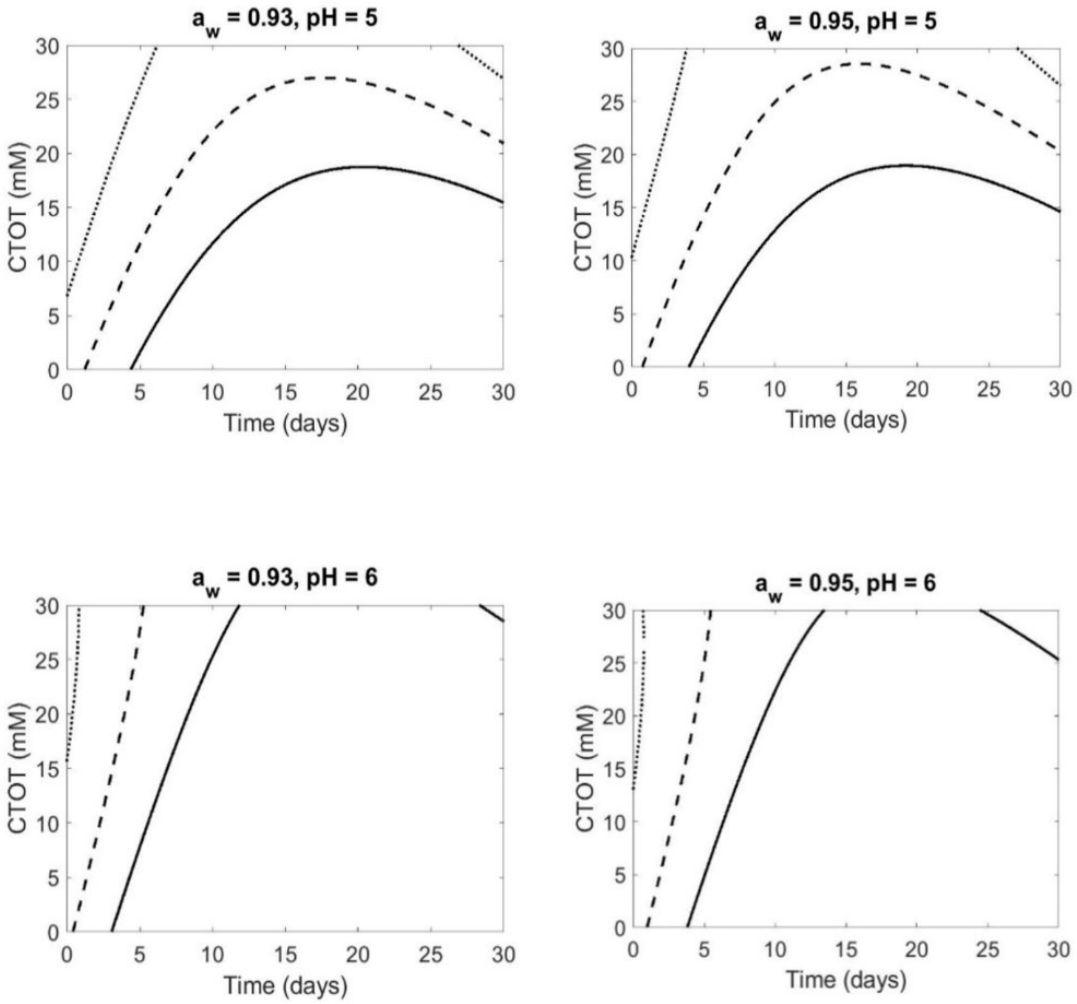

Figure 32 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line). *Cladosporium ramotenellum* is considered.

Penicillium brevicompactum (Figure 33) is sensitive to water activity variations. Growth of *Penicillium brevicompactum* results lower at lower water activity; MIC_{TOT} (Table 15) at pH 5 and water activity 0.95 is 26 mmole/L of MEA, and at water activity 0.93 is 18 mmole/L of MEA. *Penicillium brevicompactum* is also partially sensible to pH variations: at water activity 0.93 and pH 5, MIC_{TOT} is 18 mmole/L of MEA. At the same water activity but higher pH (5.5), MIC_{TOT} is 24 mmole/L of MEA; at pH 6, MIC_{TOT} is higher than 26.9 mmole/L of MEA.

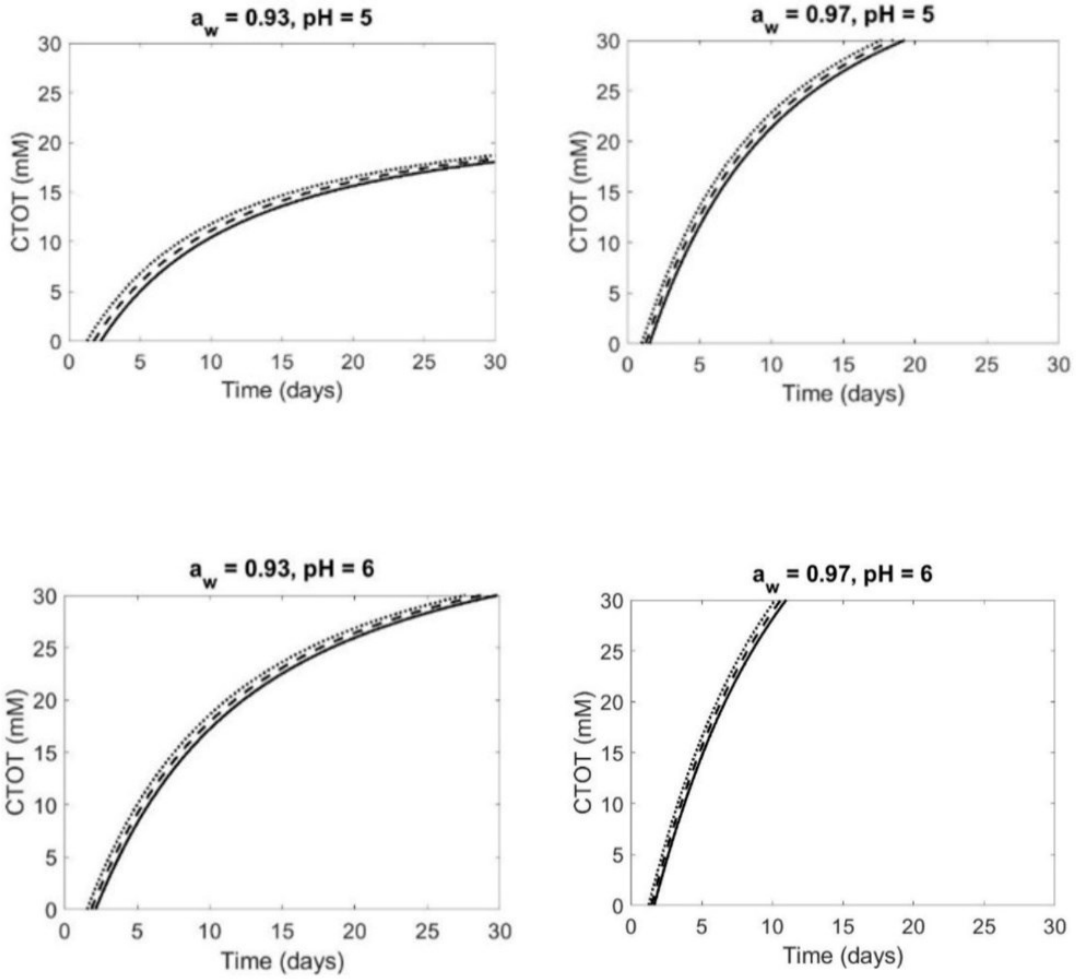

Figure 33 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line). *Penicillium brevicompactum* is considered.

Penicillium roqueforti (Figure 34) has almost no influence on growth with different water activity and pH. No variation of MIC_{TOT} was observed (Table 15). In any case (except pH5 and water activity 0.93), MIC_{TOT} is higher than 26.9 mmole/L of MEA.

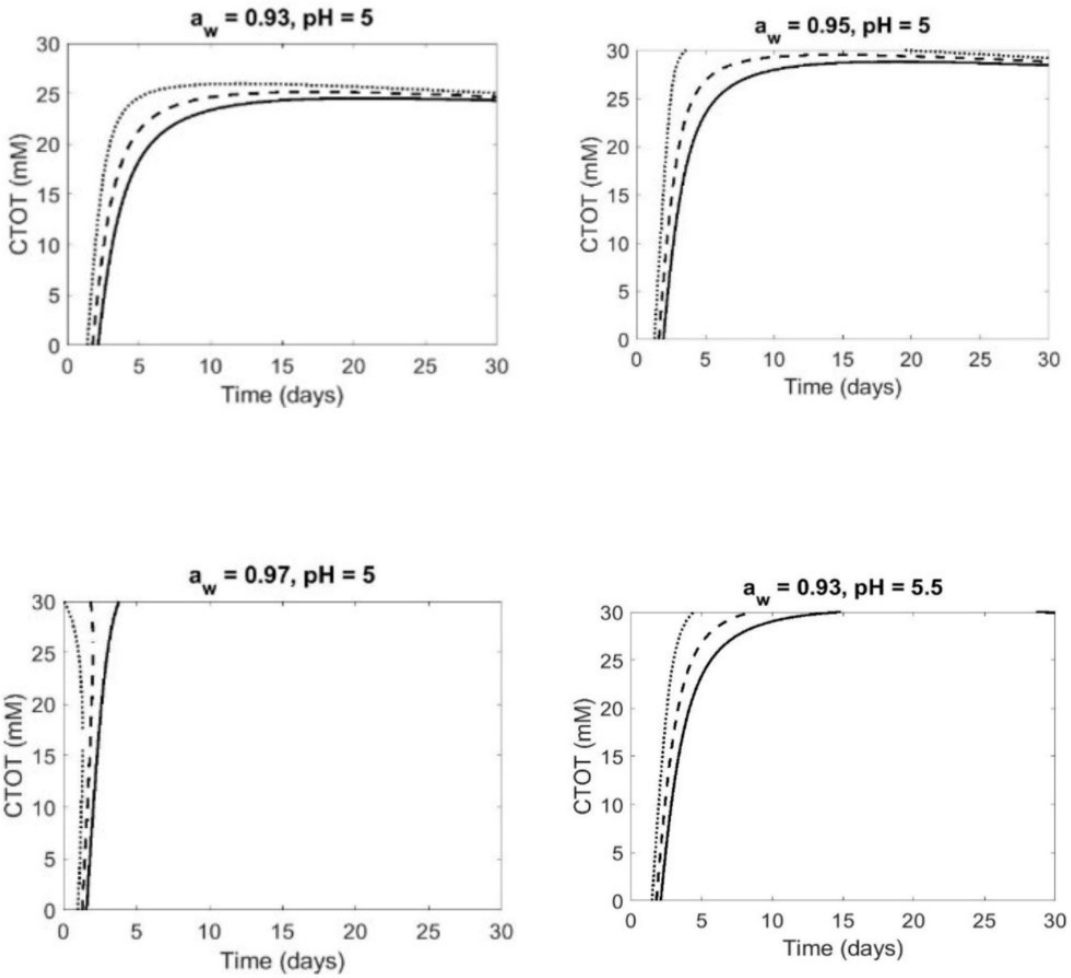

Figure 34 Effect of water activity (0.93, 0.95, and 0.97) and pH (5, 5.5, and 6) on the Growth/No-Growth limits of propionic acid. Lines represent model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), p = 0.1 (dotted line). *Penicillium roqueforti* is considered.

4.8. Minimal inhibitory concentration and impact of water activity, pH, and sorbic acid in *in-vitro* Growth/No-Growth predicted models

In table 16, minimal inhibitory concentration for sorbic acid, in function of water activity and pH is shown for *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR).

Table 15 Minimal inhibitory concentration in in-vitro (MIC_{TOT} (mmole/L MEA) of sorbic acid in function of water activity and pH. Six molds were considered: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Cladosporium ramotenellum* (CR), *Aspergillus ruber* (AR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR)

		***In vitro* (MEA)**					
		AN	**AM**	**AR**	**CR**	**PB**	**PR**
water activity	**pH**	**Sorbic acid**					
0.93	**5.0**	7	3	4	4	5	5
	5.5	13	6.5	5	3	8	8
0.95	**5.0**	7	1	3.5	5.5	5	5
	5.5	13	5	5	4	8	8
0.97	**5.0**	7	NG	2.5	6.5	6	5
	5.5	13	NG	5	5	10	8

NG: No Growth observed

As shown in table 14, *in vitro*-sorbic acid C_{TOT} (mmole/L MEA) used were 0 (0 mg/kg MEA), 4.5 (500 mg/kg MEA), 8.9 (1000 mg/kg MEA), 13.4 (1500 mg/kg MEA), 17.8 (2000 mg/kg MEA).

From the data in Table 16, sorbic acid's antifungal effect is more remarkable than propionic acid's antifungal effect in MEA.

Antifungal activity of sorbic acid was not related to the water activity in *Aspergillus niger* and *Penicillium roqueforti*: at pH 5, MIC_{TOT} (mmole/L MEA) for *Aspergillus niger* was 7 mmole/L MEA for water activity 0.93, 0.95, and water activity 0.97. MIC_{TOT} of *Penicillium roqueforti*, at pH 5 and water activity 0.93, 0.95, and 0.97 was 5 mmole/L MEA for all three water activities. Considering pH 5.5, the MIC_{TOT} of *Aspergillus niger* and *Penicillium roqueforti* was 13 and 8 mmole/L MEA, respectively.

Also, for *Penicillium brevicompactum*, the MIC_{TOT} remained the same even at different water activities considering pH 5.

Cladosporium ramotenellum, on the other hand, as water activity increases (both at pH 5 and pH 5.5), MIC_{TOT} concentration also increases. At pH 5, MIC_{TOT} turns out to be 4, 5.5, and 6.5, respectively (for water activity 0.93, 0.95, and 0.97, respectively). At pH 5.5, on the other hand, we have MIC_{TOT} values of 3, 4, and 5 (water activity 0.93, 0.95, and 0.97, respectively) mmole/L MEA. If pH increases, considering propionic or sorbic acid, MIC_{TOT} also increases. The only case where this event does not occur is with *Cladosporium ramotenellum*, which, as pH increases from 5 to 5.5 (sorbic acid), requires a lower MIC_{TOT}. At water activity 0.93, *Cladosporium ramotenellum* goes from MIC_{TOT} 4 to 3 mmole/L MEA, at water activity 0.95, it goes from 5.5 to 4 mmole/L MEA, and at water activity 0.97, it goes from 6.5 to 5 mmole/L MEA.

Aspergillus ruber, at pH 5.5, has the same MIC_{TOT} (5 mmole/L MEA) for the three water activities. At pH 5, on the other hand, MIC_{TOT} for the three water activities decreases from 4 to 3.5, going down to 2.5 mmole/L MEA.

Aspergillus montevidensis, at pH 5, shows a decrease in the concentration of MIC_{TOT}, from 3 to 1 mmole/L MEA for water activity 0.93 and 0.95, respectively. If we consider pH 5.5, the trend turns out to be the opposite, from 6.5 to 5 mmole/L MEA for water activity 0.93 and 0.95, respectively. Considering pH 5 or 5.5 and a water activity of 0.97, *Aspergillus montevidensis* does not grow.

4.9. Bread and cake validation, Shelf Life

In the Growth/No Growth models, six molds, *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR) were considered

The amount of propionic acid for bread and sorbic acid for cakes is shown in Table 17. 1500 mg/kg (theoretical) for bread and 3000 mg/kg (theoretical) for cake and lemon cake are not represented.

Table 16 Amounts (C_{TOT} mg/kg) of propionic acid for bread and sorbic acid used for cakes,

Theoretical C_{TOT} mg/kg	**0**	**500**	**1000**	**1500**	**2000**	**3000**
Bread C_{TOT} mg/kg	0	497	990	/	1967	2901
Cake C_{TOT} mg/kg	0	493	1004	1488	1988	/
Lemon cake C_{TOT} mg/kg	0	475	951	1429	1897	/

/: Experiments not performed at these C_{TOT}

C_{TOT} was expressed by mmole/L for MEA, but this is impossible with bread, cake, and lemon cake. C_{TOT} for bread and cakes were shown as mmole/L of H_2O (Table 13).

4.9.1. Shelf life of bread

Bread's shelf life (in days) in function of different concentrations of propionic acid is shown in Figure 35.

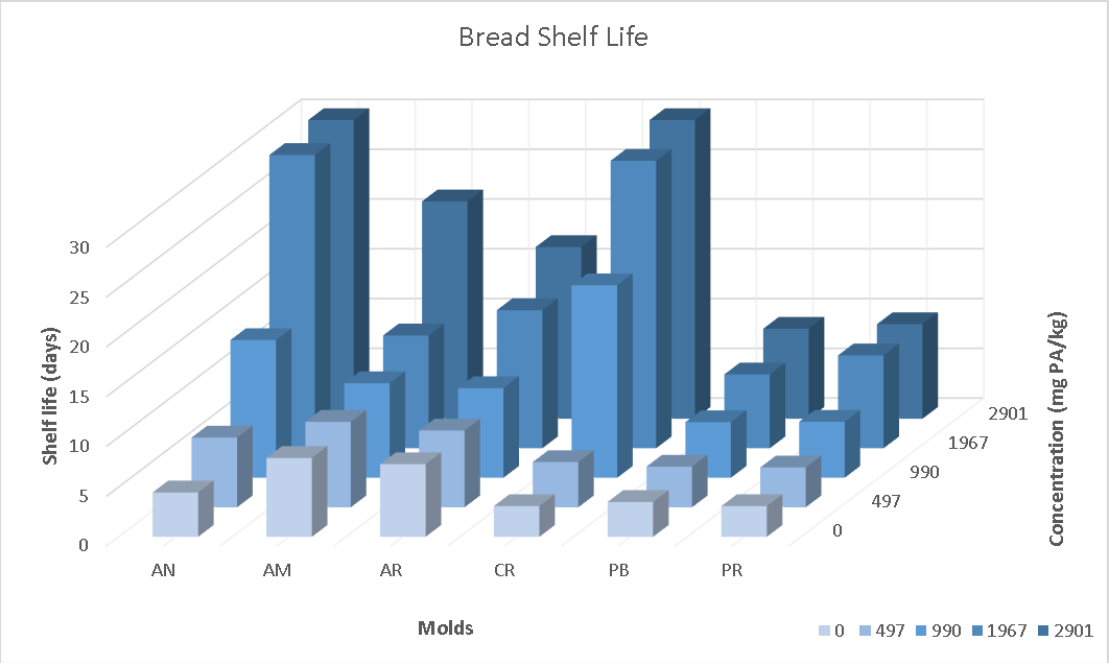

Figure 35 Mean bread's shelf life (in days) in function of different concentrations of propionic acid (mg /kg). Bread was inoculated with six molds: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR).

Table 18 shows the shelf life of bread slices at different concentrations of propionic acid and inoculated with the six molds.

Table 17 Shelf life (mean and standard deviation) of bread slices inoculated with *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR). Concentrations of propionic acid (0, 497,990, 1967, 2901) are expressed in mg sorbic acid/kg.

	0 mg propionic acid/kg	**497 mg propionic acid/kg**	**990 mg propionic acid/kg**	**1967 mg propionic acid/kg**	**2901 mg propionic acid/kg**
AN	4,46 ± 0,51	7,00 ± 1,29	13,83 ± 6,31	29,46 ± 2,65	30,00 ± 0,00
AM	7,92 ± 1,25	8,58 ± 0,50	9,50 ± 0,51	11,33 ± 1,00	21,83 ± 7,71
AR	7,29 ± 0,69	7,71 ± 0,81	9,00 ± 1,50	13,88 ± 7,58	17,25 ± 8,47
CR	3,08 ± 0,28	4,54 ± 0,51	19,33 ± 10,0	28,88 ± 3,96	30,00 ± 0,00
PB	3,50 ± 0,51	4,08 ± 0,97	5,58 ± 0,72	7,42 ± 0,68	9,04 ± 1,04
PR	3,08 ± 0,28	4,00 ± 0,00	5,63 ± 0,71	9,33 ± 1,81	9,50 ± 1,62

Penicillium brevicompactum, Cladosporium ramotenellum, and *Penicillium roqueforti*, with a shelf life 3.50 ± 0.51 days, 3,08 ± 0,28, and 3.08 ± 0.28 days respectively, grow very fast on bread with no preservatives. As shown in Figure 34, *Penicillium brevicompactum* and *Penicillium roqueforti* are not only very fast-growing on bread but also are very resistant to propionic acid.

These results match Brazil's Garcia et al., 2019's study. *Penicillium roqueforti* was found in all types of the analysed moldy bread in this study. *Penicillium roqueforti,* as reported by Garcia et al. (2019), is the main specie responsible for bread spoilage and was recovered in all the raw materials, in the air of processing areas, and bread industry.

Since the amount of fat in the bread is practically insignificant (± 0.4% oil in the flour), the calculation of $C_{HA,\ standard}$ and $C_{HA,\ model\ 2}$ (obtained through the standard Henderson-Hesselbalch equation and the one proposed by Wemmenhove et al. (2018), respectively) should be essentially the same as found in this thesis (Table 13).

With high propionic acid concentrations, Aspergillus niger and Cladosporium ramotenellum are partially inhibited. With 3000 ppm of propionic acid, the shelf life of the bread was found to be 30 days, so the visible growth of the two molds is zero. At propionic acid concentrations of 500ppm, the growth of *Aspergillus niger* and especially *Cladosporium ramotenellum* was still remarkable; in fact, the shelf life of the bread was 7.00 ± 1.29 days and 4.54 ± 0.51 days, respectively. By increasing the propionic acid concentration to 1000ppm (990 ppm), the average shelf life of both breads significantly increased (13.83 ± 6.31 days for *Aspergillus niger* and 19.33 ± 10.05 days for *Cladosporium ramotenellum*).

Aspergillus ruber and *Aspergillus montevidensis* grow relatively slowly without propionic acid on bread. As the amount of propionic acid increased, the growth of both molds decreased less: the shelf life of bread slices inoculated with *Aspergillus ruber* and in the absence of propionic acid was 7.29 + 0.69; at 500 ppm propionic acid, the shelf life became 7.71 + 0.81 days, and at 1000ppm the shelf life became 9.00 + 1.5 days. The same behavior was observed for *Aspergillus montevidensis*.

By increasing the propionic acid concentration by twice as much (reaching 2000ppm), the shelf life increased by only two days in both cases.

Penicillium brevicompactum and *Penicillium roqueforti* could be controlled with maximum amounts of propionic acid for about ten days, after which they show up. Since *Penicillium brevicompactum* and *Penicillium roqueforti* were always present on bread, the shelf life of bread with propionic acid (within the legal limits) rarely exceeds ten days.

At 3000 ppm, from most to least resistant molds in bread: *Penicillium roqueforti*, *Penicillium brevicompactum*, *Aspergillus ruber*, *Aspergillus montevidensis*, *Cladosporium ramotenellum*, and *Aspergillus niger*.

4.9.2. Shelf life of cake (pH 7.2)

The cakes and bread were inoculated with six molds: *Aspergillus niger*, *Aspergillus montevidensis*, *Aspergillus ruber*, *Cladosporium ramotenellum*, *Penicillium brevicompactum*, *Penicillium roqueforti*. Sorbic acid was used as a preservative. The shelf-life bar chart of the cake (pH 7.2) is shown in Figure 36.

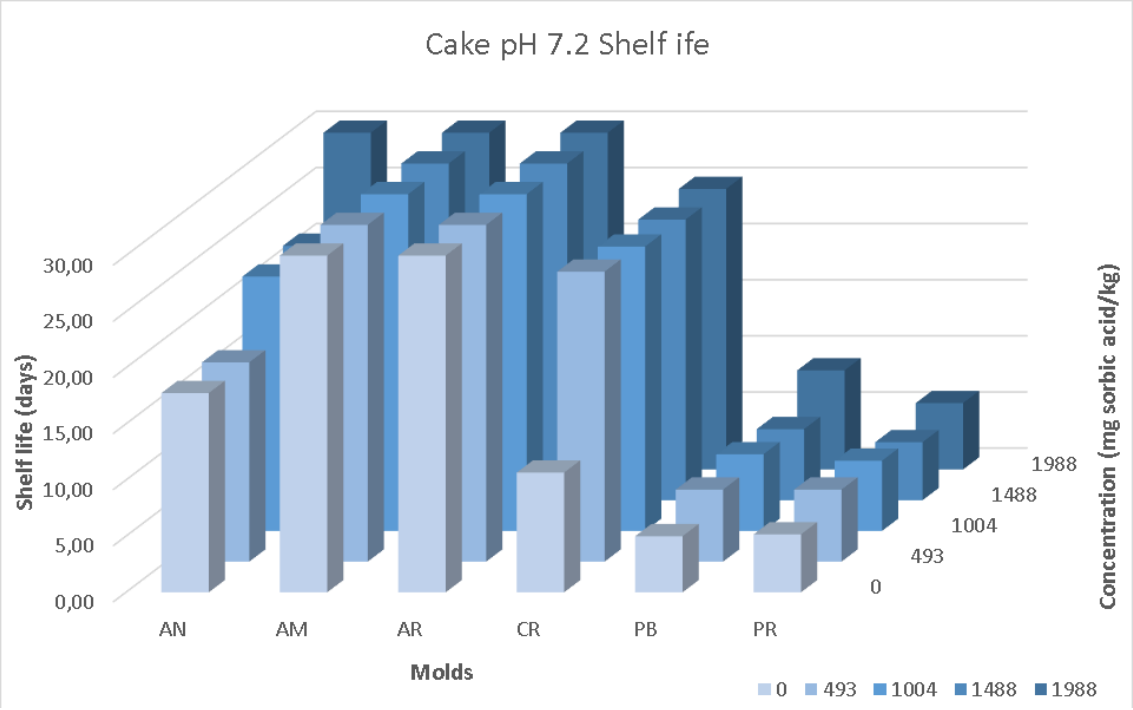

Figure 36 Bar chart of mean cake's (pH 7.2) shelf life (in days), in function of different concentrations of sorbic acid (mg /kg). Cake was inoculated with six molds: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR).

The mean and standard deviation of the cake's shelf life (pH 7.2) are shown in Table 19.

Table 18 Shelf life (mean and standard deviation) of cake slices (pH 7.2), inoculated with *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR). Concentrations of sorbic acid (0, 493,1004, 1488, 1988) are expressed in mg sorbic acid/kg.

	0 mg sorbic acid/kg	**493 mg sorbic acid/kg**	**1004 mg sorbic acid/kg**	**1488 mg sorbic acid/kg**	**1988 mg sorbic acid/kg**
AN	17,75 ± 2,6	17,75 ± 2,6	22,67 ± 5,52	22,67 ± 5,42	30,00 ± 0,00
AM	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00
AR	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00
CR	10,67 ± 2,61	25,83 ± 3,95	25,33 ± 5,58	25,00 ± 4,47	25,00 ± 4,47
PB	5,00 ± 0,00	6,42 ± 0,51	6,83 ± 0,39	6,33 ± 0,65	8,83 ± 0,58
PR	5,17 ± 0,39	6,42 ± 0,67	6,25 ± 0,87	5,17 ± 0,39	5,92 ± 0,67

Aspergillus montevidensis and *Aspergillus ruber* did not grow in any inoculated cake slices. The shelf life of the cake slices for each sorbic acid concentration used (0 to 1988 mg sorbic acid/kg) was 30 days.

Regarding the cake slices inoculated with *Aspergillus niger*, an increase in the shelf life of the slices was found. The shelf life of the cake slices increased from 17.75 + 2.6 days (0 and 493 mg sorbic acid/kg) to 22.67 ± 5.52 days (1004 and 1488 mg sorbic acid/kg) to 30 days shelf life at a preservative concentration of 1988 mg sorbic acid/kg.

As for cake slices inoculated with *Cladosporium ramotenellum*, the shelf life of cake slices increased from 10.67 ± 2.61 days (0 mg sorbic acid/kg) to about 25 days for the remaining concentrations of sorbic acid used.

Sorbic acid did not affect *Penicillium brevicompactum* and *Penicillium roqueforti* in the analyzed conditions.

4.9.3. Shelf life of lemon cake (pH 5.2)

The shelf-life bar chart of the lemon cake (pH 5.2) is shown in Figure 37.

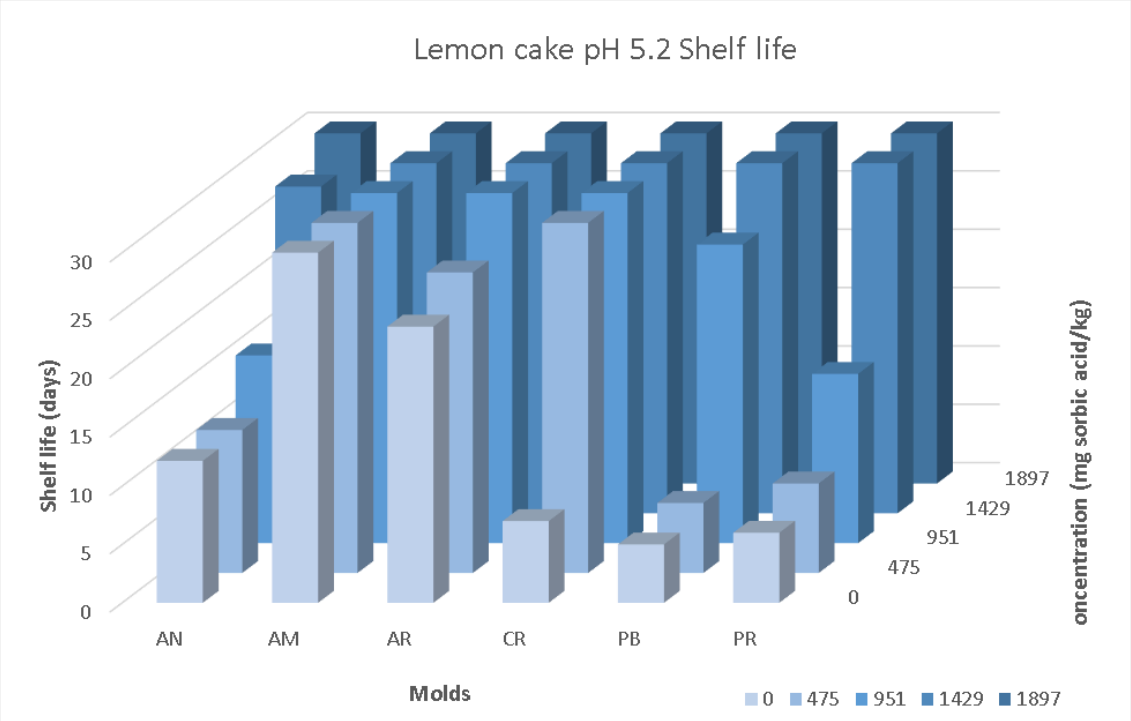

Figure 37 Bar chart of mean lemon cake's (pH 5.2) shelf life (in days), in function of different concentrations of sorbic acid (mg /kg). Lemon cake was inoculated with six molds: *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR).

Table 20 shows the shelf life of lemon cake slices (pH 5.2), inoculated with the six molds and containing different concentrations of sorbic acid.

Table 19 Shelf life (mean and standard deviation) of lemon cake slices (pH 5.2), inoculated with *Aspergillus niger* (AN), *Aspergillus montevidensis* (AM), *Aspergillus ruber* (AR), *Cladosporium ramotenellum* (CR), *Penicillium brevicompactum* (PB), *Penicillium roqueforti* (PR). Concentrations of sorbic acid (0, 493,1004, 1488, 1988) are expressed in mg sorbic acid /kg.

	0 mg sorbic acid/kg	**475 mg sorbic acid/kg**	**951 mg sorbic acid/kg**	**1429 mg sorbic acid/kg**	**1897 mg sorbic acid/kg**
AN	12,17 ± 1,34	12,25 ± 2,7	16,08 ± 2,07	28,00 ± 3,62	30,00 ± 0,00
AM	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00
AR	23,67 ± 5,69	25,75 ± 4,58	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00
CR	7,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00	30,00 ± 0,00
PB	5,00 ± 0,00	6,00 ± 0,00	25,58 ± 8,1	30,00 ± 0,00	30,00 ± 0,00
PR	6,00 ± 0,00	7,67 ± 0,78	14,50 ± 0,9	30,00 ± 0,00	30,00 ± 0,00

In lemon cake, the concentration of 1897 mg sorbic acid/kg prevented the growth of molds. All the inoculated slices showed no mold growth for thirty days (at the highest sorbic acid concentration).

Aspergillus montevidensis growth did not occur in the lemon cake at any sorbic acid concentration. The shelf life of these cake slices was always at least 30 days.

In contrast to the cake at pH 7.2, in the lemon cake at pH 5.2, the growth of *Aspergillus ruber* occurred at concentrations of 0 mg sorbic acid/kg and 475 mg sorbic acid/kg. The two shelf lives were 23.67 $\pm$ 5.69 and 25.75 $\pm$ 4.58, respectively. By further increasing the sorbic acid concentration, the shelf life was 30 days.

As for the slices of cake inoculated with *Cladosporium ramotenellum*, in both cakes (pH 7.2 and lemon cake at pH 5.2), the addition of sorbic acid increased the shelf life. In the case of the lemon cake, however, the addition of sorbic acid immediately prevented mold growth, increasing the shelf life from 7 days to 30 days.

The addition of lemon also seems to have had a positive effect on the shelf life of the cake slices inoculated with *Penicillium brevicompactum* and *Penicillium roqueforti*, especially at a concentration of at least 951 mg sorbic acid/kg, the antifungal effect was found to be significant, bringing the shelf life of lemon cake slices to 25.58 $\pm$ 8.1 and 14.50 $\pm$ 0.9 days, respectively, for *Penicillium brevicompactum*, *Penicillium roqueforti* inoculum. Further increase in the sorbic acid concentration caused an increase in the shelf life (in both cases) that reached thirty days.

It is possible to rank the molds tested for sorbic acid resistance in pH 5.2 cake (lemon cake) from the most resistant mold to the least resistant as follows *Aspergillus niger*, *Penicillium roqueforti*, *Penicillium brevicompactum*, *Aspergillus ruber*, *Cladosporium ramotenellum* (*Aspergillus montevidensis* is not considered because of no growth at 0 ppm sorbic acid).

The antifungal action exerted by sorbic acid in the pH 7.2 cake was unsatisfactory. For the slices inoculated with *Penicillium brevicompactum* and *Penicillium roqueforti*, the cake's shelf life was very low for each concentration of sorbic acid used. The reason could be the high dissociation of the weak acid at neutral pH; high dissociation is translated into a lower anti-mycotic effect. The dissociation was 0.1 mmol of non-dissociated sorbic acid/L H_2O in the pH 7.2 cake, compared with 13.1 mmol of non-

dissociated sorbic acid/L H_2O in the lemon cake (both concentrations calculated by model 2 of the Henderson-Hasselbalch equation). Regarding slices inoculated with *Aspergillus montevidensis* and *Aspergillus ruber*, the shelf life was always 30 days, regardless of the amount of sorbic acid used.

5. Conclusions

From the data in Table 16, sorbic acid's antifungal effect is more remarkable than propionic acid's antifungal effect in MEA.

The main differences between the *in-vitro* (MEA) and *in-vivo* (bread and cakes) experiments can be attributed to the different storage temperatures. Petri dishes were stored at higher temperatures (26°C) than the storage temperature of bread and cake slices (about 20°C). For example, *Aspergillus niger* and *Cladosporium ramotenellum*, which have an optimal growth temperature greater than 20°C, grew better *in-vitro* (Passamani et al., 2014).

For *Aspergillus ruber*, *Penicillium brevicompactum,* and *Penicillium roqueforti*, the antifungal action expressed by propionic acid was found to be similar both *in-vitro* and in bread validation. In the latter, *Penicillium brevicompactum* and *Penicillium roqueforti*, even when subjected to the highest dose of propionic acid, developed within the first 10 days of shelf life (9.04 $\pm$ 1.04 and 9.50 $\pm$ 1.62 days, respectively), demonstrating how these two microorganisms are among the top bread degradation problems (Garcia et al. (2019)).

In bread, *Aspergillus montevidensis*, up to 2000ppm of propionic acid, is quite resistant (11.33 $\pm$ 1.00 days), bringing the amount of propionic acid to 3000ppm, the resistance decreases (21.83 $\pm$ 7.71 days).

Molds are acid-tolerant microorganisms. The shelf life of cake slices at pH 5.2, inoculated with *Aspergillus niger* and *Cladosporium ramotenellum* and especially *Penicillium brevicompactum,* and *Penicillium roqueforti*, was found to be much longer than the shelf life of cake slices at pH 7.2, inoculated with the same molds. This means that the antimicrobial effect found in the cake at pH 5.2 compared to the cake at pH 7.2 is not to be attributed directly to the pH of the matrix, but rather, pH plays an indirect effect; by lowering the pH of the medium it increases the undissociated acid component (which is the main antifungal component).

Three Henderson-Hesselbalch equations have been provided to measure the undissociated component of an acid in a matrix. The different amounts of undissociated acid obtained from the three Henderson-Hesselbalch equations in the in-vitro experiments were very similar.

The Henderson-Hesselbalch equation model 1, in-vivo, on the one hand, often gave different results than expected; for example, values of undissociated acid were greater than values of total acid (Table 13). Henderson-Hesselbalch equation, standard, and model 2, on the other hand, were more reliable, providing very similar results for non (or low) fat matrices, such as MEA or bread. For fat components, the Henderson-Hesselbalch model 2 equation provided the best results. This result is in line with Wemmenhove et al., who proposed this equation in cheese, a very fat food.

For low-fat products, such as bread, it can be concluded that kp (partitioning coefficient of the acid) does not affect the final concentration of undissociated acid. Therefore, the partitioning coefficient of the acid for low-fat foods may not be considered. In this case, the standard Henderson-Hasselbalch equation can be used. In contrast, for foods with medium to high-fat content, such as cakes, the acid partition coefficient must be considered to obtain plausible C_{HA} values. To do this, model 2 of the Henderson-Hasselbalch equation must be considered. It will be necessary for future studies to investigate better the breakdown and dissociation behavior of weak acids in fat-rich foods; to be able to use these preservatives most appropriately and amount in the various food matrices, being able to keep mold development and mycotoxin production under control.

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