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Recent trends in detecting, controlling and detoxifying of patulin mycotoxin using biotechnology methods

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Abstract

Patulin (PAT) is a mycotoxin that can contaminate many foods and especially fruits and fruit based products. Therefore, accurate and effective testing is necessary to enable producers to comply with regulations and promote food safety. Traditional approaches involving the use of chemical compounds or physical treatments in food have provided practical methods that have been used to date. However, growing concerns about environmental and health problems associated with these approaches call for new alternatives. In contrast, recent advances in biotechnology have revolutionized the understanding of living organisms and brought more effective biological tools. This review, therefore, focuses on the study of biotechnology approaches for the detection, control and mitigation of PAT in food. Future aspects of biotechnology development to overcome the food safety problem posed by PAT were also examined. We find that biotechnology advances offer novel, more effective and environmentally friendly approaches for the control and elimination of PAT in food compared to traditional methods. Biosensors represent the future of PAT detection and use biological tools such as aptamer, enzyme, and antibody. PAT prevention strategies include microbial biocontrol, the use of antifungal biomolecules and the use of microorganisms in combination with antifungal molecules. PAT detoxification aims at the breakdown and removal of PAT in food by using enzymes, microorganisms and various adsorbent biopolymers. Finally, biotechnology advances will be dependent on the understanding of fundamental biology of living organisms regarding PAT synthesis and resistance mechanisms.

Keywords: Patulin, Bio-adsorbents, Biocontrol, Biosensors, Microbes, Enzymes, Biopolymers.

1 INTRODUCTION

Patulin (PAT; 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin that is synthesized by many molds including some species of *Byssochlamys*, *Aspergillus*, and *Penicillium* (Zheng et al., 2018). PAT has been detected in numerous countries and is known to occur worldwide (Iqbal, Malik, Asi, Selamat, & Malik, 2018; Saleh & Goktepe, 2019). PAT has been identified in a variety of agricultural crops, such as tomatoes, peppers, various fruits (red fruits, pears, grapes, apples, longans, figs), seafood, cereals (ground cereals, rice). The high water and sugar content in fruits promotes the occurrence of PAT in fruits (Saleh & Goktepe, 2019; Iqbal et al., 2018; Wright, 2015; Zhong, Carere, Lu, Lu, & Zhou, 2018; Solairaj, Legrand, Yang, & Zhang, 2020). In addition, PAT has recently been detected in several manufactured products, including dehydrated (dried) products such as figs, longans, raisins, apricots, plums, peaches, sliced bananas and dates; juices, including orange, lemon, apple, blueberry and mango juices; jams, including apple, strawberry and pear jams; pineapple and blueberry jams (Biango-Daniels, Snyder, Worobo, & Hodge, 2019; Zheng et al., 2018; Saleh & Goktepe, 2019). PAT has also been found in fruit concentrates (Biango-Daniels et al., 2019), in juice and compote blends and commercial apple-based drinks, and in baby food (Saleh & Goktepe, 2019). Therefore, the greatest risk of human exposure to PAT comes from fruits and fruit based products (Saleh & Goktepe, 2019).

PAT can result in acute and subacute toxicity and chronic symptoms (De Souza Sant'Ana, Rosenthal, & de Massaguer, 2008). The LD50 (lethal dose, 50%) for PAT is between 29 and 170 mg/kg body weight (bw) for rodents and poultry, respectively. Signs of acute toxicity such as anxiety, dyspnea, oedema and ulceration, pulmonary congestion, hyperemia and distension of the gastrointestinal tract, intestinal inflammation, epithelial cell degeneration, intestinal haemorrhage and convulsions have been reported (Puel, Galtier, & Oswald, 2010; De Souza Sant'Ana et al., 2008). Signs of subacute toxicity in rats showed weight reduction,

gastric modification, intestinal alteration, impaired renal function, inhibition of several enzymes, gastrointestinal disturbances associated with ulceration, distension and bleeding, and exceptionally alterations in renal function (De Souza Sant'Ana et al., 2008). In the case of chronic symptoms, they include genotoxicity, dermal toxicity, hepatotoxicity, enterotoxicity, oncogenicity, neurotoxicity, steroid toxicity, teratogenicity, nephrotoxicity, embryotoxicity, and immunotoxicity (Saleh & Goktepe, 2019; Ramalingam, Bahuguna, & Kim, 2019). Furthermore, PAT is reported to cause lesions in almost all organs/tissues of the body, including the kidneys, heart, liver, spleen, ovaries, brain, testes, bones, skin, lungs, thyroid and embryos. Doses generally ranged from 0.06 µg/kg to 350 µg/kg depending on the route of administration and the organisms involved. Cell toxicity due to PAT has been explored in culture using human intestinal epithelial Caco-2 cells and human hepatoma HepG2 cells (Ramalingam et al., 2019; Saleh & Goktepe, 2019). PAT may cause damage to proteins through the reaction of electrophilic groups on PAT with sulfhydryl groups on proteins. Increased oxidative stress may also be a mechanism of cellular toxicity, with targets of PAT shown to include cell junction proteins, mitochondrial proteins, cytoplasmic proteins and DNA fragmentation (Ramalingam et al., 2019; Saleh & Goktepe, 2019). Figure 1 illustrates the toxicity mechanisms of PAT at the cellular level.

In consideration of the high level of consumer exposure and the high toxic potential of PAT, the Commission Regulation No 1881/2006, of the European Union, has set the maximum PAT content at 50 µg/kg for apple products, 25 µg/kg for non-liquid apple based-foods and 10 µg/kg for apple-based infant foods. The Food and Drug Administration of the USA (FDA) and China (CFDA) have published the standard-setting limits for PAT in food at 50 µg/kg (50 µg/L). Consequently, limiting the occurrence of PAT in food and minimizing consumer exposure is crucial. The accumulated knowledge in the fields of microbiology, enzymology and the study of biomolecules has led to the development of new and impressive biological

tools to manage PAT in food. In addition, public pressure for green technologies to mitigate environmental problems is leading to the replacement of traditional chemical approaches, such as the use of fungicides, with biotechnological approaches. In this regard, biotechnology, i.e. the use of biological processes, living systems or their derivatives to create or adjust products or processes, has rapidly developed promising and effective strategies compared to traditional chemical strategies. Today, this biotechnology revolution has led to numerous applications for the control of PAT in food.

In this review, we describe recent advances in biotechnology tools and approaches and discuss strategies for the detection, control, and mitigation of PAT in food. The challenges and opportunities of biotechnology development to control PAT in food are also discussed.

2 CURRENT APPLICATION OF BIOTECHNOLOGY IN THE FACE OF THE FOOD CHALLENGE POSED BY PAT MYCOTOXIN

2.1 Detection of PAT in food

Biotechnological approaches can be appropriate for high throughput PAT detection in the food industry and regulatory laboratories. We describe below some examples of biotechnological advances in the detection of PAT in the food sector.

2.1.1 Early detection of PAT-producing fungi

Early detection of PAT-producing fungi is important to control PAT (Pinu, 2016). PAT-producing species of *Aspergillus* include *A. longivesica*, *A. clavatus*, and *A. giganteus*. There are 13 PAT-producing species in the genus *Penicillium*, including: *P. vulpinum*, *P. sclerotigenum*, *P. paneum*, *P. marinum*, *P. griseofulvum*, *P. gladioli*, *P. glandicola*, *P. dipodomyicola, P. expansum*, *P. coprobium*, *P. concentricum*, *P. clavigerum* and *P. carneum* (Barad, Sionov, & Prusky, 2016). The examination of all *Byssochlamys* as well as related *Paecilomyces* species by the use of a polyphasic strategy has shown that just *B. nivea* and a

few strains belonging to *Paecilomyces saturatus* are able to produce PAT (Puel et al., 2010). Given the multiplicity of producers, biotechnology approaches can be useful in determining the critical points to be controlled, by detecting early the presence of PAT-producing fungi.

PCR-based methods have shown high sensitivity for detection of PAT-producing fungi strains in food samples and can serve as a routine approach for fungal detection in agri-food HACCP procedures. The *idh* gene encodes the enzyme isoepoxydon dehydrogenase, which is crucial for PAT biosynthesis. In general, PCR protocols that have been developed for detection focus on selectively amplifying the *idh* sequence reported in the genome of some fungi species like *P. expansum*, with excellent sensitivity and specificity (El Sheikha, 2019). However, some complex food constituents inhibit PCR. These compounds interfere with the cellular lysis essential for DNA extraction, capture or degradation and inhibit the action of the polymerase essential for DNA amplification. An appropriate choice of nucleic acid extraction protocol could, fortunately, overcome this limitation (Luque et al., 2011). With regard to sensitivity, Rodríguez et al. (2011) developed the RTi-PCR tests using the *idh* gene for quantification of PAT-producing molds. The newly developed RTi-PCR SYBR Green as well as TaqMan probes have shown an appreciable sensitivity when applied in food. Both RTi-PCR methods reached the detection limit of 10 conidia/g of food matrices tested with a good linear relationship in the quantity of the *idh* gene units and Ct values (Rodríguez et al., 2011). Subsequently, Rodríguez, Rodríguez, Martín, Nuñez, and Córdoba (2012) reported the effectiveness of real-time quantitative PCR (qPCR) in detecting and quantifying toxic molds in food before mycotoxin production. In parallel, Isabel et al. (2012) developed a sensitive and effective TaqMan qPCR multiplex method to estimate many mycotoxins as well as PAT-producing fungi. For further development, Hosoya et al. (2012) successfully accomplished a PCR with typical primer sets (*B. nivea1F/1R*) developed to generate PCR products specific for *B. nivea*. Likewise, other amplification reactions utilizing typical primer set *B. lag 1F/1R*, *B. fulva 1F/1R*, and *B. zol3*

143 *F/R* successfully generated PCR products specific to *B. laguncularіae*, *B. fulva*, and *B.*
144 *zollerniae*, respectively. Early detection is important to determine the critical steps to eliminate
145 PAT-producing molds and thus limit PAT in food.

146 Early detection of PAT producers in food using PCR methods will help to prevent post-
147 production PAT contamination. Molecular methods based on PCR and real-time PCR are
148 developing rapidly with the availability of primers designed from PAT biosynthesis genes.
149 However, the primers available today are not specific to all PAT-producing fungi as they are
150 limited by the fundamental search for PAT biosynthetic mechanisms on all possible PAT
151 producers and not only on *P. expansum*.

152 **2.1.2 Biosensors in the detection of PAT**

153 Traditionally, several chromatographic procedures, such as gas chromatography, high-
154 performance liquid chromatography, thin-layer chromatography, and others were implemented
155 for the determination of PAT in a variety of food commodities (Vidal et al., 2019). These
156 approaches are sensitive and specific. However, they involve the use of costly tools and highly
157 skilled operators (Pennacchio, Varriale, Esposito, Staiano, & D'Auria, 2015). Unlike chemical
158 methods, the recent development of biosensor technologies proposes some "cleaner" methods
159 for detecting PAT in apple juice. Biosensors have some undeniable advantages over traditional
160 methods used for food analysis, such as selectivity ensuring immediate detection of the analyte
161 without any or minimal pre-treatment. They do not require highly qualified personnel and are
162 easy to use (Rotariu, Lagarde, Jaffrezic-Renault, & Bala, 2016). The biosensor technologies
163 are based on the use of a particular bio-recognition component in conjunction with a transducer
164 to process the signal. Their effectiveness will depend on the affinity between the bio-
165 recognition component and the PAT molecule. In addition, the ability to transmit the weakest
166 modification signal (generally electro-chemical signal) after PAT bio-recognition will

167 determine the sensitivity of the biosensors. We present below some examples of recently used
168 biosensors.

169 Competitive immuno-assay is an interesting approach to examine PAT in food.
170 Generally, the generation of polyclonal antibodies involves conjugation of a PAT derivative to
171 bovine serum albumin. Pennacchio et al. (2014) developed an innovative strategy by coupling
172 the immunological recognition of PAT with an optical procedure known as surface plasmon
173 resonance (SPR). Interactions between test and target molecular particles were initiated by a
174 Laser beam within the region of gold surface of the biochip. This induction leads to the change
175 in resonance conditions and thus to slight but effectively recognisable alter of reflectivity. This
176 approach was reported as a cost-effective and productive immunoassay approach to determine
177 PAT. In apple juice, the limit of detection (LOD) of this test was 1.54×10^{-2} µg/L (Pennacchio
178 et al., 2014). However, to detect PAT in apple juice without pre-treatment of sample,
179 Pennacchio, et al. (2015) engineered a new approach of fluorescence polarisation which uses
180 promising near-infrared (NIR) fluorescence sensors. It is characterised by the increase in
181 fluorescence polarisation emission of a PAT derivative labelled by fluorescence, on binding to
182 specific antibodies. PAT competes with the fluorescence-labelled PAT derivative and allows
183 PAT to be identified with a LOD of 6×10^{-2} µg/L. The technique proposed by Funari et al.
184 (2015) was inspired from the unique properties of quartz or crystal materials. Funari et al.
185 (2015) used photonics immobilisation technique to immobilise tethered oriented antibodies on
186 the gold-plated surface of a quartz-equipped microbalance. This biosensor has reached the PAT
187 detection limit of 21.56 µg/L. To render the micro-balanced nano-sized analytes detectable,
188 they were weighed down by a "sandwich protocol" using an additional antibody. Also, with
189 the aim to design a simple luminescent sensor to detect PAT, Zhang et al. (2017) have
190 developed a nanosensor based on manganese-doped ZnS quantum dots to selectively
191 discriminate PAT through phosphorescence. This nanosensor specifically recognises PAT

192 from various mycotoxins and allows PAT recognition in the range of 66.22-1001 µg/L with a
193 LOD of 49.31 µg/L. Much of the recent work is centred on the study to address the problem of
194 restoring biosensor activity after use. To this end, Soldatkin et al. (2017) developed a
195 conductometric urease-based biosensor to monitor PAT inhibitory activity. This biosensor was
196 noted for its relatively high PAT sensitivity, high selectivity, excellent signal reproducibility,
197 and is suitable for measuring PAT concentrations above 50 µg/L in apple juices. However,
198 some difficulties arise with the presence of heavy metals. In fact, heavy metals can, like PAT,
199 form strong covalent bonds with enzyme sulfhydryl groups. Recently, a PAT aptamer has been
200 developed and defined by Wu, Duan, Zhang, Zhao, and Wang (2016). The oligonucleotide
201 aptamer refers to a monocatenary DNA (or RNA) sequence. Aptamers are generally selected
202 by a well-known method SELEX (systematic evolution of ligands by exponential enrichment).
203 ssDNA aptamers present, generally, a high-affinity to PAT and interesting characteristics such
204 as simple synthesis and labelling, non-immunogenicity, inexpensive production process, high
205 stability, affinity, and outstanding specificity in target binding. This selected aptamer was later
206 used as the selective component in a PAT detection method based on a chromogenic enzymatic
207 substrate system. The results were very impressive; the colorimetric aptasensor gave a linear
208 detection range of 5×10^{-2} to 2.5 µg/L, and the detection limit was 4.8×10^{-2} µg/L (Wu et al.,
209 2016). To increase the transmission of signal of biosensors, lanthanide-doped rare earth-doped
210 up-conversion nanoparticles (UCNPs) have received considerable attention (Kwon et al.,
211 2016). Compared to traditional down-conversion luminescent devices, near-infrared (NIR)-to-
212 visible UCNPs have many benefits, including a near-zero auto-fluorescence background for
213 signal-to-noise ratio improvement, as well as high photostability and low toxicity, high Stokes
214 offsets, a tunable wavelength of fluorescence, a deep tissue infiltration. Applications of
215 biosensors in PAT detection in food are provided in Table 1.

Biosensors use biological tools for recognition, such as enzymes, aptamers and antibodies, and are linked to transducing system. Aptamers are gaining popularity in this field as they have a remarkable ability to recognize PAT at low concentrations and to actively modify their absorption properties, allowing the detection of PAT at the very low level. They can be used for on-line PAT control in food industries. The main drawbacks of biosensors are the low stability of the bio-recognition component (which limits the long-term storage stability of the biosensors), the weak selectivity (mainly in the case of biosensors based on enzymatic inhibition) and the high cost of the antibodies (compared to synthetic recognition elements) (Rotariu et al., 2016).

2.2 Prevention of PAT in food

Recently, there has been an upward trend for organic food consumption due to environmental and health considerations. Consumers are encouraged to eat fresh fruit and vegetables (Apaolaza, Hartmann, D'Souza, & López, 2018; Rana & Paul, 2017). Consequently, the quality of the fruits at the post-harvest stage is becoming very crucial, as, during this stage, the fruits are subject to high risks of infection by PAT-producing molds (Do Amparo, Cavichon, Baratto, Tondo, & Gelinski, 2012). The reduction of fungal infections is one of the main strategies for food security. Traditionally, producers use chemical compounds, such as chlorine dioxide (ClO_2) treatments (Zhang, Mahunu, Castoria, Yang, & Apaliya, 2018) or apply physical techniques such as UV-C (Syamaladevi et al., 2015) to inactivate the pathogenic molds. However, chemical techniques generally produce toxic residues and lead to the emergence of strains resistant to the antifungal treatments, while physical techniques require an extra energy source. In contrast, biotechnological approaches are more compatible with living systems and can comply with environmental requirements. Recently, biotechnological research has focused on the use of antagonist microbes, and antifungal biomolecules to control PAT-producing molds.

2.2.1 Microbial biocontrol

Microbial biocontrol is based on the use of harmless and beneficial microbial antagonists. It can be achieved by using different kinds of microbes such as yeasts, bacteria, and fungi (Nguyen, Strub, Fontana, & Schorr-Galindo, 2017).

Yeast species are largely reported to control PAT-producing molds at the postharvest stage (Spadaro & Droby, 2016; Mahunu, Zhang, Yang, Li, & Zheng, 2016a; Zhang et al., 2017; Zhang et al., 2018). The mechanism of action involves the yeast's ability to compete with the pathogenic molds for both space and nutrients, to directly inhibit spore germination and mold growth. The mechanisms by which yeasts compete with pathogenic molds include iron limitation, biofilm formation, "quorum sensing", release of volatile/diffusible antimicrobial compounds, parasitism, production of hydrolases (glucanases, proteases, chitinases) (Spadaro & Droby, 2016). PAT degradation cannot be considered as a yeast based mechanism in the control of pathogenic molds, as PAT is not yet recognized as an infection factor, but rather as a cultivar-dependent aggressiveness factor (Snini et al., 2016). However, yeasts can induce defence mechanisms in the fruit and limit the PAT-producing mold infection. In a previous recent report from our research group, we highlighted the potential of *Meyerozyma guilliermondii* to control *P. expansum*. We found that *M. guilliermondii* significantly reduced blue mold decay without affecting pear quality (Yan, Zheng, Apaliya, Yang, & Zhang, 2018). This yeast induced defensive response in the pear fruits leads to the production of important transcription factors such as *WRKY31*, and other genes correlated to pathogenesis (*Major allergen Pyr c1*, etc.). Also, we showed that the proteomic profile of the pear fruits changed following *M. guilliermondii* treatment, suggesting the defensive response of the pear fruits against *P. expansum* infection (Yan et al., 2018). This induction of defensive response is in agreement with Spadaro and Droby (2016) who noted that yeast antagonists are able to react with host tissue to enhance the wound and tissue repair process. In apples sprayed with *Pichia*

caribbica, the incidence of blue mold decay was substantially reduced, with higher concentrations of the antagonist yeast considerably raising the level of preservation. *P. caribbica* restrained the development of this disease after apples were stored at either 20°C or 4°C. After incubating the apples with *P. caribbica* for 15 days at room temperature (20°C), PAT formation was substantially decreased. Also, using an *in vitro* test, we showed that, *P. caribbica* can directly decompose PAT and thereby limit PAT occurrence in apples (Cao, Zhang, Yang, & Ren, 2013). However, the efficiency of antagonist yeasts can vary depending on the fruit cultivar, as cultivar type can affect the general efficacy of a biological control strategy (Tannous et al., 2018). For example, several yeast strains were tested on various apple cultivars ('Red Chief', 'Royal Gala', 'Granny Smith' and 'Golden Delicious'). Among them, one antagonistic yeast identified as *Metschnikowia fructicola* (AL27) has been effective in controlling *P. expansum* growth as well as PAT production. Notably, AL27 was found to be more efficient in controlling blue mold decay on 'Golden Delicious' apples compared to other cultivars tested. Also, AL27 reduced *in vitro* conidial germination and length of the germ tube of *P. expansum*. The use of AL27 was comparable to the traditional chemical approach in terms of efficacy (Spadaro, Lorè, Garibaldi, & Gullino, 2013). Generally, fruits and vegetables are stored at a cold temperature, firstly, to reduce the physiological activity of the fruit, and secondly, to limit microbiological contamination. An antagonist microbe that can proliferate at this range of 0-4°C can improve the control of the PAT-producing molds. This has led to the evaluation of certain psychrotrophic microbes, specifically beneficial Antarctic yeasts. These yeasts were studied for three months under cold conditions, alternating with storage at 25°C. It was found that the strain *Candida sake* 41E considerably restricted the fungal growth of apple fruits at room and cold temperature. These yeasts have protected apple fruits against *P. expansum*, at temperatures close to 1°C. Also, *Candida sake* 41E reduced PAT at 25°C in apple juice (Alvarez et al., 2019). In other reports, *Rhodosporidium paludigenum* and *Pantoea*

agglomerans have also controlled PAT-producing molds in fruit (Morales, Sanchis, Usall, Ramos, & Marín, 2008; Zhu et al., 2015).

Among bacteria, *Bacillus* species are mostly used to control PAT-producing molds. The prominence of their use is due to some key characters. *Bacillus* species are widespread and are mainly found in the plant root system. They are very heat tolerant; grow quickly in fluid cultures, and easily produce resistant spores (Calvo, Marco, Blanco, Oria, & Venturini, 2017). *Bacillus amyloliquefaciens* represents one of the promising strains belonging to *Bacillus* genera, which successfully limited the proliferation of the PAT-producing molds on fruits. *B. amyloliquefaciens* strain BUZ-14 decreased the incidence of *P. expansum* in apples by between 20 and 100% (Calvo et al., 2017). A few years before, the biocontrol efficacy of *Bacillus cereus* was also shown against PAT-producing molds (Wang et al., 2015b). *B. cereus* AR156 was tested on blue mold decays in sweet cherries, and produced a substantial decrease in the incidence and development of *P. expansum* disease. The particularity of *B. cereus* treatment is that it considerably improved the chitinase and β-1, 3-glucanase levels in the fruit. Interestingly, *B. cereus* AR156 acted by damaging the integrity of the *P. expansum* cytomembrane. In particular, *B. cereus* caused leakage of pathogenic mycelium proteins and sugars *in vitro* (Wang et al., 2015b). Other species of bacteria, such as *Agrobacterium tumefacien*, *Rhodobacter sphaeroides* and *Bacillus subtilis* have also been shown to control *P. expansum in vitro* and *in vivo*. The mechanism of action against *P. expansum* has been suggested to involve attachment to hyphae (Wang, Yuan, Liu, Zhang, & Yue, 2016). Compared to *Bacillus* species, lactic acid bacteria are rarely used for the biocontrol against PAT-producing molds, although their potential in the preservation of fresh vegetables and fruits has since been well demonstrated (Rouse, Harnett, Vaughan, & Sinderen, 2008; Trias, Bañeras, Montesinos, & Badosa, 2008).

315 Nowadays, the biological control of the PAT-producing molds using harmless
316 antagonist molds is an interesting and growing field. *Aspergillus flavus* HFB1 is a promising
317 atoxigenic mold strain in this regard. The biocontrol efficacy of *A. flavus* HFB1 has been
318 demonstrated against a new PAT-producing mold strain *A. terreus* HAP1. *A. terreus* HAP1
319 was isolated from Egyptian apple fruits and was reported to produce a high level of PAT in
320 fruits. Importantly, the investigation *in vivo* showed that *A. flavus* HFB1 decreased the PAT
321 levels produced by *A. terreus* HAP1 by approximately 59% and 62% in golden and Egyptian
322 apple fruits, respectively (Ammar, Awny, & Fahmy, 2017). However, before its practical use,
323 more studies are required to define the toxicity of all the metabolites that may be produced by
324 atoxigenic *A. flavus* HFB1.

325 Although biocontrol agents can reduce disease and PAT contamination in fruit, some
326 antagonistic microbes can, unfortunately, increase the amount of PAT produced by each
327 fungus, which the authors of the relevant case study defined as specific mycotoxigenic activity
328 in ng PAT/μg fungal DNA (Zheng et al., 2017). The study was carried out with two biocontrol
329 yeasts, *Rhodotorula kratochvilovae* strain LS11 and *R. mucilaginosa* strain 3617 used against
330 *P. expansum* on apples stored at 20°C. Both microbial biocontrol agents diminished the total
331 PAT contamination but increased the specific rate of production of this mycotoxin (Zheng et
332 al., 2017).

333 In summary, the advantages of microbial approaches are their self-maintenance under
334 appropriate conditions and their range of mechanisms of action from competition for space and
335 nutrients to the production of antifungal compounds. Yeasts are most commonly applied to
336 fruits compared to bacteria and fungi. This is due to the fact that yeasts are resistant to extreme
337 environmental situations before and after harvest (humidity, temperature, oxygen content and
338 pH). Also, yeasts are particularly well adapted to the micro-environment of the fruit (high

osmotic pressure and high sugar concentrations). Moreover, unlike filamentous fungi, yeasts do not produce mycotoxins or allergenic spores. Finally, they have simple nutritional requirements that facilitate their proliferation on dry surfaces over a long period of time (Spadaro & Droby, 2016). Nevertheless, the strategy using bacteria and fungi needs to be developed and the combination of fungi, yeasts and bacteria needs to be taken more into account as they have different biocontrol mechanisms. The disadvantage of microbial approaches remains low efficacy compared to fungicides. Strategies to control PAT are mainly applied on apples, whereas PAT is present on a wide range of foods. The microbial control approaches used for apples may also be useful for other fruits. However, this variation in the type of application product and any other significant changes in factors will result in reduced and inconsistent performance of the biocontrol product when used under commercial conditions (Droby, Wisniewski, Teixidó, Spadaro, and Jijakli, 2016). Microbial antagonists used to control PAT-producing molds are presented in Table 2.

2.2.2 Antifungal biomolecules

Some biotechnological approaches promote the use of naturally occurring molecules from various organisms against the PAT-producing molds. Salas, Reynoso, Céliz, Daz, and Resnik (2012) showed that flavanones and some of their glucoside esters (prunin 6″-O-decanoyl ester, 6″-O-lauroyl ester, 6″-O-butyryl ester) can inhibit PAT-producing molds and resulted in 95% reduction of PAT accumulation in fruits. Chitosan, a sugar derived from chitin from the hard outer coat of shellfish, has been shown to reduce the formation of the decay at 25°C in jujube infected by *P. expansum* (Wang, Wu, Qin, & Meng, 2014). Chitosan also diminished the detrimental effect of *P. expansum* on the quality of jujube fruits stored at cold temperatures. Mycelial growth, as well as spore germination of *P. expansum,* were substantially reduced by the increasing concentrations of chitosan (Wang, Wu, Qin, & Meng, 2014). Glucosinolates, which are sulphur containing compounds found in cruciferous

vegetables, have been shown to inhibit the development of the PAT-producing strain of *P. expansum* in wheat tortillas (Saladino et al., 2016). Numerous plants release essential oils which can inhibit PAT-producing molds. For example, 12×10^4 µg/L decanal, which is found in citrus fruits, reduced the germination rate of *P. expansum* from 85% to 10%, the length of the germ tube by about half, and the diameter of the colony by about 25% *in vitro* (Zhou et al., 2018). Interestingly, 24×10^4 µg/L decanal showed a strong control effect on blue mold decay in apples and pears as well as on PAT biosynthesis (Zhou et al., 2018). It was found that about two-thirds of the differentially expressed (DEG) genes in decanal treated *P. expansum* were downregulated. Of these, DEGs associated with the inhibition of *P. expansum* were implicated in oxidative phosphorylation, translation, and transcription (Zhou et al., 2018). Also, analysis of DEG and differentially expressed proteins (DEP) showed that the decanal interfered with the biosynthesis of secondary metabolites. As mycotoxins are secondary metabolites, this fact may explain the reduction in PAT accumulation (Zhou et al., 2018).

Antifungal biomolecules can have similar efficacy to chemical compounds. Their efficacy depends on the concentration applied. Their attraction remains their ability to disrupt the vital molecular mechanisms of pathogens and reduce PAT synthesis. However, to ensure a sustainable supply of antifungal biomolecules, good natural resource management and production strategies will be necessary. Table 3 presents some antifungal biomolecules used to control PAT-producing molds.

2.2.3 Integrated strategy combined microbial antagonists with biomolecules

Whilst the use of microbial biocontrol agents can lead to a significant reduction of the PAT-producing mold infection, their efficacy is not always as high or as reliable as that of chemical fungicides. Therefore, emphasis has recently been put on combining microbial biocontrol agents with other tools, to improve their reliability and/or performance. Such approaches include integrated control, which consists of the combined application of reduced

389 rates of fungicide(s) with microbial biocontrol agents. These approaches lower the amount of
390 chemical residues released into the environment and the selective pressure on the pathogen
391 population that may result in the onset of fungicide-resistant *P. expansum* strains (Lima et al.,
392 2011). Currently, the trend in this field of research follows the use of microbial biocontrol
393 enhancers, which are natural molecules that can increase the effectiveness of biocontrol action.
394 Our research group has made some contributions to this recent topic. Bamboo leaf extract, in
395 powder form, contains a large number of flavonoids and has the potential to induce antioxidant
396 reactions in the host under stressful situations. *P. caribbica* (1×10^8 cells/mL), combined with
397 flavonoids (0.01% w/v) of bamboo leaf, was effective for biodegradation of PAT to an
398 undetectable level *in vitro* after 96h in NYDB medium. Also, PAT accumulation in apples was
399 close to nil at 20 days (at 20°C) of storage. The lignin content of the apple's wound was
400 substantially increased within 15 days of storing, while the pH has been modified (> 4.0) in the
401 host, which could result in increased degradation of PAT (Mahunu et al., 2018). Phytic acid
402 (PA), which we revealed as a potential enhancer of yeast against *P. expansum*, is recognized
403 in food preservation because of its antioxidant potential (Yang, Zhang, Zhang, Zheng, & Qian,
404 2015). Our initial trial successfully showed that PA can enhance the biocontrol activity of
405 *Rhodotorula mucilaginosa* (Yang et al., 2015). To confirm these findings, we combined $2 \times$
406 10^8 cells/mL of *P. caribbica* with 0.2% v/v of PA. We noted considerable improvement in the
407 inhibition of *P. expansum* infection on apples, compared to yeast or PA alone (Mahunu et al.,
408 2016b). Also, we showed that disease development in treated fruit was significantly reduced
409 from almost 95% to less than 30% when stored for 10 days (20°C). Further studies on the
410 mechanism of fruit resistance induction by yeasts enhanced by PA are needed before its proper
411 application in food (Mahunu et al., 2016b). β-glucan is a structural polysaccharide found in the
412 cell walls of molds and yeasts, but mainly in plants. It is formed by glucose units mainly linked
413 by β-1,3 bonds. The β-glucans are well known to exhibit some interesting antioxidant activities.

Therefore, we combined β-glucans with an antagonist yeast named *Cryptococcus podzolicus* and showed an increased capacity of *C. podzolicus* biocontrol against PAT-producing molds (Wang et al., 2018). Treatment of apples with *C. podzolicus* (1×10^8 cells/mL) grown in NYDB supplemented with 0.5% β-glucan produced a disease incidence of only 40.8% while that of CK was 100% ($P < 0.05$). This efficacy did not affect the general quality parameters of the apple fruits. When enriched with β-glucan, *C. podzolicus* grows best in the apple wounds at 20°C or 4°C. In the apples tested, the defensive activities of peroxidase, catalase, and polyphenol oxidase increased (Wang et al., 2018). The use of antifungal biomolecules with some antioxidant properties gave more information about the role of oxidative burst (release of a large quantity of reactive oxygen species (ROS)) at the wound level following the colonisation of antagonistic cells. Oxidative burst intervenes in the signalling pathways leading to activation of the fruit resistance system (Spadaro & Droby, 2016). The possible role of antifungal molecules with antioxidant properties, such as β-glucans, is to help antagonist cells tolerate high levels of oxidative stress. Castoria, Caputo, De Curtis, and De Cicco (2003) were the first to point out that the ability of yeast antagonists (*R. glutinis* and *C. laurentii* LS-28) to achieve post-harvest biocontrol was related to their ability to withstand relatively high rates of ROS. The findings of Castoria et al., (2003) have revealed the part played by oxidative stress in biocontrol mechanisms and its possible critical effects on antagonistic cells and fruit tissues. Additionally, the fruit of the *Adansonia digitata L* (Baobab tree in Africa) was considered by our research group in the development of a yeast enhancer for biocontrol against PAT-producing molds. In this way, we tested *Sporidiobolus pararoseus Y16* in combination with Baobab active extract (1×10^8 µg/L) against *P. expansum*. Importantly, a considerable reduction in the development of *P. expansum* was observed. This reduction was more than for yeast or Baobab alone when stored at 4°C or 20°C for 30 days. *S. pararoseus Y16* and Baobab also substantially decreased the incidence of blue mold disease and lesion diameter (*in vivo*),

and colony diameter (*in vitro*). *S. pararoseus Y16* either in combination with Baobab or not did not affect the quality parameters when stored with fruits (Abdelhai et al., 2019). The extract of *Lentinula edodes*, an edible mushroom, was also reported to enhance *Cryptococcus laurentii* against *P. expansum* (Tolaini et al., 2010). Following these interesting results obtained with the yeasts combined with the enhancers, recent efforts were directed to find the mechanism behind the enhancement phenomenon. Accordingly, Zhang et al. (2017) used glycine betaine (GB) as a biocontrol enhancer and showed *Pichia caribbica*'s effectiveness in controlling blue mold on apples. They found that treatment with GB improved tolerance to oxidative stress of *P. caribbica*, leading to better antagonist activity against *P. expansum*. Also, a proteomic investigation conducted by Zhang et al. (2017) has shown that 51 proteins have been differently expressed in *P. caribbica* following GB exposure. Increased regulation of the proteome contributed to the efficacy of *P. caribbica* in the biological control against *P. expansum*. It was linked to metabolism (isocitrate lyase), stress response and regulation (peroxysomal catalase), and carbohydrate transport. Nature is very rich in biomolecules that can be used as enhancers of the microbial biocontrol agents against PAT-producing molds. Table 4 summarizes the combinations of microbial antagonists and biomolecules used against PAT-producing molds.

Probably the most exciting approach to control PAT-producing fungi is the integration of microbes with natural antifungal agents. By combining antifungal biomolecules with microbes, mainly synergistic effects can be achieved (Yu, Li, & Zheng, 2007). Possibly underpinning this synergistic action are the antioxidant properties of the biomolecules that help the microbial antagonist resist the "oxidative burst" (Castoria et al., 2003; Yang et al., 2020). However, the choice of the antifungal agent to be combined is essential because some antifungal agents, such as chitosan, can also be harmful to yeast. Figure 2 summarizes the control of PAT-producing molds in foods using biotechnology methods.

2.3 Mitigation of PAT in food

Reduction of PAT is of primary interest since patulin affects the quality and safety of the foods, especially apple based products, but patulin mitigation has become more complex due to stricter control measures and environmental considerations (Do Amparo et al., 2012). The development of biotechnological approaches could provide improved and environmentally friendly approaches for the elimination of PAT from food. Using biotechnological methods, PAT can be eliminated from food by degradation or removal.

2.3.1 PAT degradation

Traditionally, chemical methods to degrade PAT involve ozone. Physical methods are also widely used such as UV radiation, high hydrostatic pressure (HHP), pulsed light (PL), ultrasound and microwaves all can eliminate or effectively degrade PAT in food (Ioi, Zhou, Tsao, & Marcone, 2017). Nevertheless, additional studies are required to assess the identity and toxicity of the breakdown product(s) of PAT (Diao, Hou, Hu, Dong, & Li, 2018a; Diao, Ren, Liu, Zhang, Hu, & Hou, 2018b; Hao, Zhou, Kuchma, Wu, and Warriner, 2016). For example, the prolonged application of UVC (200 to 280 nm) in food could generate furans, which are classified as possible human carcinogens (Hu, G. et al., 2018). Also, the loss of quality, high costs, and environmental pollution are major drawbacks of traditional methods (Diao et al., 2018a; Diao et al., 2018b; Hao et al., 2016). These growing food safety concerns about traditional methods led to the development of promising biotechnological methods to degrade PAT, including the use of enzymes and microbes.

2.3.1.1 Enzymes for PAT degradation

Enzymes are biocatalysts which, compared to traditional methods, have very high substrate specificity and can be used under moderate reaction conditions. Over the past decade, there has been growing attention on the utilization of enzymes to degrade PAT due to their safety and detoxification efficacy (Hassan & Zhou, 2018). Thus, several approaches have been

488 developed such as porcine pancreatic lipase (PPL) immobilized with calcium carbonate (Tang,
489 Peng, Li, Meng, & Liu, 2018; Li et al., 2017), which degraded 99% of PAT during 3h at pH
490 5.0, 30°C. The procedure has shown appreciable performance when applied under pH 6.0
491 conditions, for 42h, and at a temperature of 40°C (Li et al., 2017). A different activity was
492 observed by Liu, Peng, and Meng (2018), who found that PPL can degrade above 90% of PAT
493 in aqueous solution at pH 7.5, 40°C for 48h and identified the residual product as $C_7H_{11}O_4^+$. In
494 apple juice, the degradation rate of PAT was greater than 70% from an initial level of 1×10^3
495 µg/L. The best condition was 40°C for 18h for immobilized PPL at 3×10^4 µg/L. The sensorial
496 and nutritional quality of apple juice was not significantly altered. Orotate
497 phosphoribosyltransferase has also been reported as a new substance for PAT degradation
498 (Tang, Li, Zhang, Meng, & Liu, 2019). The purified degrading enzyme was obtained from the
499 yeast *Rhodotorula mucilaginosa*. Addition of this catalyst, at the concentration of 1.5×10^5
500 µg/L, could accomplish a detoxification of 80% of 1×10^3 µg/L PAT at 25°C for 18h.
501 Importantly, this activity occurs without significant changes in nutrient content of apple juice
502 (Tang et al., 2019).

503 The use of enzymes is a promising approach for its specificity and speed of degradation
504 of PAT in contaminated fruit juices. However, the constant supply of enzymatic material
505 increases the cost of the operation. It is therefore necessary to search for specific microbes with
506 high enzyme production and to design an appropriate strategy for the recovery of enzymes after
507 use as well as for enzyme immobilization (Tang et al., 2018; Li et al., 2017).

508 **2.3.1.2 Microbes for PAT degradation**

509 In recent years several microorganisms, including bacteria, molds and yeasts have been
510 shown to have the ability to degrade PAT and be safely applied to food processing. Eighty
511 percent of PAT was broken down by the activity of *Lactobacillus plantarum* cells, after

512 incubation for 4h with 1×10^{10} cells/mL (37°C), forming Z- and E-ascladiol (Figure 3) (Hawar
513 et al., 2013). Z- and E-ascladiol have been shown to be non-cytotoxic in studies conducted on
514 human cell lines (Caco-2 colon carcinoma cells, HEK-293 embryonic kidney cells, HepG2
515 hepatocellular carcinoma cells, HL-60 promyeloblast cells) (Tannous et al., 2017; Tannous et
516 al., 2018). Toxicological analysis of microbial and human cells revealed that ascladiol induced
517 a very low production of reactive oxygen species and induced cellular apoptosis to a lower
518 extent than PAT (Zheng et al., 2018). Nevertheless, further toxicological studies are needed to
519 determine the toxicity of ascladiol and of all eventual metabolites identified after PAT
520 degradation. *L. plantarum* is a strong potential candidate for biodegradation due to its resistance
521 to very high concentrations of PAT ($\geq 1 \times 10^5$ µg/L) (Hawar et al., 2013). In the case of yeasts,
522 the presence of PAT can induce production of enzymes that have the ability to degrade PAT
523 (Zheng et al., 2016). PAT degradation was demonstrated to be achieved by the biocontrol yeast
524 *R. kratochvilovae* LS11 (Castoria et al., 2005 and 2011), with formation of desoxypatulinic
525 acid as the main product. This yeast was extraordinarily resistant to PAT ($>2.5 \times 10^5$ µg/L). A
526 study with ^{13}C labelled PAT showed that desoxypatulinic acid was a product of PAT
527 degradation by LS11, and that it was less genotoxic and cytotoxic than PAT to human cell
528 lines. This was probably due to the rupture of the lactone ring and the loss of reactivity with
529 thiol groups of the antioxidant peptide glutathione (Castoria et al., 2011, Pinedo et al., 2018).
530 Other basidiomycete yeasts such as *Rhodotorula mucilaginosa* and *Rhodosporidium*
531 *paludigenum* (Zhu et al., 2015; Li, Tang, Yang, Meng, & Liu, 2019) transformed PAT into
532 deoxypatulinic acid (Figure 3). Li, Chen, Zhang, Zhang, and Peng (2018) provisionally
533 identified E-ascladiol as the product of biodegradation of PAT by *Saccharomyces cerevisiae*.
534 Zheng et al. (2016) reported that enzymes would be produced in contact with PAT by
535 *Sporobolomyces sp.*. Ianiri, Pinedo, Fratianni, Panfili, and Castoria (2017) noted that important
536 enzymes in PAT biodegradation are induced by the mycotoxin in the basidiomycete yeast

Sporobolomyces sp. whose products of degradation were desoxypatulinic acid and ascladiol (Figure 3). Further studies with this same yeast showed that biodegradation of PAT was carried out by two phases; the first one involving PAT tolerance followed by pathways for biodegradation of PAT (Ianiri et al., 2013; Ianiri, Idnurm, & Castoria, 2016). Wang et al. 2019 recently noted that *P. caribbica* was capable of decomposing PAT as a response to being stressed by PAT. Wang et al. (2019) demonstrated the key role of the gene PcCRG1 in this process. Deletion and overexpression of this gene led respectively to a decrease or acceleration in PAT decomposition. With regard to molds, Zhao et al. (2018) recently reported that *Byssochlamys nivea* FF1-2 is a filamentous fungus with outstanding PAT biodegradation potential. Apple puree, that has been deliberately contaminated with high doses of PAT (1.25×10^5-5×10^5 µg/L), has been incubated with FF1-2 for 10 days, resulting in more than 97% of PAT decomposition. Microbes used for PAT degradation are summarised in Table 5.

Microbial approaches to degrading PAT are of interest because they have an autonomous reproduction. However, their disadvantage is that they modify the final quality of food by their metabolism, which involves use of nutrients and release of metabolites in the food where they are applied. Figure 3 illustrates the degradation of PAT by microbes.

2.3.2 Removal of PAT from contaminated food

PAT degradation methods may leave residues of degradation products in food, which is not the case for systems that rely on the removal of PAT, especially through adsorbents. Traditionally, chemical adsorbents are more often used, such as the use of propylthiol-functionalized SBA-15 silica (Appell, Jackson, & Dombrink-Kurtzman, 2011), sulfhydryl-terminated magnetic bead separation (Bayraç & Camizci, 2019), or magnetic carbon nanotubes (Fe3O4-MWCNTs adsorbent) (Zhang, Zeng, & Peng, 2019). Chemical adsorbents are of interest because of their practicality. Some chemical adsorbents can negatively affect the final

quality parameters (clarity, colour, Brix, titratable acidity and total sugar) and the overall sensorial quality. But, the main consumer concern about chemical adsorbents is that prolonged contact with food may cause an exchange of toxic compounds from the chemical adsorbent to the food or an uncontrolled reaction between the food ingredients and the chemical adsorbent. This is not the case for bio-adsorbents (inactivated microbes or biopolymers) derived from living systems and chosen for their lack of nocivity to humans.

Recently, biotechnology methods are proposed as an alternative for the removal of PAT. This can be achieved by the use of biomolecule-based adsorbents and microbe-based adsorbents.

2.3.2.1 Biomolecule-based adsorbents

This is probably one of the most innovative groups of methods (Figure 4 and Table 6). These include the removal by adsorption of PAT into aqueous solution by means of a thiourea-modified chitosan resin. The study for its pilot application indicated that adsorption can be characterized by a Freundlich isothermal model. This model is usually implemented for the design of the adsorption process (Liu, B. et al., 2015). As PAT reacts easily with SH groups, a special sulphurous material has been generated to remove PAT from the aqueous mixture. The chitosan beads have been produced by the reverse suspension crosslinking method, and used to extract PAT from the aqueous mixture. The optimum time of contact for PAT high adsorption was 24h and the capacity for adsorption was increased with the PAT concentration (Table 6) (Liu, B. al., 2015). PAT bio-adsorption was tested in kiwi fruit juice by using a superior magnetic chitosan. The efficient superior magnetic chitosan was prepared in juice at the ratio 1:1 of Fe_3O_4 particles to chitosan. This resulted in an adsorption of PAT of 89% and a recuperation of the adsorbent of nearly 100% (Luo, Li, Yuan, & Yue, 2016). Particles of chitosan-coated Fe_3O_4 have been designed as magnetic adsorbents and shown to be efficient in

PAT removal with a potential of adsorption up to 6.67×10^6 µg/kg in 5h when adding 300 µg of adsorbents in 10mL of 200 µg/L of PAT in aqueous solution. As with kiwi juice, the recovery rate of the chitosan-coated Fe_3O_4 adsorbent was 99.95% after 60min (Luo, Zhou, & Yue, 2017). For PAT-contaminated apple juice, an efficient PAT biosorption with cross-linked xanthan chitosan resin (CXCR) was implemented. Interestingly, optimal adsorption of CXCR for PAT was obtained at a pH of 4 (30°C) over 18h. Adsorption data were adjusted with a Freundlich isothermal model and a pseudo-second-order kinetic model. This was an indication that CXCR was an appropriate adsorbent for removing PAT from apple juice (Peng et al., 2016).

Several biopolymers can remove PAT from juices. However, the best approach is one that combines a bio-adsorbent material with chemical immobilization materials, resulting in exciting absorption and recovery properties.

2.3.2.2 Microbe-based adsorbents

Microbe-based adsorption represents another possibility for eliminating PAT from food. With regard to recent research, Guo et al. (2013) implemented the PAT biosorption in juice of apple by caustically treated cider yeast biomass. Advances in biotechnology have enabled immobilization of inactivated cells (*C. utilis* CICC1769) on coated magnetic Fe_3O_4@CTS nanoparticles to obtain an innovative biosorbent. The latter reduces PAT in orange juice by more than 90% without any significantly detrimental effect on quality parameters such as content of vitamin C, sugar and titratable acids (Ge, Xu, Li, Peng, & Pan, 2017). Some bacteria, such as *Enterococcus faecium* and *Lactobacillus rhamnosus*, have also been identified to reduce PAT in fruit juices. The inactivated form of these bacteria can reduce PAT in juice by adsorbing more than 60% of the initial PAT content, but the adsorption process depends on strain, temperature, initial concentration of bacterial cells and also the initial PAT concentration. The cell wall has been identified by FTIR analysis as playing a major role

(Hatab, Yue, & Mohamad, 2012a). Other lactic acid bacteria such as *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* were also successfully tested for their ability to reduce PAT in aqueous solution (Hatab, Yue, & Mohamad, 2012b; Li et al., 2020). There are some advantages to conducting more tests with lactic acid bacteria, as they are generally recognized as safe and can be used as additives in food. The mechanism of adsorption of bacteria cells is related to the chemical and physical properties of the cell surface, including cell wall volume, specific surface area, nitrogen/carbon (N/C) ratio, hydrophobicity, and functional groups (Wang et al. 2015a; Ioi et al., 2017). The adsorption capacity of yeast depends on the surface area, volume and thickness of the cell wall, as well as the content of 1,3-β-glucan. The 1,3-β-glucan content and cell wall thickness play a decisive role in all these factors. The adsorption capacity of PAT is also changed by the 3D network structure of the cell wall consisting of 1,3-β-glucan (Luo et al., 2015; Ioi et al., 2017).

The effectiveness of microbial adsorbents remains reduced compared to chemical sorbents. However, microbial adsorbents are well accepted in food compared to chemical adsorbents because of their biological origin. It is therefore essential to seek appropriate combinations of chemical (acid-base) and physical (high temperature) treatments compatible with the microbial adsorbent to improve its ability to adsorb PAT in contaminated fruit juices (Li et al., 2020). Table 6 indicates microbial and biomolecule-based adsorbents used to reduce PAT in different solutions. Figure 4 illustrates the removal of patulin by biotechnological methods.

3 FUTURE ASPECTS FOR BIOTECHNOLOGY DEVELOPMENT TO OVERCOME THE FOOD CHALLENGE POSED BY MYCOTOXIN PAT

To further increase the applications of biotechnology to the food challenges posed by PAT, biotechnologists could increasingly take advantage of improved knowledge of molecular mechanisms, finding new areas of PAT reduction application and improving the existing biotechnologies (biocontrol microbes, enzymes, bio-adsorbents, etc.).

Basic research underpins future advances in biotechnology. For instance, orotate phosphoribosyltransferase is an enzyme successfully used for PAT detoxification of apple juice. The purified form of this enzyme comes from the study of the degradation mechanism of the *yeast Rhodotorula mucilaginosa* (Tang et al., 2019). The use of primers for the early detection of PAT-producing fungi, also, was developed through understanding the molecular process of PAT biosynthesis (Li et al., 2019). The identification of the key yeast genes involved in PAT degradation may be useful for transforming apples to confer the property of degrading PAT, as was the case for transgenic rice plants and zearalenone (Higa et al., 2003, Ianiri et al., 2013). Therefore, advances in biotechnology should take into account the importance of fundamental mechanisms by continuing to give priority to its development. Research on molecular mechanisms can ensure the sustainable development of biotechnology by serving as an inexhaustible source of information. Some current trends in the elucidation of mechanisms are presented: the mechanism of PAT degradation by yeast (Ianiri et al., 2013; Yang et al., 2018; Wang et al., 2019; Chen et al., 2017), mechanism of PAT biosynthesis (Tannous et al., 2018; Li et al., 2019), mechanism of PAT toxicity (Figure 1) (Ramalingam, Bahuguna, & Kim, 2019), the biocontrol mechanism (Zhang et al., 2018), mechanism of PAT adsorption by bacteria (Wang et al., 2015a). There are confirmed biotechnological methods that are less used in PAT control. For example, the clustered, regularly interspaced, short palindromic repeat (CRISPR) and CRISPR associated protein 9 (Cas9) system is a gene-editing biotechnological tool, mimicking the bacterial immune system, used by many researchers to understand living organisms. It has demonstrated its effectiveness in the analysis of genes related to dysfunctions

or potential diseases in many organisms (Bortesi & Fischer, 2015). It can be used to identify genes involved in the induction of fruit defence mechanisms, mold biosynthesis of PAT or PAT degradation by yeasts. Also, peptide arrays are powerful and simple tools for studying protein-protein and protein-drug interaction. Peptide networks can be useful in PAT control because post-translational modifications (PTMs) are important in the cellular mechanism (Mauser & Jeltsch, 2019). For example, peptide arrays could be helpful in the study of PTMs during PAT degradation by *R. mucilaginosa* (Yang et al., 2018).

The risk posed by PAT-producer fungi should be taken seriously by biotechnology advances to prevent PAT occurrence in food. Currently, the number of PAT-producing fungi and the number of biocontrol microbial agents is underestimated (Puel et al., 2010). Biotechnology research should suggest a new list of PAT-producing species as well as potential biocontrol microbial agents, for example, after exploration of the aquatic ecosystem (Vansteelandt et al., 2012) or by considering that some strains of bacteria can also produce PAT (Lackner, Partida-Martinez, & Hertweck, 2009). Biotechnology should build on the successes of its application in other areas. For instance, metagenomic techniques have already proven their ability to study microbial communities in particular ecosystems. Also, functional metagenomics is now representing a more attractive approach for the discovery of new enzymes. Functional metagenomics can stimulate the discovery of knowledge from basic research. It can also provide important innovative bioprocesses for the development of new and effective biotechnological tools to control PAT (Bahram et al., 2018). A major contribution to the control of PAT production in food is the study of biomolecules and environment factors in the PAT production by fungi. Research on biotechnology should benefit from trans-omics analysis, dynamic omics analysis, and cross-species omic analysis to understand how environmental factors and biomolecules influence PAT production in fungi (Kawata et al., 2018; Tannous et al., 2018). Bio-adsorbents, biosensors, and biocontrol agents have some

limits compared to analytic approaches: they are less practical, less accessible and less versatile compared to traditional chemical or physical approaches. The commercialization of these biotechnological tools is less frequent than laboratory research activities (Kunzelmann, Solscheid, & Webb, 2014). Many of them still have high costs related to key technical barriers that characterize them. In addition, these biotechnological approaches are often designed for a limited number of food specimens.

4 CONCLUSIONS

This review presents recent advances in biotechnology for the detection, prevention and detoxification of PAT in the food chain. Biotechnological trends in detection have focused on biosensors, which can be highly accurate and sensitive. Biosensors represent the future of PAT detection given their as yet unexplored potential. They use living tools for biological recognition such as enzymes, aptamers and antibodies. Aptamers, in particular, appear promising due to high sensitivity of signalling coupled to high specificity of PAT recognition. Biosensors associated with aptamers are now one step ahead of chemical detectors. However, food contamination by PAT can also occur after food production due to incapacity to detect PAT producers in the foods, which can cause safety problems for the food industry. This limitation justifies the parallel interest in the early detection of PAT producers in food by PCR approaches. Molecular methods based on PCR and real-time PCR are developing rapidly with the availability of primers designed from PAT biosynthesis genes. However, the primers available today are not specific to all PAT-producing fungi as they are limited by the scarcity of fundamental research into PAT biosynthetic mechanisms.

PAT prevention strategies to date include microbial biocontrol, the use of antifungal biomolecules and the use of microorganisms in combination with antifungal molecules.

Microbial approaches have the advantage of utilizing the ability of microbes to proliferate at the expense of pathogens, so that their action is self-sustaining under appropriate conditions. In addition, they act through various mechanisms that make them a serious alternative for PAT prevention. The use of yeasts is the most promising in this field, and a research effort is needed to develop their full potential against PAT-producing species. However, the efficacy of microbial biocontrol is much lower than that of antifungal biomolecules, such as essential oils from plants and chitosan from animal crustaceans. Antifungal biotechnology, therefore, remains promising mainly because it disrupts the essential molecular mechanisms of pathogens. Its disadvantage is that the cost of a constant supply of material will have to be taken into account. The most exciting prevention approach is the integration of microbes with natural antifungal agents. Indeed, by combining antifungal biomolecules with microbes, synergistic effects can be obtained. Through the understanding of molecular mechanisms, we now know that antioxidant activities of these biomolecules are responsible for these interesting effects. However, the choice of the antifungal agent to be combined is essential, so as to not disrupt the activity of the microbial antagonist.

PAT detoxification is necessary when, despite preventive efforts, PAT is detected in food. Biotechnological research has made significant progress in this area. It is oriented towards the degradation and elimination of PAT in food. These approaches use enzymes, microorganisms and various adsorbent biopolymers. Autonomous reproduction is an advantage of microbial approaches but microbial metabolism may alter the final quality of the food. Enzymes, such as orotate phosphoribosyltransferase, produced by microorganisms can hydrolyse PAT. However, the disadvantage of using enzymes is the constant supply of enzymatic material, which increases the cost of the operation. Also, the residues from the enzymatic hydrolysis of PAT, although less toxic, are not absolutely proven to be harmless in the long term. For this reason, many biotechnological strategies are used for the removal of

PAT from food. These new approaches are based on the use of bio-adsorbents. They have a clear advantage over chemical sorbents in that they are better accepted in food because they use both live and inactivated microbes as well as biopolymers such as chitosan. They are therefore convenient for the elimination of PAT in fruit juices. However, their effectiveness is reduced compared to chemical absorbents. Therefore, the best approach is one that combines a bio-adsorbent material with chemical materials, which gives better absorption and recovery properties.

Biotechnology has made many advances in the detection, prevention and mitigation of PAT in food compared to traditional chemical methods. We anticipate that progress will be more rapid in the future. However, the lack of general rules for the validation, evaluation and selection of new biotechnological approaches is the main limitation to their progress. The accumulation of successes in applied and developed biotechnology for the control of PAT will provide appropriate rules for the validation of methods. To control PAT in food, the rational design of candidate biotechnology tools or methods with the desired properties should be based on knowledge of molecular mechanisms such as biosynthesis, biodegradation and biocontrol of PAT. The critical challenge is to move biotechnology advances from the laboratory to the field by overcoming the barriers between discovery and commercial applications.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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TABLE 1

Biosensor methods for the detection of patulin

TABLE 2

Microbial antagonists used to control PAT-producing molds

TABLE 3

Antifungal biomolecules used to control PAT-producing molds

TABLE 4

Integrated strategy combining microbial antagonists and biomolecules against PAT-producing molds

TABLE 5

Microbes used for PAT degradation

TABLE 6

Bio-adsorbents used for patulin removal

Figure legends

Figure 1: Mechanism of patulin driving toxicity at the cellular level.

This scheme is an original synthesis of the schemes proposed by Ramalingam et al. (2019). Patulin toxicity at the cellular level is mediated by oxidative stress, leading to the production of reactive oxygen species (ROS) and the reduction of glutathione.

P21-cyclin-dependent kinase inhibitor 1, PIG3-p53-inducible gene 3, UPR-Unfolded Protein Response, p62-sequestosome-1, GADD34-growth arrest and DNA damage-inducible protein 34, Cas.9-Caspase 9, Cas.6-Caspase 6, Cas.7-Caspase 7, Cas.3-Caspase 3, Cleaved cas.6-Cleaved Caspase 6, Cleaved cas.7-Cleaved Caspase 7, Cleaved cas.8-Cleaved Caspase 8, ATP-6 & 8-ATP synthase subunits, LC-3-light chain 3, LC-1 light chain (MAP1B-LC1), Cyt. c-Cytochrome C, COX-17-cytochrome C oxidase copper chaperone, Bcl-2-B-cell lymphoma-2, Bax-Bcl-2 associated X protein, Apaf-1-Apoptotic peptidase activating factor 1, c-DFF45-DNA fragmentation factor is a heterodimeric protein of 45 kDa subunits, Bcl-2-B-cell lymphoma 2, Bcl-xl-B-cell lymphoma extra-large, MCL1-myeloid cell leukemia sequence 1, BAD-phosphorylated Bcl-2 associated death promoter, BAD-Bcl-2 associated death promoter, GRP78-glucose-regulated protein (78 kDa), NSP70-family of genes/proteins related to heat shock proteins 70 (Hsp70), ZO-1-Tight junction protein (receptor), MLC-2-myosin light chain 2, CTSB&D-Cathepsin B and cathepsin D, CAT-Catalase, PARP-poly (ADP-ribose)

polymerase, p-PARP-phosphorylated PARP, MLCK-Myosin light-chain kinase, P38-mitogen-activated protein kinases, PERK-Protein kinase RNA-like endoplasmic-reticulum kinase, IRE1 α-Inositol requiring ER-to-nucleus signal kinase 1α, p-PERK-phosphorylated-protein kinase RNA-like endoplasmic reticulum kinase, IERI α-phosphorylated-inositol requiring ER-to-nucleus signal kinase-1α, ERK 1/2-extracellular signal-regulated protein kinases 1 and 2, MAPK-Mitogen-activated protein kinases, PKR-Protein kinase RNA-activated, JNK-Junamino terminal kinases, p38-mitogen-activated protein kinases, p-ERK-phosphoextracellular signal regulated kinase, ERK-extracellular signal-related kinases, p-JNK-JNK phosphorylation, DEP-1-density-enhanced phosphatase-1, PTP-Protein tyrosine phosphatases, PPAR-δ-peroxisome proliferator-activated receptor gamma, TER-DNA replication terminus site-binding protein (replication factors), PPAR-δ-peroxisome proliferator-activated receptor (transcription factors), P53-tumor protein 53, eIF2 α-eukaryotic initiation factor 2α, p-eIF2 α-phosphorylated-eukaryotic initiation factor 2α, EGR-1- early growth response protein 1, p-EGR1-EGR-1 phosphorylated, XBPIu-unspliced X-box-binding protein 1, XBPIs-spliced X-box-binding protein 1, CHOP-transcription factor C/EBP homologous protein, ATF3-Activating transcription factor 3, Factor ATF-2-transcription activator factors (Thr71), c-Jun-transcription factor AP-1, c-Fos-transcription factor encoded by the c-fos gene, NF-Kb-Nuclear Factor Kappa Beta, p-p38-p38 phosphorylation.

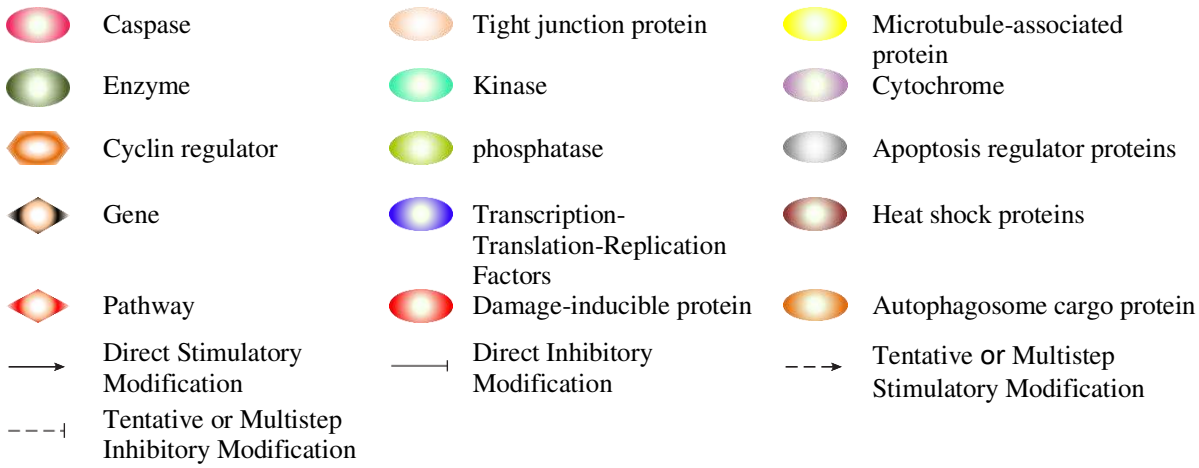

Figure 2: Scheme to control patulin-producing molds in food using biotechnological methods. The biocontrol agents and antifungal molecule act through one or more mechanisms to prevent patulin biosynthesis in foods: (1) inhibition of spore production, (2) inhibition of spore germination, (3) inhibition of mold growth, (4) inhibition of patulin biosynthesis. "X" means inhibition.

Figure 3: Scheme to degrade patulin using biotechnological methods

Figure 4: Scheme to remove patulin using biotechnological methods