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Development of a Taxon-Specific Real-Time Polymerase Chain Reaction Method to Detect *Trichoderma reesei* Contaminations in Fermentation Products

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Abstract: Recently, a genetically modified microorganism (GMM) detection strategy using real-time PCR technology was developed to control fermentation products commercialized in the food and feed chain, allowing several unexpected GMM contaminations to be highlighted. Currently, only bacterial strains are targeted by this strategy. Given that fungal strains, like *Trichoderma reesei*, are also frequently used by the food industry to produce fermentation products, a novel real-time PCR method specific to this fungal species was developed and validated in this study to reinforce the GMM detection strategy. Designed to cover a sequence of 130 bp from the translation elongation factor alpha 1 (Tef1) gene of *T. reesei*, this real-time PCR method, namely TR, allows for the screening of commercial fermentation products contaminated with *T. reesei*, genetically modified or not, which is one of the major fungal species used as an industrial platform for the manufacturing of fermentation products. The developed real-time PCR TR method was assessed as specific and sensitive (LOD$_{95\%}$ = eight copies). In addition, the developed real-time PCR TR method performance was confirmed to be in line with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories. The validated real-time PCR TR method was also demonstrated to be applicable to commercial microbial fermentation products. Based on all these results, the novel real-time PCR TR method was assessed as valuable for strengthening the current GMM detection strategy regarding major fungal species used by the food industry to produce microbial fermentation products.

Keywords: food control; fungal fermentation products; real-time PCR; detection; *Trichoderma reesei*

1. Introduction

Both bacterial and fungal strains, genetically modified or not, are broadly used by the food industry for the production of fermentation products, including enzymes and additives. Among these microbial species of interest, *Bacillus subtilis* and *B. licheniformis* for bacterial species, and *Trichoderma reesei*, *Aspergillus niger*, and *A. oryzae* for fungal species, are the majority used [1–12].

Recently, a real-time PCR strategy was developed to detect genetically modified bacterial strains, and numerous commercial fermentation products were unexpectedly notified for genetically modified bacterial contamination, including DNA and viable cells [13–22]. In addition to the subsequent associated traceability concerns, potential public health concerns were raised. Indeed, since genetically modified microorganisms (GMMs) used for the production of fermentation products generally carry antimicrobial resistance genes as selection markers, there were also inquiries about the potential horizontal transfer of such antimicrobial resistance genes to gut microbiota and pathogens [23–33].

Currently, the developed GMM detection strategy focuses exclusively on bacterial strains. However, given that approximately half of fermentation products are made using fungal strains [2,3], the proposed GMM detection strategy needs to be reinforced regarding

fungal contamination. For this purpose, a novel real-time PCR method was developed in this study to screen for the presence of *T. reesei*, one of the major fungal species used as an industrial platform for manufacturing fermentation products [2,3,34,35]. This developed taxon-specific real-time PCR method, namely TR, was assessed for its performance, including its specificity, sensitivity, and applicability. The real-time PCR TR method was also evaluated for its compatibility with the "Minimum Performance Requirements (MPR) for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL) to assess its suitability for enforcement purposes [36].

2. Materials and Methods

2.1. Materials

DNA from an artificially synthetized control plasmid (Genecust) carrying a single copy of the *T. reesei* sequence targeted by the real-time PCR TR method was used. DNA from *Homo sapiens* (G3041 from Promega), *Zea mays* (ERM-BF413ak from JRC IRMM), wild-type (WT) microbial species, and genetically modified bacterial strains (*B. subtilis* RASFF2014.1249 and *B. velezensis* RASFF2019.333) was obtained as previously reported (Tables 1–3). All WT microbial species were collected from the BCCM (Belgian Coordinated Collection of Microorganisms) Consortium (collection number starting by IHEM, MUCL, and LMG), the American Type Culture Collection (ATCC), the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), the CBS-KNAW Fungal Biodiversity Centre (collection number starting with CGS), and Sciensano (collection number starting with TIAC and RASFF). The associated strain collection numbers are indicated in Table 2. DNA from 10 microbial fermentation products (samples n°1–10) commercialized on the European market was extracted using the NucleoSpin Food kit (Macherey-Nagel), as previously reported (Table 4). DNA concentration and purity were measured and evaluated as previously described [15–22].

Table 1. Targeted *T. reesei* sequence and oligonucleotides from the newly developed real-time PCR TR method targeting *T. reesei*.

Targeted *T. reesei* Sequence			
agtcacccaacgtcatcaacgcagcagttttcaatcagcgatgctaacc atattccctcgaacaggaagccgccgaactcggcaagggttccttcaagtacgcgtgggttcttgacaagctcaaggccga			
Oligonucleotides		Annealing Temperature	Expected Amplicon Sizes
Names	Sequences		
TR-F TR-P TR-R	AGTCACCCAACGTCATCA FAM-ATATTCCCTCGAACAGGAAGCCGC-TAMRA TCGGCCTTGAGCTTGT	60 °C	130 bp

On the targeted *T. reesei* sequences, the positions of the used primers and probe are underlined.

Table 2. Specificity evaluation of the newly developed real-time PCR TR method.

Kingdom	Genus	Species	Strain Number	Real-Time PCR TR Method
Fungi	*Aspergillus*	*acidus*	IHEM 26,285	-
	Aspergillus	*aculeatus*	IHEM 5796	-
	Aspergillus	*brasiliensis*	IHEM 3766	-
	Aspergillus	*costaricaensis*	IHEM 21,971	-
	Aspergillus	*fijiensis*	IHEM 22,812	-
	Aspergillus	*flavus*	IHEM 932	-
	Aspergillus	*flavus*	IHEM 2465	-
	Aspergillus	*flavus*	IHEM 5785	-
	Aspergillus	*heteromorphus*	IHEM 5801	-
	Aspergillus	*ibericus*	IHEM 23498	-
	Aspergillus	*melleus*	IHEM 25956	-

Table 2. *Cont.*

Kingdom	Genus	Species	Strain Number	Real-Time PCR TR Method
	Aspergillus	*neoniger*	IHEM 2463	-
	Aspergillus	*neoniger*	IHEM 21592	-
	Aspergillus	*niger*	IHEM 25485	-
	Aspergillus	*niger*	IHEM 5296	-
	Aspergillus	*niger*	IHEM 3415	-
	Aspergillus	*niger*	IHEM 5622	-
	Aspergillus	*niger*	IHEM 5788	-
	Aspergillus	*niger*	IHEM 5844	-
	Aspergillus	*niger*	IHEM 2312	-
	Aspergillus	*oryzae*	IHEM 25836	-
	Aspergillus	*oryzae*	IHEM 27253	-
	Aspergillus	*oryzae*	IHEM 4381	-
	Aspergillus	*oryzae*	IHEM 4382	-
	Aspergillus	*oryzae*	IHEM 5782	-
	Aspergillus	*oryzae*	IHEM 5789	-
	Aspergillus	*piperis*	IHEM 5316	-
	Aspergillus	*tubingensis*	IHEM 1941	-
	Aspergillus	*tubingensis*	IHEM 6184	-
	Aspergillus	*tubingensis*	IHEM 5615	-
	Aspergillus	*vadensis*	IHEM 26351	-
	Aspergillus	*welwitschiae*	IHEM 2864	-
	Aspergillus	*welwitschiae*	IHEM 2969	-
	Candida	*cylindracea*	MUCL 41387	-
	Candida	*rugosa*	IHEM 1894	-
	Chaetomium	*gracile*	MUCL 53569	-
	Cryphonectria	*parasitica*	MUCL 7956	-
	Disporotrichum	*dimorphosporum*	MUCL 19341	-
	Fusarium	*venenatum*	MUCL 55417	-
	Hansenula	*polymorpha*	MUCL 27761	-
	Humicola	*insolens*	MUCL 15010	-
	Kluyveromyces	*lactis*	IHEM 2051	-
	Leptographium	*procerum*	MUCL 8094	-
	Mucor	*javanicus*	IHEM 5212	-
	Penicillium	*camemberti*	IHEM 6648	-
	Penicillium	*chrysogenum*	IHEM 3414	-
	Penicillium	*citrinium*	IHEM 26159	-
	Penicillium	*decumbens*	IHEM 5935	-
	Penicillium	*funiculosum*	MUCL 14091	-
	Penicillium	*multicolour*	CBS 501.73	-
	Penicillium	*roqueforti*	IHEM 20176	-
	Pichia	*pastori*	MUCL 27793	-
	Rhizomucor	*miehei*	IHEM 26897	-
	Rhizopus	*niveus*	ATCC 200757	-
	Rhizopus	*oryzae*	IHEM 26078	-
	Saccharomyces	*cerevisiae*	IHEM 25104	-
	Sporobolomyces	*singularis*	MUCL 27849	-
	Talaromyces	*cellulolyticus/pinophilus*	IHEM 16004	-
	Talaromyces	*emersonii*	DSMZ 2432	-
	Trametes	*hirsute*	MUCL 30869	-
	Trichoderma	*atroviride*	IHEM 745	-
	Trichoderma	*citrinoviride*	IHEM 25858	-
	Trichoderma	*harzianum*	IHEM 5435	-
	Trichoderma	*longibrachiatum*	IHEM 935	-
	Trichoderma	*reesei*	IHEM 5264	+ (C_q: 20.0)
	Trichoderma	*reesei*	IHEM 5476	+ (C_q: 20.4)
	Trichoderma	*reesei*	IHEM 5648	+ (C_q: 20.7)

Table 2. *Cont.*

Kingdom	Genus	Species	Strain Number	Real-Time PCR TR Method
	Trichoderma	*reesei*	IHEM 5652	+ (C$_q$: 22.7)
	Trichoderma	*reesei*	IHEM 4122	+ (C$_q$: 19.1)
	Trichoderma	*viride*	IHEM 4146	-
Bacteria	*Arthrobacter*	*ramosus*	LMG 17309	-
	Bacillus	*amyloliquefaciens*	LMG 12331	-
	Bacillus	*brevis*	LMG 7123	-
	Bacillus	*cereus*	ATCC 14579	-
	Bacillus	*circulans*	LMG 6926T	-
	Bacillus	*coagulans*	LMG 6326	-
	Bacillus	*firmus*	LMG 7125	-
	Bacillus	*flexus*	LMG 11155	-
	Bacillus	*lentus*	TIAC 101	-
	Bacillus	*licheniformis*	LMG 7558	-
	Bacillus	*megaterium*	LMG 7127	-
	Bacillus	*pumilus*	DSMZ 1794	-
	Bacillus	*smithii*	LMG 6327	-
	Bacillus	*subtilis*	LMG 7135T	-
	Bacillus	*subtilis*	GMM RASFF2014.1249	-
	Bacillus	*velezensis*	LMG 12384	-
	Bacillus	*velezensis*	GMM RASFF2019.3332	-
	Cellulosimicrobium	*cellulans*	LMG 16121	-
	Corynebacterium	*glutamicum*	LMG 3652	-
	Enterococcus	*faecium*	LMG 9430	-
	Escherichia	*coli*	LMG 2092T	-
	Geobacillus	*caldoproteolyticus*	DSMZ 15730	-
	Geobacillus	*pallidus*	LMG 11159T	-
	Geobacillus	*stearothermophilus*	LMG 6939T	-
	Klebsiella	*pneumonia*	LMG 3113T	-
	Lactobacillus	*casei*	LMG 6904	-
	Lactobacillus	*fermentum*	LMG 6902	-
	Lactobacillus	*plantarum*	LMG 9208	-
	Lactobacillus	*rhamnosus*	LMG 18030	-
	Lactococcus	*lactis*	LMG 6890T	-
	Leuconostoc	*citreum*	LMG 9824	-
	Microbacterium	*imperiale*	LMG 20190	-
	Paenibacillus	*alginolyticus*	LMG 18723	-
	Paenibacillus	*macerans*	LMG 6324	-
	Protaminobacter	*rubrum*	CBS 574.77	-
	Pseudomonas	*amyloderamosa*	ATCC 21262	-
	Pseudomonas	*fluorescens*	LMG 1794T	-
	Pullulanibacillus	*naganoensis*	LMG 12887	-
	Streptomyces	*aureofaciens*	LMG 5968	-
	Streptomyces	*mobaraensis*	DSMZ 40847	-
	Streptomyces	*murinus*	LMG 10475	-
	Streptomyces	*netropsis*	LMG 5977	-
	Streptomyces	*rubiginosus*	LMG 20268	-
	Streptomyces	*violaceoruber*	LMG 7183	-
	Streptoverticillium	*mobaraense*	CBS 199.75	-
Plantae	*Oryzae*	*sativa*	/	-
Animalia	*Homo*	*sapiens*	/	-

The presence or absence of amplification are, respectively, symbolized by + and -. For each result, the experiment was performed in triplicate on 10 ng of each sample. The mean values of the observed C$_q$ values are given in brackets.

Table 3. Sensitivity evaluation of the newly developed real-time PCR TR method.

	Estimated Target Copy Number						
	50	**20**	**10**	**5**	**1**	**0.1**	**0**
Real-Time PCR TR Method	+ (12/12) (C_q: 34.6)	+ (12/12) (C_q: 36.0)	+ (12/12) (C_q: 36.9)	+ (9/12) (C_q: 38.2)	+ (5/12) (C_q: 39.9)	- (0/12)	- (0/12)

The presence or absence of amplification are symbolized by + and -, respectively. For each target copy number tested, 12 replicates were used. The number of positive replicate(s) out of the 12 replicates tested is indicated, and the mean values of the observed C_q values are given in brackets.

Table 4. Applicability evaluation of the newly developed and in-house validated real-time PCR TR method using commercial food enzyme products.

	Samples	Labeled Microbial Production Sources	Forms	Applications	Brands	Real-Time PCR Methods	
						BSG	**TR**
1	Alpha-amylase, protease, cellulase, xylanase, beta-glucanase—RASFF2019.3332	*Aspergillus* sp., *Bacillus* sp., *Trichoderma* sp.	Solid	Distillery, brewing	A	+′ (C_q: 20.6)	+ (C_q: 24.0)
2	Beta-glucanase	*Trichoderma* sp.	Solid	Unknown	B	+ (C_q: 36.7)	+ (C_q: 28.1)
3	Neutral protease—RASFF2019.3332	*Bacillus* sp.	Solid	Baking, distillery, brewing	A	+′ (C_q: 19.5)	+ (C_q: 30.2)
4	Alpha-amylase—RASFF2020.2582	Unknown	Solid	Distillery, brewing	C	+′ (C_q: 31.2)	+ (C_q: 32.9)
5	Alpha-amylase	*Bacillus* sp.	Liquid	Unknown	B	+′ (C_q: 22.9)	+ (C_q: 33.5)
6	Alpha-amylase—RASFF2020.2846	Bacteria	Liquid	Distillery, brewing	D	+′ (C_q: 19.8)	+ (C_q: 35.4)
7	Alpha-amylase—RASFF2020.2579	Bacteria	Solid	Distillery, brewing	E	+′ (C_q: 22.6)	- *
8	Alpha-amylase—RASFF2020.2577	Unknown	Solid	Distillery	F	+′ (C_q: 19.4)	- *
9	Alpha-amylase—RASFF2020.2577	Unknown	Solid	Distillery	G	+′ (C_q: 19.5)	- *
10	Alpha-amylase	Unknown	Liquid	Distillery, brewing	H	-′	-

For all these food enzyme samples, available labeling information associated with the intended areas of use as well as with the microbial production sources is provided. Moreover, the information related to the form of the sample (solid or liquid) is given, and the brand names are anonymously symbolized by A–H. If applicable, the associated RASFF notification number is cited. These samples were analyzed in duplicate at a concentration of 10 ng using the real-time PCR BSG and TR methods. The averages of the observed C_q are indicated under brackets. The real-time PCR results were generated either in this study or previously (indicated by ′) [14]. The absence or presence of PCR amplification are, respectively, represented by - and +. Only PCR amplification signals above the LOD$_{95\%}$ of the tested real-time PCR method were considered to be positive, ensuring consequently consistent and reproducible analysis results. If below the LOD$_{95\%}$ of the tested real-time PCR method, the PCR amplification signal was symbolized by -*. The LOD$_{95\%}$ is at 8 estimated target copies for the real-time TR method (experimental C_q at 36.9 for 10 estimated target copies) (Tables 3 and S4) and at 22 estimated target copies for the real-time BSG method (experimental C_q at 38.2 for 100 estimated target copies) [14].

2.2. Development and Validation of the Real-Time PCR TR Method

Based on previous studies [37–39], the translation elongation factor alpha 1 (Tef1) gene was selected to develop a taxon-specific real-time PCR method targeting *T. reesei* species. Using the Primer3 (v. 0.4.0) software, a set of primers and probes was designed, allowing for the amplification of 130 bp of the *T. reesei* Tef1 gene (Table 1) [40,41]. Each

real-time PCR assay was applied as previously described. The real-time PCR program comprised an annealing/extension step at 60 °C. Each real-time PCR run included a no-template control (NTC) and a positive control (DNA from the *T. reesei* IHEM 5264 strain) (Table 2).

2.2.1. Specificity Evaluation

First, the in silico specificity of the newly developed real-time PCR TR method was tested. On the one hand, the sequence amplified by the real-time PCR TR method targeting *T. reesei* (Table 1) was blasted against the NCBI nucleotide collection (nr/nt) database (access on June 2022; default parameters) as well as against the NCBI Ref-Seq Genome database (access on June 2022; default parameters; Fungi (taxid:4751)) (Tables S1 and S2). On the other hand, the hybridization properties of the targeted regions and the designed set of primers and probes were examined using SCREENED v1.0 [22,42]. The used parameter settings were (i) maximum 10% for mismatches in the annealing sites, (ii) minimum 90% for the length of the alignment in the annealing sites, and (iii) no mismatch in the last five nucleotides at the 3′ end for primers. The targeted regions were collected from a sequence dataset of the NCBI Genome database (access on June 2022; filter: *Trichoderma*). The database contained 90 items, including *T. afroharzianum, T. arundinaceum, T. asperelloides, T. asperellum, T. atrobrunneum, T. atroviride, T. brevicompactum, T. brevicrassum, T. citrinoviride, T. cornu-damae, T. erinaceum, T. gamsii, T. gracile, T. guizhouense, T. hamatum, T. harzianum, T. koningii, T. koningiopsis, T. lentiforme, T. lixii, T. longibrachiatum, T. oligosporum, T. parareesei, T. pleuroti, T. pseudokoningii, T. reesei, T. semiorbis, T. simmonsii, T. virens,* and *T. viride.*

The specificity of the developed real-time PCR TR method was then experimentally tested in triplicates on 10 ng of DNA from positive and negative materials (Table 2). For the positive materials, DNA extracted from 5 WT *T. reesei* strains was used. For the negative materials, DNA extracted from animals (*Homo sapiens*), plants (*Zea mays*), 113 WT microbial strains, bacterial and fungal species often used by the food industry to manufacture fermentation products, and 2 genetically modified *Bacillus* strains producing vitamin B_2 or protease (RASFF2014.1249 and RASFF2019.3332) was used [15–22].

The amplicon generated from the *T. reesei* IHEM 5264 strain using the developed real-time PCR TR method was purified and sequenced as previously described [20]. Using the Clustal Omega multiple sequence alignment software (v1.2.4) with default parameters, the generated sequence was aligned against the targeted *T. reesei* reference sequence (Tables 1 and S3) [43].

2.2.2. Sensitivity Evaluation

Using DNA from the artificially synthetized control plasmid carrying a single copy of the targeted *T. reesei* sequence, serial dilutions were prepared ranging from 50 to 0 estimated target copy numbers. Each dilution point was then tested in 12 replicates using the developed real-time PCR TR method (Table 3). Based on the control plasmid size (3161 bp), the estimated target copy numbers for each dilution point were calculated, as previously described [20]. The limit of detection ($LOD_{95\%}$) was determined as previously described (Table S4) [20,44–46]. The plausibility check for the probability of detection (POD) curve presented no irregularities. Moreover, the POD curve was associated with a limit of detection ($LOD_{95\%}$) below 25 estimated target copies.

2.2.3. Applicability Evaluation

Several commercial food enzyme products were used to evaluate the applicability of the developed real-time PCR TR method (Table 4). For each sample (n°1–10), the real-time PCR TR method was applied in duplicate on 10 ng of DNA. These food enzyme products, in liquid or solid forms, were collected from different brands and are designed to be used in different sectors such as brewing, distillery, and baking. These food enzyme products were labeled as containing beta-glucanase, alpha-amylase, protease, cellulase, and xylanase.

In samples n°1, 3–9, an unauthorized contamination with genetically modified bacterial strains was previously detected (RASFF2019.3332, RASFF2020.2577, RASFF2020.2579, and RASFF2020.2582). In addition to the real-time PCR TR method, these 10 food enzyme samples were also investigated for the presence of DNA from the *Bacillus subtilis* group, as previously described [22].

3. Results and Discussion

3.1. Development of the Real-Time PCR TR Method

According to the FEDA (Food Enzyme Database—accessed in May 2023), 150 food enzyme dossiers using GMMs are currently submitted for evaluation by EFSA. Of those food enzymes obtained from GMMs, 40% are produced by bacterial strains and 60% are produced by fungal strains. Among these fungal strains, the majority belong to only three species: *A. niger* (41.1%), *T. reesei* (27.8%), and *A. oryzae* (16.7%) [2,3,47]. The detection of such fungal species represents, therefore, a warning signal of possible contamination with producer organisms, including genetically modified strains, in food enzyme samples.

However, although more than half of the genetically modified microbial strains used to produce food enzymes belong to fungal species, the GMM detection strategy recently proposed that the control of GMM contamination in commercial microbial fermentation products nowadays only targets genetically modified bacterial strains. Moreover, to our knowledge, no real-time PCR method, being the most popular technology to control GMOs in the food and feed chain, was developed or validated to target these three key fungal species in commercial microbial fermentation products. Therefore, a taxon-specific real-time PCR targeting *T. reesei* was designed, developed, and validated in-house in this study.

Based on previous studies [37–39], the translation elongation factor alpha 1 (Tef1) gene from *T. reesei* was selected to develop the newly developed real-time PCR TR method (Table 1). Using the software Primer3, a set of primers and probes was designed, allowing for the amplification of 130 bp of the Tef1 gene.

3.2. Specificity Assessment of the Real-Time PCR TR Method

The specificity of the newly developed real-time PCR TR method was first confirmed in silico (Tables S1 and S2). On the one hand, in blasting the sequence generated by the real-time PCR TR method against the NCBI nucleotide collection (nr/nt) database, 30 hits of 100% in terms of coverage and identity were observed, all belonging to *T. reesei* (Table S1). Moreover, among all the fungal species genomes from the NCBI RefSeq Genome Database, a hit of 100% in terms of coverage and identity was only observed with *T. reesei* (Table S2). On the other hand, using SCREENED on a dataset composed of all *Trichoderma* sp. genome sequences extracted from the NCBI Genome database, a theoretical PCR amplification with the developed real-time PCR TR method was predicted only for *T. reesei* (Genbank: CP016234.1 *T. reesei* QM6a chromosome III).

The specificity of the newly developed real-time PCR TR method was then experimentally demonstrated using bacterial and fungal species often used by the food and feed industry to manufacture microbial fermentation products [2,3,15–22,47]. As positive controls, five WT *T. reesei* strains were used. As negative controls, 108 WT microbial strains and 2 genetically modified bacterial strains (RASFF2014.1249 and RASFF2019.3332) were used. In addition, one plant material and one animal material were tested. Among the 108 WT microbial strains, 43 bacterial species and 65 strains from 49 fungal species not belonging to *T. reesei* were included (Table 2). As expected, all the positive controls presented an amplification, while no amplification was observed for all the negative controls. Moreover, the sequence generated from the *T. reesei* IHEM 5264 strain, used as a positive control, showed 100% identity and coverage with the target *T. reesei* reference sequence (Table S3).

As a positive signal was exclusively detected in the samples containing the targeted *T. reesei* sequences and no false positive signals or false negative signals were reported, the developed real-time PCR TR method was consequently assessed as specific.

3.3. Sensitivity Assessment of the Real-Time PCR TR Method

The sensitivity of the newly developed real-time PCR TR method was assessed according to the international standard (ISO Standard 16140-2:2014). Using a control plasmid carrying a single copy of the target sequence from the *T. reesei* Tef1 gene, serial dilutions of DNA from the control plasmid, ranging from 50 to 0 estimated target copy numbers, were tested (Table 3).

At as low as 10 estimated target copies, an amplification signal was detected for all 12 replicates. Moreover, up to one estimated target copy, a positive signal was detected. Based on all positive and negative signals observed for all 12 replicates at each serial dilution point tested, the $LOD_{95\%}$ of the real-time PCR TR method was calculated and established at eight estimated target copies (Table S4). Presenting an $LOD_{95\%}$ lower than 25 estimated target copies, the newly developed real-time PCR TR method was assessed as sensitive.

This taxon-specific method is the first real-time PCR method designed to specifically screen for the presence of DNA from *T. reesei* in microbial fermentation products, with performance complying with the "MPR for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories, which is the standard used by GMO enforcement laboratories [36].

3.4. Applicability Assessment of the Real-Time PCR TR Method

The applicability of the developed and in-house validated real-time PCR TR method was assessed using several commercial food enzyme products (Table 4). In liquid or solid forms, these products were collected from different brands and were intended for various sectors, such as brewing, distillery, and baking. These food enzyme products were labeled as containing beta-glucanase, alpha-amylase, protease, cellulase, or xylanase. Previously, all these food enzyme samples, except sample n°10, were reported for the presence of DNA specific to the *B. subtilis* group, using the real-time PCR BSG method (Table 4). In addition, contaminations of most of these samples (n°1, 3–9) with genetically modified *Bacillus* strains were identified previously (RASFF2019.3332, RASFF2020.2577, RASFF2020.2579, and RASFF2020.2582).

Among the 10 investigated food enzyme samples, the presence of DNA specific to *T. reesei* was detected in 6 samples (n°1–6) with an amplification signal above the $LOD_{95\%}$ of the real-time PCR TR method (Table 4). For samples n°1–2, *T. reesei* was labeled as being the food enzyme-producing microbial species. These samples presented the lowest C_q values observed for the real-time PCR TR method. This is consistent with the product information available on the label. For sample n°4, a C_q value was also observed for the real-time PCR TR method, although *T. reesei* was not labeled as being the food enzyme-producing microbial species. For samples n°3, 5–6, only bacterial species, including from the *Bacillus* genus, were labeled as being the food enzyme-producing species. These samples showed low C_q values for the real-time PCR BSG method, in line with the labeled product information. However, a C_q value for the real-time PCR TR method was also observed. The origin of such *T. reesei* contaminations in samples n°3 and 5 could potentially be related to the production chain because these samples belong to the same brands as samples n°1 and 2, respectively. Regarding sample n°6, the origin of *T. reesei* contamination is unknown based on the available information. It could also be related to the production chain since mixes of food enzymes are manufactured using both *Bacillus* and *Trichoderma* species, as illustrated by sample n°1.

In 4 out of the 10 tested food enzyme samples (n°7–10), no *T. reesei* DNA was detected since either no amplification signal or an amplification signal below the $LOD_{95\%}$ of the real-time PCR TR method were observed, indicating that no impurity with DNA from

T. reesei was present (Table 4). For these four food enzyme samples, the labeling did not indicate that *T. reesei* was used for their manufacture. The food enzyme-producing microbial species were either labeled as belonging to the bacterial kingdom for sample n°7 or were non-labeled (unknown) for samples n°8–10.

According to all these results, the newly developed real-time PCR TR method was confirmed to be applicable to commercial food enzyme products. In addition, contamination with DNA specific to *T. reesei* with an amplification signal above the $LOD_{95\%}$ was observed in several samples (n°1–6) (Table 4).

4. Conclusions

In this study, the real-time PCR TR method specific to the *T. reesei* species, whose genetically modified strains are widely used by the food industry to manufacture microbial fermentation products, was developed and validated in-house. This method was successfully evaluated as being specific since no false positive or false negative results were observed. In addition, in line with the "MPR for Analytical Methods of GMO Testing", the method was assessed as being sensitive, allowing for the detection of *T. reesei* contaminations even at the trace level. Finally, the applicability of this real-time PCR method was demonstrated on several commercial microbial fermentation products. On this basis, the unexpected presence of DNA from *T. reesei*, genetically modified or not, was discovered, highlighting the relevance of this real-time PCR method to control unexpected biological impurities in the food and feed chain. In the future, additional real-time PCR methods specific to the *A. niger* and *A. oryzae* species, whose genetically modified strains are also frequently used by the food industry, could be developed to strengthen the control of unexpected fungal impurities in the food and feed chains. However, such real-time PCR methods allow only the screening of suspicious samples containing DNA specific to key fungal species. To clearly demonstrate the presence of genetically modified fungal strains, further investigations of the identified suspicious samples need to be performed to identify unnatural associations of sequences [19,48,49]. For this purpose, a whole-genome sequencing strategy may be considered. Here, a prior isolation of GMM strains, usually carried out by classical microbiology, is mandatory. However, for such GMMs used to produce microbial fermentation products, both bacterial and fungal strains, genetic information, including sequencing data, is confidential, which critically hampers the controls performed by enforcement laboratories to guarantee the traceability of commercial microbial fermentation products. Therefore, without publicly available information on the GMM strains of interest, this isolation step is particularly challenging due to the enormous list of microbial growth conditions to be tested, including possible auxotrophic mutations [15,48,50–54]. In the absence of prior knowledge, a high-throughput sequencing strategy, like metagenomics, represents an interesting and promising option, as recently demonstrated [49,55–60]. Nonetheless, metagenomics for the detection of GMMs in fermentation products is not yet mature enough to be implemented at the level of enforcement laboratories. In addition, its performance in terms of sensitivity is currently expected to be limited. To overcome this latter issue, a targeted sequencing strategy involving a prior enrichment step of key sequences is possible but consequently requires a minimum of publicly available information on the GMM strains used to manufacture fermentation products [19,20,61–66].

reference Tef1 gene sequence from *T. reesei* (NW_006711153.1); Table S4: Calculation of LOD$_{95\%}$ according to the POD curve for the newly developed real-time PCR TR method.

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