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Pilot market surveillance of GMM contaminations in alpha-amylase food enzyme products: A detection strategy strengthened by a newly developed qPCR method targeting a GM *Bacillus licheniformis* producing alpha-amylase

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ABSTRACT

Using high-throughput metagenomics on commercial microbial fermentation products, DNA from a new unauthorized genetically modified microorganism (GMM), namely the GM *B. licheniformis* strain producing alpha-amylase (GMM alpha-amylase2), was recently discovered and characterized. On this basis, a new qPCR method targeting an unnatural association of sequences specific to the GMM alpha-amylase2 strain was designed and developed in this study, allowing to strengthen the current GMM detection strategy. The performance of the newly developed qPCR method was assessed for its specificity and sensitivity to comply with the minimum performance requirements established by the European Network of GMO Laboratories for GMO analysis. Moreover, the transferability of the *in house* validated qPCR method was demonstrated. Finally, its applicability was confirmed by a pilot market surveillance of GMM contaminations conducted for the first time on 40 alpha-amylase food enzyme products labelled as containing alpha-amylase. This pilot market surveillance allowed also to highlight numerous contaminations with GMM alpha-amylase2, including frequent cross-contaminations with other GMM strains previously characterized. In addition, the presence of full-length AMR genes, raising health concerns, was also reported.

1. Introduction

Several unexpected contaminations of unauthorized genetically modified microorganisms (GMM) in commercial fermentation products, including food enzymes (FE) and food and feed additives, have recently been reported in the European market. However, currently, no GMM, whether viable strains or associated recombinant DNA, are authorized in the food and feed chain (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Deckers et al., 2021a; Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021a; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Paracchini et al., 2017; RASFF portal; Regulation (EC) No 1829/2003, Regulation (EC) No 1830/2003). In addition to the European legislation, considering that antimicrobial resistance (AMR) genes are often harboured by such GMM contaminations, public health concerns, associated with the potential risk of AMR horizontal transfer to gut microbiota and pathogens, were raised (Florez-Cuadrado et al., 2018; Nadeem et al., 2020; Tóth et al., 2020; von Wintersdorff et al., 2016; von Wrighta and Bruce, 2003). Consequently, a GMM detection strategy based on qPCR technology was developed for enforcement laboratories to support the Competent Authorities to ensure the food safety and traceability (Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021a; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). This GMM detection strategy is currently used by several European enforcement laboratories to control commercial microbial fermentation products for which sample analyses are regularly requested by the Competent Authorities. The presence of GMM is firstly

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screened by targeting key genetic elements commonly found in genetically modified (GM) bacteria used for the production of fermentation products, including the *Bacillus subtilis* group (16S-23S region), three AMR genes conferring resistance to chloramphenicol (*cat*), tetracycline (*tet-l*) or kanamycin (*aadD*), as well as the pUB110 shuttle vector carrying *aadD* (Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2021b; Fraiture et al., 2022). After obtaining a positive signal for the screening step, an identification step is conducted to demonstrate the presence of specific GMM strains by targeting unnatural associations of sequences specific to known GMM strains, as performed for any genetically modified organisms (GMO) (ENGL, 2015). All negative and positive amplifications observed by the enforcement laboratories are reported to the Competent Authorities allowing them to take the most appropriate action for the products concerned (Fraiture et al., 2020d; Fraiture et al., 2021c; Fraiture et al., 2021d). Currently, no GMM production strains are authorized to be present in the food and feed chain. Consequently, in contrast to GMO intended for human consumption and for animal feed, no methods specific to GMM events are distributed to enforcement laboratories, while sequence information is also confidential. Therefore, unknown sequences allowing the identification of each GMM need to be experimentally identify and characterized. Up to now, using various sequencing strategies (e.g., whole-genome sequencing, metagenomics, DNA walking sequencing), unnatural associations of sequences specific to five unknown GM bacterial strains have been characterized, including the GM *B. subtilis* strain producing vitamin B_2 (GMM vitamin B_2), the GM *B. velezensis* strain producing protease (GMM protease1), the GM *B. amyloliquefaciens* strain producing protease (GMM protease2), the GM *B. amyloliquefaciens* strain producing alpha-amylase (GMM alpha-amylase1) and the GM *B. licheniformis* strain producing alpha-amylase (GMM alpha-amylase2) (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Buytaers et al., 2021; D'aes et al., 2021; D'aes et al., 2022; Fraiture et al., 2020d; Fraiture et al., 2021b,c,d; Paracchini et al., 2017). The discovery of these sequences has allowed the development of qPCR methods for the identification by enforcement laboratories of all these GM bacterial strains, with the exception of the GMM alpha-amylase2 strain that was very recently discovered through metagenomic sequencing. These qPCR methods were validated in accordance with the minimum performance requirements (MPR) for GMO analysis, a gold standard for qPCR method validation, ensuring an harmonized and reliable control by all enforcement laboratories (Barbau-Piednoir et al., 2015b; D'aes et al., 2022; ENGL, 2015; Fraiture et al., 2020d; Fraiture et al., 2021c, Fraiture et al., 2021d). Therefore, at the present time, enforcement laboratories have the full panel of qPCR methods targeting all GMM strains currently discovered and characterized, except for the GMM alpha-amylase2 strain. Moreover, in the absence of a qPCR method targeting the GMM alpha-amylase2 strain, no information is currently available on the prevalence and level of contamination in the food and feed chain with the GMM apha-amylase2 strain.

In this study, based on the recently characterized unnatural associations of sequences specific to the GMM alpha-amylase2 strain, we have designed, developed and validated a new qPCR method targeting specifically the GMM alpha-amylase2 strain in order to strengthen the current GMM detection strategy. This qPCR method specific to the GMM alpha-amylase2 strain was designed to be compatible with the qPCR conditions used for all GMM methods previously developed for the GMM detection strategy. The performance of the qPCR method specific to the GMM alpha-amylase2 strain, including specificity and sensitivity, was assessed with respect to the MPR for GMO analysis (ENGL, 2015). The *in house* validated method was also for transferability, allowing other enforcement laboratories to control GMM alpha-amylase2 contaminations on the market. Moreover, the applicability of the new qPCR method was assessed through a pilot market surveillance of GMM contaminations applied on 40 commercial FE products labelled as containing alpha-amylase. This FE is one of the most popular FE used by the food industry for a wide range of applications (e.g., starch processing, brewing, baking, fruit processing, digestive aids) (de Souza and de Oliveira Magalhães, 2010; Deckers et al., 2021a; Deckers et al., 2021b; Raveendran et al., 2018). Through this pilot market surveillance applied for the first time on such FE products, we have investigated the presence of GMM alpha-amylase2 contaminations as well as additional cross-contaminations with other GMM strains previously characterised. In addition, as all these GMM strains carried AMR genes as selective marker and as DNA from AMR genes was detected by qPCR during this pilot market surveillance, the presence of full-length AMR genes, raising health concerns, was also investigated.

2. Materials and methods

2.1. Materials

DNA from the control plasmid (Genecust, France), artificially synthetized to carry one copy of the sequence targeted by the developed GMM alpha-amylase2 qPCR method, was used. DNA from microbial, human, plant and commercial FE materials (Tables 1–4) were obtained as previously described (D'aes et al., 2022; Deckers et al., 2021; Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). For each commercial FE sample, the DNA extraction was carried out independently. DNA concentration and purity were measured as previously described (Fraiture et al. 2021d; Fraiture et al., 2022).

2.2. qPCR assays

Each qPCR assay was performed using 1X SsoAdvanced universal probes supermix (Bio-Rad, USA) as previously described (Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). The qPCR program included an annealing-

Table 1
Oligonucleotides of the qPCR method targeting the GMM alpha-amylase2. On the targeted sequence, the locations of the used oligonucleotides are indicated in bold. The targeted sequence is composed of a part belonging to the downstream region of *B. licheniformis catA* (dashed underline) and a part belonging to *B. licheniformis amyS* (dotted underline).

Targeted sequence

AACGATCGATCGCAGAAATCGCTTCAATGGTAGGCATTGCGA**ATGCGGAATATCAAGCTTATCGGGC**CGCTCTAGAACTAGTGGATCACCCGCGATACCGTCATTT**TCGACACATTTGC TTTCTTTG**

Oligonucleotides Names	Sequences	Annealing temperature	Expected amplicon size
GMM alpha-amylase2-F	AACGATCGATCGCAGAAATC	60 °C	127 bp
GMM alpha-amylase2-P	FAM-ATGCGGAATATCAAGCTTATCGGGC-TAMRA		
GMM alpha-amylase2-R	CAAAGAAAGCAAATGTGTCGA		

Table 2
Specificity assessment of the qPCR method targeting the GMM alpha-amylase2. The presence and absence of amplification are respectively symbolized by "+" and "-". For each result, the experiment was carried out in duplicate.

Kingdom	Genus	Species	Strain number	GMM alpha-amylase2 method
Fungi	*Aspergillus*	*acidus*	IHEM 26,285	–
	Aspergillus	*aculeatus*	IHEM 05,796	–
	Aspergillus	*brasiliensis*	IHEM 3766	–
	Aspergillus	*costaricaensis*	IHEM 21,971	–
	Aspergillus	*fijiensis*	IHEM 22,812	–
	Aspergillus	*flavus*	IHEM 932	–
	Aspergillus	*heteromorphus*	IHEM 5801	–
	Aspergillus	*ibericus*	IHEM 23,498	–
	Aspergillus	*melleus*	IHEM 25,956	–
	Aspergillus	*neoniger*	IHEM 2463	–
	Aspergillus	*niger*	IHEM 25,485	–
	Aspergillus	*oryzae*	IHEM 25,836	–
	Aspergillus	*piperis*	IHEM 5316	–
	Aspergillus	*tubingensis*	IHEM 1941	–
	Aspergillus	*vadensis*	IHEM 26,351	–
	Aspergillus	*welwitschiae*	IHEM 2864	–
	Candida	*cylindracea*	MUCL 041,387	–
	Candida	*rugosa*	IHEM 01,894	–
	Chaetomium	*gracile*	MUCL 053,569	–
	Cryphonectria	*parasitica*	MUCL 007,956	–
	Disporotrichum	*dimorphosporum*	MUCL 019,341	–
	Fusarium	*venenatum*	MUCL 055,417	–
	Hansenula	*polymorpha*	MUCL 027,761	–
	Humicola	*insolens*	MUCL 015,010	–
	Kluyveromyces	*lactis*	IHEM 02,051	–
	Leptographium	*procerum*	MUCL 008,094	–
	Mucor	*javanicus*	IHEM 05,212	–
	Penicillium	*camemberti*	IHEM 06,648	–
	Penicillium	*chrysogenum*	IHEM 03,414	–
	Penicillium	*citrinium*	IHEM 26,159	–
	Penicillium	*decumbens*	IHEM 05,935	–
	Penicillium	*funiculosum*	MUCL 014,091	–
	Penicillium	*multicolor*	CBS 501.73	–
	Penicillium	*roqueforti*	IHEM 20,176	–
	Pichia	*pastori*	MUCL 027,793	–
	Rhizomucor	*miehei*	IHEM 26,897	–
	Rhizopus	*niveus*	ATCC 200,757	–
	Rhizopus	*oryzae*	IHEM 26,078	–
	Saccharomyces	*cerevisiae*	IHEM 25,104	–
	Sporobolomyces	*singularis*	MUCL 027,849	–
	Talaromyces	*cellulolyticus/pinophilus*	IHEM 16,004	–
	Talaromyces	*emersonii*	DSMZ 2432	–
	Trametes	*hirsuta*	MUCL 030,869	–
	Trichoderma	*atroviride*	IHEM 745	–
	Trichoderma	*citrinoviride*	IHEM 25,858	–
	Trichoderma	*harzianum*	IHEM 5435	–
	Trichoderma	*longibrachiatum*	IHEM 00,935	–
	Trichoderma	*reesei*	IHEM 05,651	–
	Trichoderma	*viride*	IHEM 04,146	–
Bacteria	*Arthrobacter*	*ramosus*	LMG 17,309	–
	Bacillus	*amyloliquefaciens*	LMG 12,331	–
	Bacillus	*brevis*	LMG 7123	–
	Bacillus	*cereus*	ATCC 14,579	–
	Bacillus	*circulans*	LMG 6926 T	–
	Bacillus	*coagulans*	LMG 6326	–
	Bacillus	*firmus*	LMG 7125	–
	Bacillus	*flexus*	LMG 11,155	–
	Bacillus	*lentus*	TIAC 101	–
	Bacillus	*licheniformis*	LMG 7558	–
	Bacillus	*licheniformis*	LMG 6934	–
	Bacillus	*licheniformis*	LMG 6933 T	–
	Bacillus	*licheniformis*	LMG 7634	–
	Bacillus	*licheniformis*	LMG 7631	–
	Bacillus	*megaterium*	LMG 7127	–
	Bacillus	*pumilus*	DSMZ 1794	–
	Bacillus	*smithii*	LMG 6327	–
	Bacillus	*subtilis*	LMG 7135 T	–
	Bacillus	*subtilis*	GMM RASFF2014.1249	–
	Bacillus	*velezensis*	LMG 12,384	–
	Bacillus	*velezensis*	GMM RASFF2019.3332	–
	Cellulosimicrobium	*cellulans*	LMG 16,121	–
	Corynebacterium	*glutamicum*	LMG 3652	–
	Enterococcus	*faecium*	LMG 9430	–

(*continued on next page*)

Table 2 (*continued*)

Kingdom	Genus	Species	Strain number	GMM alpha-amylase2 method
	Escherichia	*coli*	LMG 2092 T	–
	Geobacillus	*caldoproteolyticus*	DSMZ 15,730	–
	Geobacillus	*pallidus*	LMG 11159 T	–
	Geobacillus	*stearothermophilus*	LMG 6939 T	–
	Klebsiella	*pneumonia*	LMG 3113 T	–
	Lactobacillus	*casei*	LMG 6904	–
	Lactobacillus	*fermentum*	LMG 6902	–
	Lactobacillus	*plantarum*	LMG 9208	–
	Lactobacillus	*rhamnosus*	LMG 18,030	–
	Lactococcus	*lactis*	LMG 6890 T	–
	Leuconostoc	*citreum*	LMG 9824	–
	Microbacterium	*imperiale*	LMG 20,190	–
	Paenibacillus	*alginolyticus*	LMG 18,723	–
	Paenibacillus	*macerans*	LMG 6324	–
	Protaminobacter	*rubrum*	CBS 574.77	–
	Pseudomonas	*amyloderamosa*	ATCC-21262	–
	Pseudomonas	*fluorescens*	LMG 1794 T	–
	Pullulanibacillus	*naganoensis*	LMG 12,887	–
	Streptomyces	*aureofaciens*	LMG 5968	–
	Streptomyces	*mobaraensis*	DSMZ 40,847	–
	Streptomyces	*murinus*	LMG 10,475	–
	Streptomyces	*netropsis*	LMG 5977	–
	Streptomyces	*rubiginosus*	LMG 20,268	–
	Streptomyces	*violaceoruber*	LMG 7183	–
	Streptoverticillium	*mobaraense*	CBS 199.75	–
Plantae	*Oryza*	*sativa*	/	–
Animalia	*Homo*	*sapiens*	/	–
Other	Control plasmid carrying one copy of the sequence targeted by the GMM alpha-amylase2 qPCR method			+
	Commercial alpha-amylase product (sample n°1)			+

Table 3
Sensitivity assessment of the qPCR method targeting the GMM alpha-amylase2 for the *in house* validation and transferability assays. The presence and absence of amplification are respectively symbolized by "+" and "-". For each estimated target copy number, 12 replicates were tested. The number of positive replicate(s) out of the 12 replicates tested and the averages of the observed C_q are indicated within brackets.

Estimated target copy number	GMM alpha-amylase2 method	
	***In house* validation assays**	**Transferability assays**
100	+ (12/12) (C_q: 32.3)	+ (12/12) (C_q: 33.2)
25	+ (12/12) (C_q: 34.4)	+ (12/12) (C_q: 34.8)
20	+ (12/12) (C_q: 34.7)	+ (12/12) (C_q: 35.7)
10	+ (12/12) (C_q: 36.0)	+ (12/12) (C_q: 36.4)
5	+ (11/12) (C_q: 37.1)	+ (10/12) (C_q: 37.7)
1	+ (5/12) (C_q: 37.6)	+ (4/12) (C_q: 39.1)
0.1	– (0/12)	+ (1/12) (C_q: 38.6)
0	– (0/12)	– (0/12)

extension step either at 64 °C for BSG (*B. subtilis* group), cat, tet-l, aadD, pUB110-L methods or at 60 °C for GMM protease1, GMM protease2, GMM alpha-amylase1, GMM alpha-amylase2 methods. Each qPCR assay included a No Template Control (NTC).

2.3. Development and validation of the qPCR GMM alpha-amylase2 method

In order to develop a qPCR method targeting the GMM alpha-amylase2 strain recently discovered, the recently characterized sequence from its transgenic construct was used (D'aes et al., 2022). For this GMM alpha-amylase2 qPCR method, one set of primers (forward and reverse) and probe was designed using the software Primer3, allowing to cover 127 bp of the unnatural association of sequences between a part from the downstream region of the *B. licheniformis catA* gene and a part from the *B. licheniformis amyS* gene (Table 1, Supplementary data 1) (D'aes et al., 2022). The performance of this GMM alpha-amylase2 qPCR method was then assessed.

2.3.1. Specificity

For *in silico* investigations, the amplicon sequence generated from the GMM alpha-amylase2 qPCR method was blasted against the NCBI database (nr/nt) with default parameters (Supplementary data 2) as well as, using the Clustal Omega software (v1.2.4) with default parameters, aligned to the amplicon sequences generated from the qPCR methods targeting the GMM protease1, GMM protease2 and GMM alpha-amylase1 strains (Supplementary data 3).

For the experimental investigations, the qPCR method targeting the GMM alpha-amylase2 strain was applied in duplicate on 10 ng of DNA from positive and negative controls (Table 2). For positive controls, DNA from a commercial alpha-amylase product (sample n°1 in Table 4) and DNA from the GMM alpha-amylase2 control plasmid were used. For negative controls, DNA from (i) 96 microbial strains, including 49 fungal and 47 bacterial species used by the food and feed industry for the production of fermentation products, (ii) 2 GM *Bacillus* strains, namely GM *B. subtilis* producing vitamin B_2 (RASFF2014.1249) and GM *B. velezensis* producing protease1 (RASFF2019.3332), (iii) animal (*Homo sapiens*) and (iv) plant (*Oryza sativa*) were tested. The false negative rate was calculated based on the number of misclassified known positive samples regarding the total number of known positive samples. The false positive rate was calculated based on the number of misclassified known negative samples regarding the total number of known negative samples. For sample n°1, the generated amplicon was purified, sequenced

Table 4

Pilot market surveillance of GMM contaminations, including GMM alpha-amylase2, using commercial FE products labelled as containing alpha-amylase (samples n°1-40). Available information on these samples is indicated in Supplementary data 6. These samples were either collected on the European market or provided by food companies (indicated by *). For samples n°1, 5 and 12 (indicated by **), the GMM protease1 strain was previously isolated by classical microbiology (D'aes et al., 2021). The samples were screened for the potential presence of GMM, using the BSG, cat, tet-l, aadD and pUB110-L qPCR methods, as well as tested for the presence of specific GMM strains, including GMM protease1, GMM protease2, GMM alpha-amylase1 and GMM alpha-amylase2. Each sample was tested in duplicate. The presence or absence of PCR amplification is symbolized by "+" or "-", respectively. If below the $LOD_{95\%}$ of the tested qPCR method, the positive amplification signal is reported in italic and symbolized by "(+)", as potential false positive signals cannot be discarded. The $LOD_{95\%}$ of each tested qPCR method: 22 estimated target copies for BSG (experimental C_q at 38.2 for 100 estimated target copies); 25 estimated target copies for cat (experimental C_q at 34.2 for 55 estimated target copies); 17 estimated target copies for tet-l (experimental C_q at 33.7 for 55 estimated target copies); 24 estimated target copies for aadD (experimental C_q at 34.0 for 55 estimated target copies); 9 estimated target copies for pUB110-L (experimental C_q at 32.4 for 12 estimated target copies); 3 estimated target copies for GMM protease1 (experimental C_q at 38.2 for 5 estimated target copies); 13 estimated target copies for GMM protease2 (experimental C_q at 36.3 for 20 estimated target copies); 17 estimated target copies for GMM alpha-amylase1 (experimental C_q at 36.7 for 20 estimated target copies); 6 estimated target copies for GMM alpha-amylase2 (experimental C_q at 36.0 for 10 estimated target copies) (Fraiture et al., 2020a,b,c,d; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). The averages of the observed C_q are indicated within brackets. The qPCR results were generated either in this study or previously (indicated by ') (D'aes et al., 2022; Deckers et al., 2021a; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). If applicable, the associated RASFF notification number is listed.

Samples	First-line qPCR analysis					Second-line qPCR analysis			
	BSG	cat	tet-l	aadD	pUB110-L	GMM protease1	GMM protease2	GMM alpha-amylase1	GMM alpha-amylase2
1 *RASFF2020.2557*	+' (C_q: 19.5)	–	–	+ (C_q: 17.8) Φ	+ (C_q: 19.0)	+' (C_q: 19.7)**	-'	+' (C_q: 18.1)	+ (C_q: 16.2)
2 *RASFF2020.2846*	+' (C_q: 19.8)	–	–	+ (C_q: 17.0) Φ	+ (C_q: 17.9)	+ (C_q: 20.9)	–	+ (C_q: 17.4)	+ (C_q: 17.0)
3 *RASFF2020.2579*	+' (C_q: 22.6)	+ (C_q: 32.0)	+ (C_q: 30.8) Φ	+ (C_q: 14.3) Φ	+ (C_q: 15.3)	+' (C_q: 36.4)	-'	+' (C_q: 15.2)	+ (C_q: 17.0)
4 *RASFF2020.2577*	+' (C_q: 19.4)	–	–	+ (C_q: 17.8) Φ	+ (C_q: 18.4)	+' (C_q: 19.8)	-'	+' (C_q: 18.2)	+ (C_q: 17.1)
5 *RASFF2019.3332*	+' (C_q: 20.6)	–	*(+) (C_q: 34.1)*	+' (C_q: 13.9) Φ	+' (C_q: 15.2)	+' (C_q: 14.0)**	-'	+' (C_q: 22.0)	+ (C_q: 17.2)
6 *RASFF2019.3332*	+ (C_q: 22.0)	–	–	+' (C_q: 15.8) Φ	+' (C_q: 17.0)	+ (C_q: 23.1)	–	+' (C_q: 16.4)	+ (C_q: 17.6)
7*	+ (C_q: 25.6)	–	–	+ (C_q: 33.1)	*(+) (C_q: 39.7)*	*(+) (C_q: 42.3)*	*(+) (C_q: 42.2)*	*(+) (C_q: 38.0)*	+ (C_q: 24.6)
8*	+ (C_q: 26.8)	*(+) (C_q: 37.8)*	*(+) (C_q: 37.4)*	+ (C_q: 30.1) Φ	+ (C_q: 31.1)	*(+) (C_q: 41,1)*	–	+ (C_q: 29.7)	+ (C_q: 24.8)
9 *RASFF2020.2576*	+' (C_q: 34.4)	–	–	*(+)' (C_q: 34.9)*	-'	+ (C_q: 35.2)	–	*(+)' (C_q: 39.5)*	+ (C_q: 26.1)
10*	+ (C_q: 32.5)	–	+ (C_q: 30.5) Φ	+ (C_q: 25.9) Φ	+ (C_q: 27.0)	*(+) (C_q: 38.4)*	–	+ (C_q: 26.9)	+ (C_q: 27.1)
11*	+ (C_q: 29.0)	*(+) (C_q: 38.7)*	–	+ (C_q: 30.4) Φ	*(+) (C_q: 32.9)*	*(+) (C_q: 41.2)*	–	+ (C_q: 32.4)	+ (C_q: 29.4)
12 *RASFF2020.2582*	+' (C_q: 31.2)	–	–	+ (C_q: 24.8) Φ	+ (C_q: 25.4)	+ (C_q: 30.7)**	–	+ (C_q: 25.2)	+ (C_q: 30.2)
13*	+ (C_q: 28.1)	–	–	–	–	–	–	–	+ (C_q: 30.2)
14*	+ (C_q: 22.9)	–	+ (C_q: 32.2) Φ	+ (C_q: 17.3) Φ	+ (C_q: 17.8)	+ (C_q: 38.1)	–	+ (C_q: 16.6)	+ (C_q: 30.7)
15 *RASFF2020.2572*	+' (C_q: 36.9)	–	–	+ (C_q: 33.2) Φ	*(+) (C_q: 35.5)*	+ (C_q: 37.2)	–	+ (C_q: 36.3)	+ (C_q: 32.2)
16*	*(+)' (C_q: 43.6)*	–	–	–	–	–	–	–	*(+) (C_q: 37.2)*
17 *RASFF2020.2570*	+' (C_q: 31.4)	*(+) (C_q: 36.1)*	*(+) (C_q: 35.6)*	+ (C_q: 22.1) Φ	+ (C_q: 23.2)	+ (C_q: 37.1)	–	+ (C_q: 23.8)	*(+) (C_q: 37.9)*
18 *RASFF2020.2576*	+' (C_q: 27.9)	–	+ (C_q: 26.1) Φ	+' (C_q: 18.3) Φ	+' (C_q: 20.0)	+ (C_q: 35.6)	–	+' (C_q: 20.8)	*(+) (C_q: 38.1)*
19*	+	*(+)*	+	+	+	–	–	+	*(+)*

(*continued on next page*)

Table 4 (*continued*)

Samples	First-line qPCR analysis					Second-line qPCR analysis			
	BSG	cat	tet-l	aadD	pUB110-L	GMM protease1	GMM protease2	GMM alpha-amylase1	GMM alpha-amylase2
	(C_q: 27.1)	*(C_q: 34.6)*	(C_q: 29.9) Φ	(C_q: 20.6) Φ	(C_q: 21.2)			(C_q: 19.7)	*(C_q: 38.5)*
20 *RASFF2019.3332*	*(+)'* *(C_q: 40.3)*	–	–	+' (C_q: 31.8) Φ	*(+)'* *(C_q: 33.0)*	+ (C_q: 31.4)	–	+' (C_q: 34.1)	*(+)* *(C_q: 39,6)*
21*	+ (C_q: 17.6)	*(+)* *(C_q: 38.9)*	–	–	*(+)* *(C_q: 40.8)*	–	–	–	*(+)* *(C_q: 43.4)*
22	+' (C_q: 25.1)	–	+ (C_q: 28.4) Φ	+ (C_q: 17.0) Φ	+ (C_q: 19.5)	–	–	+ (C_q: 21.1)	–
23 *RASFF2020.2870*	*(+)* *(C_q: 42.4)*	–	–	+' (C_q: 33.4)	*(+)'* *(C_q: 35.6)*	+ (C_q: 37.8)	–	+' (C_q: 36.0)	–
24	–	-'	-'	*(+)* *(C_q: 37.5)*	–	-'	–	*(+)* *(C_q: 38.0)*	–
25*	+ (C_q: 30.2)	–	–	*(+)* *(C_q: 38.2)*	*(+)* *(C_q: 39.3)*	*(+)* *(C_q: 38.9)*	–	*(+)* *(C_q: 39.2)*	–
26*	+ (C_q: 37.4)	–	–	*(+)* *(C_q: 37.7)*	*(+)* *(C_q: 40.0)*	*(+)* *(C_q: 39.5)*	–	*(+)* *(C_q: 42.4)*	–
27	+ (C_q: 22.2)	–	*(+)* *(C_q: 34.9)*	*(+)* *(C_q: 36.7)*	–	+ (C_q: 37.2)	–	–	–
28*	*(+)* *(C_q: 40.2)*	*(+)* *(C_q: 34.6)*	*(+)* *(C_q: 40.7)*	+ (C_q: 30.2) Φ	*(+)* *(C_q: 43.9)*	*(+)* *(C_q: 38.4)*	–	–	–
29*	+ (C_q: 29.9)	–	–	+ (C_q: 32.7) Φ	–	–	–	–	–
30	+ (C_q: 24.5)	-'	-'	*(+)'* *(C_q: 38.6)*	–	–	-'	–	–
31	+ (C_q: 36.9)	*(+)'* *(C_q: 35.7)*	-'	*(+)'* *(C_q: 36.3)*	–	-'	–	–	–
32	+ (C_q: 27.2)	–	–	–	–	–	–	–	–
33*	+ (C_q: 30.4)	–	–	–	–	–	–	–	–
34*	+ (C_q: 36.8)	–	–	–	–	–	–	–	–
35*	+ (C_q: 37.9)	–	–	–	–	–	–	–	–
36*	*(+)* *(C_q: 39.6)*	–	–	–	–	–	–	–	–
37*	*(+)* *(C_q: 40.7)*	–	–	–	–	–	–	–	–
38	-'	–	–	–	–	–	–	–	–
39*	–	–	–	–	–	–	–	–	–
40*	–	–	–	–	–	–	–	–	–

Φ full-length of detected AMR gene confirmed (Deckers et al., 2021a; Supplementary data 7).

and compared to the targeted reference sequence as previously described (Table 1, Supplementary data 4) (Fraiture et al., 2021d).

2.3.2. Sensitivity

Using DNA from the GMM alpha-amylase2 control plasmid serial dilutions, ranging from 100 to 0.1 estimated target copy number, were prepared. Each dilution point was tested 12-fold by the GMM alpha-amylase2 qPCR method (Table 3). The estimated target copy numbers were calculated based on the GMM alpha-amylase2 control plasmid size (4925 bp), as previously described (Fraiture et al., 2021d). The limit of detection $LOD_{95\%}$ of the GMM alpha-amylase2 qPCR method was determined as previously described (Supplementary data 5) (Fraiture et al., 2021d; Grohmann et al., 2016; Uhlig et al., 2015).

2.3.3. Transferability assays

As previously described, the *in house* sensitivity assessment described in section 2.3.2 was also tested by an external laboratory, in this case the Unità Operativa Semplice a Valenza Direzionale - Ricerca e Controllo degli Organismi Geneticamente Modificati (CROGM) at the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M.Aleandri"

(Roma, Italy) (Table 3, Supplementary data 5) (Fraiture et al., 2021d).

2.3.4. Pilot market surveillance of GMM contaminations and applicability assessment of the GMM alpha-amylase2 qPCR method

A total of 40 FE products labelled as containing alpha-amylase (samples n°1-40), collected either on the European market or provided by food companies (intended for commercialization), was used (Table 4). These FE products were labelled as containing either only alpha-amylase (samples n°1-4, 6–29, 32–40) or alpha-amylase mixed with other enzymes such as alpha-galactosidase, beta-glucanase, cellulase, hemicellulase, lactase, lipase, protease or xylanase (samples n°5, 30–31) (Supplementary data 6). These samples were screened for the potential presence of GMM, using the BSG, cat, tet-l, aadD and pUB110-L qPCR methods, as well as tested for the presence of specific GMM strains, including GMM protease1, GMM protease2, GMM alpha-amylase1 and GMM alpha-amylase2 (Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Fraiture et al., 2022). From each sample, 10 ng of DNA per assay was tested in duplicate (Table 4). The full-length of AMR genes detected by qPCR (above $LOD_{95\%}$) was assessed by conventional PCR followed by Sanger sequencing as previously described (Deckers et al., 2021a; Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c) (Supplementary data 7). If the sample was provided by a food company, the PCR results were communicated to them. If the sample was collected on the European market, the PCR results were reported to the Belgian Competent Authorities who took appropriate actions such as a RASFF (Rapid Alert System for Food and Feed) notification at the European level.

3. Results and discussion

3.1. Development of a qPCR method targeting a new unauthorized GM bacterium producing alpha-amylase

A qPCR method was developed to specifically target a recently discovered GMM producing alpha-amylase, namely GMM alpha-amylase2. The GMM alpha-amylase2 construct, recently identified and characterized by D'aes et al., 2022, includes a sequence region from the *B. licheniformis amyS* gene connected to a sequence region from the *B. licheniformis catA* gene (Supplementary data 1). At least two contiguous copies of the GMM alpha-amylase2 construct are integrated into the *B. licheniformis* host at the site of the *catA* gene. In this study, a set of primers and probe was designed within the GMM alpha-amylase2 construct to cover 127 bp of the unnatural association of sequences between a part belonging to the downstream region of the *B. licheniformis catA* gene, encoding for type A chloramphenicol O-acetyltransferase (conferring resistance to chloramphenicol), and a part belonging to the *B. licheniformis amyS* gene, encoding for alpha-amylase (Table 1, Supplementary data 1) (D'aes et al., 2022). The GMM alpha-amylase2 qPCR method was developed in accordance with the qPCR conditions from the previously developed methods targeting currently identified unauthorized GMMs, including GMM protease1, GMM protease2 and GMM alpha-amylase1 strains (Fraiture et al., 2020d; Fraiture et al., 2021c; Fraiture et al., 2021d). The developed GMM alpha-amylase2 qPCR method was further *in house* validated.

3.2. Specificity assessment

The specificity of the GMM alpha-amylase2 qPCR method was firstly investigated *in silico*. By blasting the GMM alpha-amylase2 amplicon against the NCBI nucleotide (nr/nt) database, no hit of 100 % in terms of identity and coverage was detected (Supplementary data 2). Within the GMM alpha-amylase2 amplicon sequence, 53 bp and 44 bp respectively matched to a partial sequence of the downstream region of *B. licheniformis catA* (NCBI Gene ID: 66215181) and a partial sequence of *B. licheniformis amyS* (NCBI Gene ID: 66217199), as expected (Table 1, Supplementary data 2 and 4) (D'aes et al., 2022). The sequence from the GMM alpha-amylase2 amplicon was also differentiable from the unnatural associations of sequences specific to the currently identified GMM protease1, GMM protease2 and GMM alpha-amylase1 strains (Supplementary data 3). In addition to a different GMM construct, the GMM alpha-amylase2 strain is the only one of all GMM strains discovered and characterized to date that belongs to the *B. licheniformis* species (Fraiture et al., 2020d; Fraiture et al., 2021c; Fraiture et al., 2021d).

The specificity of the developed GMM alpha-amylase2 qPCR method was further experimentally tested (Table 2). No amplification was observed from all negative controls while an amplification was observed for all positive controls, including DNA extracted from the GMM alpha-amylase2 control plasmid and a commercialized alpha-amylase product (sample n°1) (Table 2, Supplementary data 8). Furthermore, the amplicon generated from sample n°1 was identical to the GMM alpha-amylase2 reference sequence, which is of particular importance in this validation process as official reference material was unavailable (Supplementary data 4). Since these results indicating a false positive rate of 0 % and a false negative rate of 0 %, the developed GM alpha-amylase2 qPCR method was evaluated as being specific for its target.

3.3. Sensitivity assessment

According international standard (ISO Standard 16140–2:2014), the sensitivity of the developed GMM alpha-amylase2 qPCR method was investigated using different estimated target copy numbers (100, 25 20, 10, 5, 1, 0.1 and 0) of the GMM alpha-amylase2 control plasmid (Table 3, Supplementary data 8).

A positive signal was observed as low as 10 estimated target copies for all 12 replicates (Table 3). Based on modelling of the probability of detection (POD), the limit of detection ($LOD_{95\%}$) was determined at 6 estimated target copies (Supplementary data 5), complying with the criteria for GMO detection methods described in the MPR for GMO analysis of the European Network of GMO Laboratories (ENGL, 2015). Therefore, the developed GMM alpha-amylase2 qPCR method was assessed as being sensitive.

3.4. Transferability assays

Using the same experimental set up as for the *in house* sensitivity assessment, The performance of the *in house* validated GMM alpha-amylase2 qPCR method was evaluated in an external laboratory with different operators using different equipment and reagents.

The results generated by the external laboratory were equivalent with those observed during the *in house* validation (Table 3). A positive signal was observed as low as 10 estimated target copies for all 12 replicates. Based on modelling of POD, the $LOD_{95\%}$ was calculated at 8 estimated target copies, being very close to the value ($LOD_{95\%}$=6) observed during the *in house* validation (Supplementary data 5). Since comparable performance was observed between the *in house* and external assays, the transferability of the GMM alpha-amylase2 qPCR method was demonstrated.

3.5. Pilot market surveillance of GMM contaminations and applicability assessment of the GMM alpha-amylase2 qPCR method

A pilot market surveillance of GMM contaminations was conducted on a total of 40 FE products labelled as containing alpha-amylase (samples n°1-40), either exclusively or mixed with additional enzymes (e.g., alpha-galactosidase, beta-glucanase, cellulase, hemicellulase, lactase, lipase, protease or xylanase) (Table 4, Supplementary data 6). These FE products, from different brands and intended for different food sectors (e.g., distillery, brewing, baking, fruit processing or digestive aids), were collected, under liquid or solid form, either from the European market or provided by food companies.

The GMM detection strategy, described previously (Fraiture et al.,

2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021b,c,d; Fraiture et al., 2022) and strengthened in this study by the newly developed and *in house* validated GMM alpha-amylase2 qPCR method, was applied on all FE samples. More precisely, the samples were first screened for the potential presence of GMM (first-line analysis), using the BSG, cat, tet-l, aadD and pUB110-L qPCR methods. Then, the presence of specific GMM strains (second-line analysis) was tested using qPCR methods specific to GMM protease1, GMM protease2, GMM alpha-amylase1 and GMM alpha-amylase2 (Table 4). All these qPCR methods present also the advantage to be culture-independent, bypassing the GMM isolation step being especially challenging since information on GMM strains used to manufacture fermentation products is confidential. Consequently, a gigantic list of microbial growth conditions, including possible auxotrophic mutations and persistence as spores, should theoretically be tested, being however unrealistic at the practical level (D'aes et al., 2022; Deckers et al., 2020).

For the first-line analysis step, no amplification signal above $LOD_{95\%}$ for any of the screening methods was observed in 7 samples (n°16, 24, 36–40), while 33 samples (n°1-15, 17–23, 25–35) presented an amplification signal above $LOD_{95\%}$ for at least one of the screening methods, suggesting the potential presence of GMM contaminations in 82.5 % of the 40 investigated samples (33 samples). Moreover, the full-length of detected AMR genes, including *tet-l* and *aadD* conferring respectively a resistance to tetracycline and kanamycin, was confirmed in 47.5 % of the 40 investigated samples (19 samples). For the second-line analysis step, no amplification signal above $LOD_{95\%}$ was observed for any of the GMM identification methods in samples n°16, 24, 36–40, as expected. For the 33 samples suspected to contain GMM contaminations (n°1-15, 17–23, 25–35), none of the targeted GMM strains with an amplification signal above $LOD_{95\%}$ were identified in 11 samples (n°21, 25–26, 28–35), while the presence of at least one specific GMM strain with an amplification signal above $LOD_{95\%}$ was confirmed in 22 samples (n°1-15, 17–20, 22–23, 27), representing 66.7 % of the 33 suspicious samples. Among these 22 samples, the new GMM alpha-amylase2 strain was detected with an amplification signal above $LOD_{95\%}$ in 15 samples (n°1-15), corresponding to 68.2 % of the 22 samples presenting a confirmed GMM contamination. Moreover, a high contamination level for the GMM alpha-amylase2 strain ($C_q \leq 25$) was observed for 8 samples (n°1-8). These results demonstrated the applicability of the newly *in house* validated GMM apha-amylase2 qPCR method. In addition, 13 of these 15 samples presented an amplification signal above $LOD_{95\%}$ for at least one additional previously characterized GMM strain (GMM protease1, GMM protease2 and/or GMM alpha-amylase1), suggesting, on the one hand, potentially frequent combined uses of more than one alpha-amylase produced by different GMM strains, and, on the other hand, potentially frequent cross-contaminations of several GMM strains in commercial FE products labelled as containing alpha-amylase. Since all these GMM strains, especially GMM protease1, GMM alpha-amylase1 and GMM alpha-amylase2, were often observed together in the tested FE products, these results suggest also a possible common origin of such GMM contaminations.

Regarding the 11 samples (n°21, 25–26, 28–35) presenting a positive signal above $LOD_{95\%}$ for the first-line analysis that was unexplained by the second-line analysis, further investigations are necessary. For samples n°28-29, the potential presence of unknown GMM strain(s) may be strongly suspected but not demonstrated, since either both DNA from the *B. subtilis* group and AMR genes or only DNA from AMR genes were detected with an amplification signal above $LOD_{95\%}$. For samples n°21, 25–26, 30–35, only DNA from the *B. subtilis* group was detected with an amplification signal above $LOD_{95\%}$. Such amplification signals may be attributed to the presence of members of the *B. subtilis* group, GM or not. Therefore, strategies such as DNA walking and metagenomic sequencing represent interesting options to collect key information.

4. Conclusion

In order to strengthen the current set of methods targeting unauthorized GMMs, a new qPCR method was developed to cover 127 bp of an unnatural association of sequences specific to the GM *B. licheniformis* strain producing alpha-amylase, named GMM alpha-amylase2, recently discovered using high-throughput metagenomics on commercial microbial fermentation products (D'aes et al., 2022). The targeted unnatural association of sequences is composed of a part belonging to the downstream region of the *B. licheniformis catA* gene and a part belonging to the *B. licheniformis amyS* gene (Table 1). The GMM alpha-amylase2 qPCR method was designed to be compatible with methods targeting currently identified GMMs, including GMM protease1, GMM protease2 and GMM alpha-amylase1 strains (Fraiture et al., 2020d; Fraiture et al., 2021c, Fraiture et al., 2021d). Regarding its performance, the developed GMM alpha-amylase2 qPCR method was evaluated as specific, sensitive and applicable. This qPCR method also complied with the criteria for GMO detection methods as described in the MPR for GMO analysis of the European Network of GMO Laboratories (ENGL, 2015). Moreover, the transferability of the *in house* validated qPCR method was demonstrated, being a first critical step towards a full method validation aiming for method dissemination within European enforcement laboratories to ensure the traceability and safety of the food and feed chain. Finally, a pilot market surveillance of GMM contaminations was conducted for the first time on 40 commercial FE products labelled as containing alpha-amylase. Using the newly developed GMM alpha-amylase2 qPCR method together with previously developed qPCR methods targeting other GMM strains, the presence of GMM contaminations carrying AMR genes was detected with an amplification signal above $LOD_{95\%}$ in 55 % of the 40 samples (22 samples), including a contamination with the GMM alpha-amyase2 strain in 37.5 % of the 40 samples (15 samples) (Table 4). Moreover, among the 22 samples presenting a confirmed GMM contamination, 13 samples showed a high GMM contamination level ($C_q < 25$) (Table 4). These results suggest frequent GMM contaminations in such type of FE products. Moreover, the generated results indicated in samples n°28-29 the potential presence of unknown GMM strain(s) carrying AMR genes, requiring however to be demonstrated using for example DNA walking or metagenomic sequencing.

The numerous unexpected GMM contaminations found in FE products has a non-negligible impact on the European GMO regulations (Regulation (EC) No 1829/2003, Regulation (EC) No 1830/2003). Discussions are currently underway to determine whether GMM contaminations either fall under the scope of the European GMO regulations or are considered as processing aids (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Deckers et al., 2020; Fraiture et al, 2020d; Wesseler et al., 2022). In addition to these regulations, the use of GMM carrying AMR genes as markers to select the transformed cells that synthetise fermentation products is controversial. Evidence of contaminations with GMM carrying AMR genes, both viable strains and associated recombinant DNA, in various fermentation products has been demonstrated (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Deckers et al., 2021a; Fraiture et al., 2020a; Fraiture et al., 2020b; Fraiture et al., 2020c; Fraiture et al., 2020d; Fraiture et al., 2021a; Fraiture et al., 2021b; Fraiture et al., 2021c; Fraiture et al., 2021d; Paracchini et al., 2017). The pilot market surveillance carried out in this study on 40 commercial FE products labelled as containing alpha-amylase has revealed frequent contaminations, even at high levels ($C_q < 25$), of AMR genes originated from GMM. The full-length of detected AMR genes was also confirmed in 47.5 % of the 40 monitored samples. This highlights the need for an in-depth assessment of the potential health risks associated to the unexpected presence of AMR genes in the food and feed chain via fermentation products. Indeed, in addition to DNA encapsulated in a viable bacterial strain, free extracellular DNA can also be used for horizontal transfer (von Wintersdorff et al., 2016; Woegerbauer et al., 2020; Michaelis and Grohmann, 2023). It will be therefore necessary to evaluate whether the observed contamination

levels by AMR genes represent a risk of horizontal transfer of AMR genes to other microorganisms, thereby compromising the effectiveness of antibiotic treatments as well as the normal functioning of the microbiome.

This pilot market surveillance also pointed out frequent cross-contaminations with several different previously characterized GM *Bacillus* strains, including GMM protease1, GMM protease2 and GMM alpha-amylase1. Consequently, although the GMM alpha-amylase2 strain does not harbour the pUB110 shuttle vector associated with *aadD*, in contrast to the GMM protease1, GMM protease2 and GMM alpha-amylase1 strains (D'aes et al., 2021; D'aes et al., 2022, Fraiture et al., 2020d; Fraiture et al., 2021c; Fraiture et al., 2021d), their frequent cross-contaminations suggest a common contamination origin. However, to confirm this hypothesis, further investigations are necessary, such as using high-throughput sequencing followed by SNP (single nucleotide polymorphism) characterization of the GM bacterial strains (D'aes et al., 2022).

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CRediT authorship contribution statement

Marie-Alice Fraiture: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Andrea Gobbo:** Formal analysis, Writing – review & editing. **Chloé Guillitte:** Formal analysis, Writing – review & editing. **Ugo Marchesi:** Formal analysis, Writing – review & editing. **Daniela Verginelli:** Formal analysis, Writing – review & editing. **Joke De Greve:** Formal analysis, Writing – review & editing. **Jolien D'aes:** Writing – review & editing. **Kevin Vanneste:** Writing – review & editing. **Nina Papazova:** Validation, Writing – review & editing. **Nancy H.C. Roosens:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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