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Detection strategy targeting a chloramphenicol resistance gene from genetically modified bacteria in food and feed products

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ABSTRACT

Genetically modified microorganisms (GMM), harbouring commonly antimicrobial resistance (AMR) genes as selection markers, are frequently used to produce food and feed enzymes, additives and flavourings. Such commercialized microbial fermentation products should not contain GMM, or associated recombinant DNA. Although the use of AMR genes gives rise to public health and environmental concerns regarding their potential acquisitions by pathogens and gut microbiota, no method targeting AMR genes harboured by such GMM is currently available for the enforcement laboratories. In reason of the increasing interest of the competent authorities to be able to assess the potential risks related to the presence of these AMR genes in microbial fermentation products, we propose therefore for the first time a PCR-based strategy easily implementable in enforcement laboratories. This strategy targets a chloramphenicol resistance gene, highlighted by the patent analysis performed in this study as being harboured by a noteworthy part of GMM producing microbial fermentation products from the food and feed industry. First, the potential presence of the AMR gene is detected by real-time PCR. Next, its full-length is evaluated by a nested-PCR amplifying a large fragment of its sequence to determine the risks of likely AMR gene acquisition. This strategy allows thus to support the competent authorities regarding the measures to be taken in case of unexpected DNA contaminations from such GMM in commercialized microbial fermentation products.

1. Introduction

In the food and feed industry, microbiological fermentation processes are widely used to produce food and feed additives, enzymes and flavourings. For this purpose, genetically modified microorganisms (GMM) harbouring antimicrobial resistance (AMR) genes as selection markers are frequently used (von Wrighta & Bruce, 2003; Heller, 2006; Aguilera, Gomes, & Olaru, 2013; Barbau-Piednoir et al., 2015b; Guo et al., 2017; Paracchini et al., 2017; Kallscheuer, 2018; Deckers et al., 2019). However, such practices gave rise to public health and environmental concerns due to the mechanism of horizontal gene transfer that could potentially lead to AMR acquisition by pathogens and gut microbiota through the ingestion of GMM, or associated recombinant DNA, harbouring AMR genes. Therefore, in order to protect consumers against in particular such potential adverse effects, viable GMM, as well as associated recombinant DNA, producing additives, enzymes and flavourings should be absent from the final food and feed products commercialized on the European (EU) market (RASFF portal, EC/1831/2003, von Wrighta & Bruce, 2003, EC/1332/2008, EC/1333/2008, EC/1334/2008, EFSA, 2012, EFSA, 2014; Munita & Arias, 2016; Rozwandowicz et al., 2018; Xiong, Sun, & Zeng, 2018; Bacanli & Basacan, 2019). Nonetheless, unexpected contaminations originated from genetically modified (GM) *Bacillus subtilis* strains producing vitamin B2 were detected in commercialized feed additives in 2014, 2018 and 2019 (RASFF2014.1249, RASFF2014.1360, RASFF2014.1657, RASFF2018.2755 and RASFF2019.0793) (RASFF portal). In containing unauthorized GMM, such products were consequently falling under the Regulation EC/1829/2003 related to the commercialization of genetically modified organisms (GMO) as food and feed. Given that the reported unauthorized GMM were harbouring AMR genes conferring a resistance to various antibiotics, the potential related risks to public health and environment of these microbial fermentation products were highlighted (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; EFSA, 2019; Paracchini et al., 2017). The likelihood of AMR gene acquisition is increased by the presence of the full-length gene, the gene with flanking regions, the gene on mobile genetic elements and the viable GMM harbouring the gene (EFSA, 2011; EFSA, 2019). However, no method allowing to assess the presence of the full-length

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of AMR genes harboured by GMM is currently available to the enforcement laboratories. Indeed, only short DNA fragments of interest are generally targeted by enforcement laboratories using the real-time PCR technology. For example, the event-specific real-time PCR 558 method, previously developed to detect the presence of the GM *B. subtilis* RASFF2014.1249 strain, covers 76 bp of the junction between the *B. subtilis recA* gene and the chloramphenicol acetyl-transferase (*cat*) gene (EFSA, 2011, Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Paracchini et al., 2017; EFSA, 2019). A positive signal obtained via the real-time PCR technology is thus compatible with the presence of the full-length cat gene but is not able to prove it. Consequently, this crucial issue hinders the competent authorities, despite their strong interest, to conclude about the potential risks of likely AMR gene acquisition (EFSA, 2011, Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Paracchini et al., 2017; EFSA, 2019).

Among AMR genes used, chloramphenicol resistance (CmR) genes represent an interesting discriminative marker to target microbial fermentation products-producing GMM because only few bacterial strains are naturally harbouring it (Nawaz et al., 2019; Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004; Yan & Fong, 2017). Surprisingly, although the chloramphenicol antibiotic has been banned in food-producing animals as well as in other food products on the EU market in reason of its toxicity for humans at any dose, the unexpected presence of this antibiotic has previously been reported several times in microbial fermentation products (Supplementary file 1, RASFF protal, Hanekamp, Frapporti, & Olieman, 2003; EFSA, 2014). All these notifications are thus strongly supporting the likely use of the chloramphenicol antibiotic to select food and feed additives, enzymes and flavourings-producing GMM carrying CmR genes. However, apart from this indirect evidence, no clear estimation of the occurrence regarding the use of such GMM harbouring CmR genes in the food and feed industry is currently available, mainly due to the confidentially of the corresponding GMM dossiers.

In the present study, in order to verify this hypothesis by evidence-based, publicly available patents on microbial fermentation products-producing GM bacteria harbouring CmR genes were collected and analysed. Moreover, we developed a PCR-based strategy composed of two main successive steps targeting a frequently used CmR gene. More precisely, the potential presence of this AMR gene is first screened using a real-time PCR method (Turgeon, Laflamme, Ho, & Duchaine, 2008). In case a positive real-time PCR signal is obtained, the presence of the full-length of this AMR gene is then evaluated by a nested-PCR developed in this study to amplify a large fragment of its sequence. In order to be suitable for enforcement laboratories, the performance of the proposed strategy was successfully assessed regarding its specificity, sensitivity and applicability. The data generated by the proposed PCR-based strategy allow thus for the first time to support the competent authorities for the decisions to be taken regarding the level of risks

Table 1
List of wild-type microorganisms used for the specificity assessment of the real-time PCR and nested-PCR methods targeting the *cat* gene (GenBank: NC_002013.1). The presence and absence of amplification of the targeted *cat* gene are respectively symbolized by "+" and "-". The GM *Bacillus subtilis* (RASFF2014.1249) strain was used as the positive control. For each result, the experiment was carried out in quadruplicate for the real-time PCR and in duplicate for the nested PCR.

Kingdom	Genus	Species	Strain number	Real-time PCR	Nested-PCR
Fungi	*Aspergillus*	*acidus*	IHEM 26285	-	-
	Aspergillus	*aculeatus*	IHEM 05796	-	-
	Aspergillus	*fijiensis*	IHEM 22812	-	-
	Aspergillus	*melleus*	IHEM 25956	-	-
	Aspergillus	*niger*	IHEM 25485	-	-
	Aspergillus	*oryzae*	IHEM 25836	-	-
	Boletus	*edulis*	MUCL 043104	-	-
	Candida	*cylindracea*	MUCL 041387	-	-
	Candida	*rugosa*	IHEM 01894	-	-
	Chaetomium	*gracile*	MUCL 053569	-	-
	Cryphonectria	*parasitica*	MUCL 007956	-	-
	Disporotrichum	*dimorphosporum*	MUCL 019341	-	-
	Fusarium	*venenatum*	MUCL 055417	-	-
	Hansenula	*polymorpha*	MUCL 027761	-	-
	Humicola	*insolens*	MUCL 015010	-	-
	Kluyveromyces	*lactis*	IHEM 02051	-	-
	Leptographium	*procerum*	MUCL 008094	-	-
	Mucor	*javanicus*	IHEM 05212	-	-
	Penicillium	*camemberti*	IHEM 06648	-	-
	Penicillium	*chrysogenum*	IHEM 03414	-	-
	Penicillium	*citrinium*	IHEM 26159	-	-
	Penicillium	*decumbens*	IHEM 05935	-	-
	Penicillium	*funiculosum*	MUCL 014091	-	-
	Penicillium	*multicolor*	CBS 501.73	-	-
	Penicillium	*roqueforti*	IHEM 20176	-	-
	Pichia	*pastori*	MUCL 027793	-	-
	Rhizomucor	*miehei*	IHEM 26897	-	-
	Rhizopus	*niveus*	ATCC 200757	-	-
	Rhizopus	*oryzae*	IHEM 26078	-	-
	Saccharomyces	*cerevisiae*	IHEM 25104	-	-
	Sporobolomyces	*singularis*	MUCL 027849	-	-
	Talaromyces	*cellulolyticus/pinophilus*	IHEM 16004	-	-
	Talaromyces	*emersonii*	DSM 2432	-	-
	Trametes	*hirsuta*	MUCL 030869	-	-
	Trichoderma	*citrinoviride*	IHEM 25858	-	-
	Trichoderma	*longibrachiatum*	IHEM 00935	-	-
	Trichoderma	*reesei*	IHEM 05651	-	-
	Trichoderma	*viride*	IHEM 04146	-	-

(*continued on next page*)

Table 1 (*continued*)

Kingdom	Genus	Species	Strain number	Real-time PCR	Nested-PCR
Bacteria	*Arthrobacter*	*ramosus*	LMG 17309	-	-
	Bacillus	*amyloliquefaciens*	LMG 9814	-	-
	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 6933T	-	-
	Bacillus	*megaterium*	LMG 7127	-	-
	Bacillus	*pumilus*	DSMZ 1794	-	-
	Bacillus	*smithii*	LMG 6327	-	-
	Bacillus	*subtillis*	LMG 7135 T	-	-
	Bacillus	*subtillis*	W04-510	-	-
	Bacillus	*subtillis*	E07-505	-	-
	Bacillus	*subtillis*	S10005	-[a]	-
	Bacillus	*subtillis*	SUB033	-	-
	Bacillus	*subtillis*	BNB54	-	-
	Bacillus	*subtilis*	GMM from RASFF2014.1249	+	+
	Cellulosimicrobium	*cellulans*	LMG 16121	-	-
	Corynebacterium	*glutamicum*	LMG 3652	-	-
	Enterococcus	*faecium*	LMG 9430	-	-
	Escherichia	*coli*	LMG2092T	-	-
	Geobacillus	*caldoproteolyticus*	DSM 15730	-	-
	Geobacillus	*pallidus*	LMG 11159T	-	-
	Geobacillus	*stearothermophilus*	LMG 6939T	-	-
	Klebsiella	*pneumoniae*	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*	LMG1794T	-	-
	Pullulanibacillus	*naganoensis*	LMG 12887	-	-
	Streptomyces	*aureofaciens*	LMG 5968	-	-
	Streptomyces	*mobaraensis*	DSM 40847	-	-
	Streptomyces	*murinus*	LMG 10475	-	-
	Streptomyces	*netropsis*	LMG 5977	-	-
	Streptomyces	*rubiginosus*	LMG20268	-	-
	Streptomyces	*violaceoruber*	LMG 7183	-	-
	Streptoverticillium	*mobaraense*	CBS 199.75	-	-

[a] One of the four real-time PCR replicates presented a very late signal (C_q: 40).

associated with the potential AMR gene acquisition in case of unexpected DNA contaminations from additives, enzymes and flavourings-producing GMM in commercialized food and feed products.

2. Materials and methods

2.1. Patent inventory and related analysis

All GM bacterial species used for the production of food and feed additive, enzyme and flavouring products mentioned in EFSA (2018b) were identified. For the most frequently used GM bacterial species (being *B. subtilis* and *B. licheniformis*), related patents were inventoried using the "lens.org" database (https://www.lens.org/lens/) with the following query: title:"X" AND abstract:"Y", where X is the bacterial species and Y is the additive/enzyme/flavouring product (Supplementary file 2). Based on the generated list of patents, the patents and associated patents (family documents) reporting the use of GM *B. subtilis* and GM *B. licheniformis* producing microbial fermentation products were collected and analysed (Supplementary file 2).

For the analysis of the collected CmR gene sequences from plasmids mentioned in the inventoried patents, the CLC Genomics Workbench (Qiagen) and the NCBI database (Refseq_genomic) were used (Supplementary files 2 and 4).

2.2. Materials

Eighty-five wild-type microbial strains, including bacteria and fungi, corresponding to the majority of microorganisms mentioned in EFSA (2018b), reporting their use in the food and feed industry to produce food and feed additives, enzymes and flavourings, are indicated in Table 1 (EFSA (2018b); Deckers, et al., 2019). These microbial strains were collected from Sciensano, the Belgian co-ordinated collections of micro-organisms (BCCM), the Research Institute for Agriculture, Fisheries and Food (ILVO), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The GM *B. subtilis* RASFF2014.1249 strain (Tables 1 and 2), described in Barbau-Piednoir et al. (2015a), Barbau-Piednoir et al. (2015b) and Paracchini et al. (2017), was collected from Sciensano. All microbial strains were cultured according to the collection recommendations on specific growth media and at optimal temperature.

Among the tested commercialized samples containing an unauthorized GMM or associated recombinant DNA, two vitamin B2 feed additive matrices sampled in 2016 and 2018 (RASFF2018.2755) on the Belgian market by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) as well as one vitamin B2 feed additive matrix sampled in 2014 (RASFF2014.1249) on the French market by the French competent authorities were selected (Table 3). In addition, a vitamin B2 feed additive matrix sampled in 2017 on the Belgian market by FASFC was used as negative control.

2.3. DNA extraction, concentration and purity

DNA from the GM *B. subtilis* RASFF2014.1249 strain was extracted from a bacterial liquid culture using the NucleoSpin® Food kit (MACHEREY-NAGEL), according to the manufacturer instructions. DNA from wild-type microorganism strains were extracted as mentioned in Deckers et al. (2019). For each of these microbial strains, 200 mg of a fresh culture were used. The DNA extraction step from wild-type microbial strains was performed independently to the GM microbial strain. DNA from the four commercialized feed additive matrices was extracted using a CTAB-based procedure (International Standard ISO 21571, 2005). For each of these commercialized samples, 1 g of the feed additive matrix was used. DNA concentration was measured by spectrophotometry using Nanodrop® 2000 (ThermoFisher) and DNA purity was evaluated using the A260/A280 and A260/A230 ratios.

2.4. Nested-PCR method targeting the cat gene

Each PCR assay was applied on a standard 25 µl reaction volume containing 1X Green DreamTaq PCR Master Mix (ThermoFisher Scientific), 400 nM of each primer (Eurogentec) and 5 µl of DNA. First, the majority of the AMR gene sequence is amplified using the couple of primers cat-F1/R1. A 1:100 dilution of the generated PCR product is then submitted to a nested-PCR using the couple of primers cat-F2/R2 in order to increase the specific amplicon yield (Fig. 2). The primers were designed in this study using "Primer3" on the *cat* gene with the NCBI accession number NC_002013.1. The PCR program consisted of a single cycle of 1 min at 95 °C (initial denaturation) followed by 35 amplification cycles of 30 s at 95 °C (denaturation), 30 s at 60 °C (annealing) and 1 min at 72 °C (extension) and finishing by a single cycle of 5 min at 72 °C (final extension). The run was performed on a Swift MaxPro Thermal Cycler (Esco). For each assay, a "No Template Control" (NTC) was included. The PCR products were visualized by electrophoresis using the Tapestation 4200 device with the associated D1000 Screen Tape and reagents (Agilent) (Supplementary files 3,5–6). The sequencing of the PCR products, earlier purified using USB ExoSAP-IT PCR Product Cleanup (Affymetrix) according to the manufacturer instructions, was performed on a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1 (Applied Biosystems). The generated sequences were analysed using CLC Genomics Workbench (Qiagen) (Supplementary file 7).

Table 2
Sensitivity assessment of the proposed strategy targeting the *cat* gene (GenBank: NC_002013.1). For each real-time PCR and nested-PCR result, the experiment was carried out in duplicate. The observation of a PCR amplification is symbolized by "+" (2/2), "+/−" (1/2) or "-" (0/2). For each tested DNA amount from GM *Bacillus subtilis* RASFF2014.1249 strain, the corresponding estimated full genome copy number is indicated. For real-time PCR results, the means of the observed C_q are indicated under brackets.

DNA amount (ng)	Estimated full genome copy number	Real-time PCR	Nested-PCR
25	5,500,000	+ (C_q: 16.6)	+
0.25	55,000	+ (C_q: 23.6)	+
0.0025	550	+ (C_q: 31.3)	+
0.00025	55	+ (C_q: 34.2)	+
0.00005	11	+ (C_q: 36.9)	+
0.000025	5	+ (C_q: 37.4)	+/−
0.0000025	1	-	-
0	0	-	-

Table 3
Applicability assessment of the proposed strategy targeting the *cat* gene (GenBank: NC_002013.1) using real-life samples. Each sample was tested at10 ng with the real-time PCR 558 and *cat* markers as well as with the *cat* nested-PCR method. The presence or absence of PCR amplification is respectively symbolized by "+" or "-". The feed additive sample collected in 2017 was used as negative control.

Real-life samples	Real-time PCR		Nested-PCR
	558	cat	cat
Feed additive - 2018 (RASFF2018.2755)	+	+	+
Feed additive - 2017	-	-	-
Feed additive - 2016	+	+	+
Feed additive - 2014 (RASFF2014.1249)	+	+	+

For the specificity assessment, the developed PCR method was applied in duplicate on eighty-five different wild-type strains of microorganisms, including bacteria and fungi, at a DNA amount of 10 ng. As positive control, 10 ng of DNA from the GM *B. subtilis* RASFF2014.1249 strain was also tested (Table 1, Supplementary file 3).

The sensitivity assessment was performed using the GM *B. subtilis* RASFF2014.1249 strain at different estimated full genome copy numbers (55×10^5, 55×10^3, 550, 55, 11, 5, 1 and 0). Each sample was tested in duplicate. The calculation of the estimated full genome copy number was based on the NCBI reported genome size of *B. subtilis* (4,214,810 bp) and the formula mentioned in Barbau-Piednoir et al. (2015b) (Table 2, Supplementary files 5).

The applicability assessment was performed using commercialized vitamin B2 feed additive matrices at a DNA amount of 10 ng (Table 3, Supplementary files 6–7).

2.5. Real-time PCR assays

The TaqMan® real-time PCR cat marker, targeting the *cat* gene, as describe in Turgeon et al. (2008), was applied in quadruplicate on all microbial strains mentioned in Table 1 at a DNA amount of 10 ng. This TaqMan® real-time PCR cat marker was also applied in duplicate on DNA from the GM *B. subtilis* strain 2014–3557 at different estimated full genome copy numbers (55×10^5, 55×10^3, 550, 55, 11, 5, 1 and 0) (Fig. 2, Table 2). The TaqMan® real-time PCR 558 marker, targeting the integration site of the *cat* gene in the GM *B. subtilis* isolate e871, as described by Paracchini et al. (2017), and the TaqMan® real-time PCR cat marker were applied in duplicate on commercialized feed additive matrices at a DNA amount of 10 ng (Fig. 2, Table 3). All runs were performed on an a CFX96 Touch Real-Time PCR Detection System (BioRad). For each assay, a NTC was included.

3. Results and discussion

3.1. Occurrence of CmR genes in GM bacteria producing food and feed microbial fermentation products

Based on EFSA (2018b) reporting the microorganisms used in the food and feed industry to produce food and feed additives, enzymes and flavourings, 37 GM bacterial strains used for the production of food and feed additives, enzymes and flavourings were inventoried. These GM bacterial strains were identified as belonging to the following bacterial species: *B. licheniformis* (45.9%), *B. subtilis* (29.7%), *Escherichia coli* (8.1%), *Streptomyces violaceoruber* (8.1%), *Corynebacterium glutamicum* (2.7%), *B. amyloliquefaciens* (2.7%) and *Pseudomonas fluorescens* (2.7%). For the most frequent GM bacterial strains, belonging to the *B.*

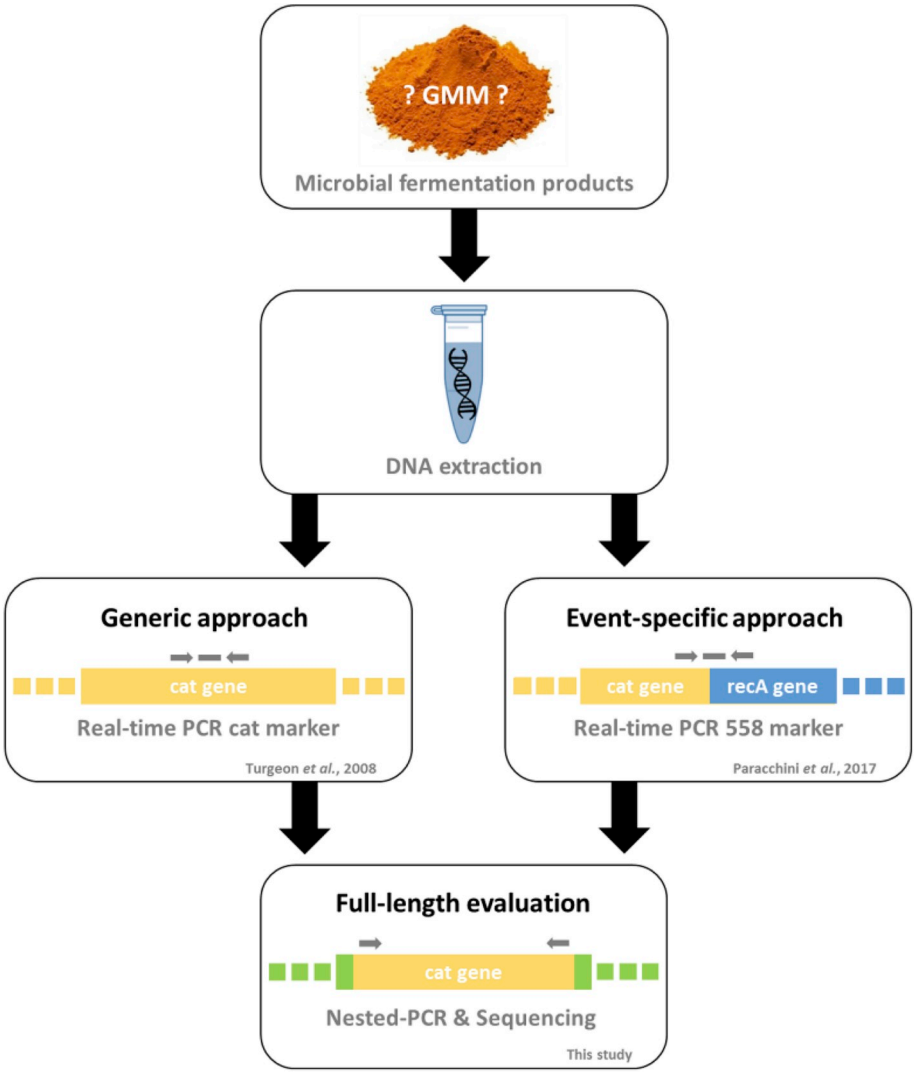

Fig. 1. Workflow of the proposed strategy confirming the presence and the full-length size of the *cat* gene. The *cat* gene is represented in yellow, the *Bacillus subtilis recA* gene is represented in blue and undefined genome sequences are represented in green. The small horizontal grey arrows and the small grey rectangles are respectively indicating the annealing sites of primers and probes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

licheniformis and *B. subtilis* species (75.6%), an inventory of related patents was performed in this study taking into account the associated microbial fermentation products previously reported in EFSA (2018b) (Supplementary file 2). More precisely, the selected microbial fermentation products were the Xylanase, Maltogenic amylase, Asparaginase, Aqualysin I, Pullulanase, Endo-1,3(4)-B-glucanase, Beta-galactosidase, Bacillolysin, Pectate lyase, 1,4-alpha-glucan, Subtilisin, Alpha-amylase, Riboflavin, Endo-1,4-beta-xylanase, Lactic acid, Pullanase, Glucan 1,4-alpha-maltotetraohydrolase, Chymotrypsin, Lactase, Beta-amylase, Acetolactate decarboxylase, Serine protease, Protease and Beta-cyclodextrin (EFSA, 2019; European Union Register of Feed Additives, Kallscheuer, 2018). On this basis, among the 241 patents collected, 30% of them (72 patents) mentioned the use of CmR genes as selection marker in microbial fermentation products-producing GMM (Supplementary file 2A). Very frequently, the CmR marker was combined to other AMR markers (Supplementary file 2A). Interestingly, the majority of these 72 patents were submitted by applicants from the United States (USA) and China. In addition, a limited number of plasmids, including mainly pC194 as well as pHT01, pJH101, pBGSC6, pBR325, pGJ148 and pL603, was identified as being the origin of the used CmR gene sequences (Supplementary file 2A). Except for pBR325, the available CmR gene sequences from these plasmids (pC194, pHT01, pJH101, pBGSC6) were identical among themselves. This sequence corresponded to the chloramphenicol acetyl-transferase (*cat*) gene with the GenBank number NC_002013.1 encoding a protein preventing the chloramphenicol antibiotic from binding to the ribosomes (Supplementary file 2B–C). It must be notified that this *cat* gene (GenBank: NC_002013.1) was previously shown to be harboured by the unauthorized GM *B. subtilis* producing vitamin B2 detected in 2014 (RASFF2014.1249), demonstrating clearly its use (Paracchini et al., 2017) (Supplementary files 6–7). Consequently, the development of a strategy allowing to detect this *cat* gene in the food and feed chain as well as to evaluate the presence of its full-length is definitely of the upmost importance.

3.2. Development of the PCR-based strategy to detect and evaluate the presence of the full-length cat gene

The proposed PCR-based strategy, targeting the *cat* gene (GenBank: NC_002013.1), was developed to be performed in two main successive steps (Fig. 1). First, the potential presence of the *cat* gene is detected using a real-time PCR cat marker. For this first line screening step, the TaqMan® real-time PCR method developed by Turgeon et al. (2008) to cover 96 bp of the targeted *cat* gene was selected. Secondly, following

A

>Chloramphenicol acetyl transferase (*cat*) gene from plasmid pC194 (GenBank: NC_002013.1)

ATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGATATTTAATCATTA<u>TTTGAA**CCAACAAACGACTTT**</u>**TAGTATAACC**ACAGAAATTGA
TATTAGTGTTTTATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCATTTATTTTCTTAgtgacaagggtgataaactcaaatacAGCTTTTAGAactggtta
caatagcgacggagagttaggtTATTgggataagttagagccactttatacaATTTTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTCCTGTAAAGAATGACTTCA
AAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATATAATGGTTCGGGGAAATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTTCTATT
ATTCCATGGACTTCATTTACTGGGTTTAACTTAAATATCAATAATAATAGTAATTACCTTCTACCCATTATTACAGCAGGAAAATTCATTAATAAAGGTAATTC
AATATATTTACCGCTATCTTTACAGGTACATCATTCTGTTT**GTGATGGTTATCATGCAGGA**TTGTTTATGAACTCTAT<u>TCAGGAATTGTCAGATAGGCC</u>TAAT
GACTGGCTTTTATAA

B

Methods	Oligonucleotide names	Oligonucleotide sequences	Expected amplicon size	References
PCR	cat-F1 cat-R1	TTTGAACCAACAAACGACTTT GGCCTATCTGACAATTCCTGA	573 bp	This study
PCR	cat-F2 cat-R2	CCAACAAACGACTTTTAGTATAACC TCCTGCATGATAACCATCAC	529 bp	This study
Real-time PCR	cat-F cat-P cat-R	GTGACAAGGGTGATAAACTCAAATAC FAM-ACCTAACTCTCCGTCGCTATTGTAACCAGT-TAMRA TGTATAAAGTGGCTCTAACTTATCCC	96 bp	Turgeon et al., 2008

Fig. 2. Oligonucleotides used for the real-time PCR and nested-PCR methods targeting the *cat* gene (GenBank: NC_002013.1**)** (A) Location of the used oligonucleotides on the *cat* gene. For the PCR methods, the couple of primers used in the first PCR round (cat-F1 and cat-R1) is underlined while the couple of primers used in the following nested-PCR (cat-F2 and cat-R2) is indicated in bold. For the real-time PCR, the oligonucleotides are indicated in lowercase. (B) Description of the used oligonucleotides and the generated amplicons.

to a positive signal for the real-time PCR cat marker, the presence of the full-length *cat* gene is evaluated via a PCR method developed in this study. For this second line step, primers were designed to amplify the largest fragment of the *cat* gene possible taking into account method performance criteria compatible with GMO routine analysis. In order to increase the yield of the generated specific amplicon, a strategy of nested-PCR was performed, including a first amplification of 573 bp, using the couple of primers cat-F1 and cat-R1, followed by a second amplification of 529 bp, using the couple of primers cat-F2 and cat-R2 (Fig. 2). The nested-PCR method was initially tested on 25 ng of DNA from a vitamin B2- producing GM *B. subtilis* strain harbouring the *cat* gene (RASFF2014.1249) that was previously fully characterized (Table 2, Supplementary file 5) (Barbau-Piednoir et al., 2015a, 2015b; Paracchini et al., 2017). The final PCR product, presenting a single amplicon with the expected size, was then sequenced in order to confirm the identity of the generated sequence by alignment to the *cat* gene reference sequence (GenBank: NC_002013.1) (Supplementary files 5 and 7). Afterwards, the performance of the proposed PCR-based strategy, including the real-time PCR cat marker and the developed nested-PCR method, was investigated.

On the one hand, the specificity was assessed using eighty-five wild-type bacterial and fungal strains commonly used in the food and feed industry to produce additives, enzymes and flavourings (EFSA (2018b); Deckers et al., 2019). Following to the real-time PCR analysis using the cat marker, no PCR amplification was observed for the tested wild-type microbial strains, except for one wild-type *B. subtilis* strain (S10005) (Table 1). More precisely, on the seven different *B. subtilis* strains tested, only one strain (S10005) presented one positive replicate out of four at very late signal (C_q: 40). Such a result could be explained by the possible presence of fragments of AMR genes in some wild-type bacterial strains, as previously described for example in Bacillus species (Agerso et al., 2019). Nonetheless, in using then the developed nested-PCR method to assess the full-length of the *cat* gene, all the tested wild-type microbial strains presented no PCR amplification, supporting the specificity of the proposed strategy to detect GMM harbouring the *cat* gene (Table 1, Supplementary file 3). In addition, this result was confirmed *in silico*. Indeed, the *cat* gene sequence used as reference (GenBank: NC_002013.1) was blasted to the wild-type strains from the NCBI reference genomic sequence database. From this *in silico* analysis, only few species presented a hit of 100% in terms of identity and recovery (Supplementary file 4). Among these species naturally harbouring this AMR gene, only *Enterococcus faecium* was reported in EFSA (2018b) as being used to produce microbial fermentation products. However, no positive signal was experimentally observed in the present study with the proposed strategy on the tested *E. faecium* strain, which could be explained by the fact that, within the *E. faecium* species, approximately half of the strains was recently estimated to harbour a CmR gene (Bi, Qin, Fan, Ma, & Gu, 2018). Therefore, according to both the experimental and *in silico* analysis, the potential observation of positive signals that are not directly related to the presence of GMM, or associated recombinant DNA, seems thus to be very limited. Nonetheless, given that the solely presence, natural or not, of AMR genes in food and feed products constitute a crucial issue for the public health and environment, the proposed strategy represents of any manner a key tool for control of the food and feed safety (Hudson et al., 2017; Tacconelli et al., 2018).

On the other hand, the sensitivity was tested on DNA, at different estimated full genome copy numbers going from 5,500,000 to 0, from the previously fully characterized vitamin B2-producing GM *B. subtilis* strain harbouring the *cat* gene (RASFF2014.1249) (Barbau-Piednoir et al., 2015a, 2015b; Paracchini et al., 2017). On this basis, as both the real-time PCR cat marker and the nested-PCR method were able to reach up to 5 estimated full genome copies, the proposed PCR strategy was thus considered as sensitive (Table 2, Supplementary file 5). However, even if these results seem compatible with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories, further analysis, such as additional PCR replicates, will be necessary to confirm it (ENGL, 2015). Moreover, to support its widespread adoption by enforcement laboratories, a full validation, including the robustness and transferability analysis, of the proposed PCR methods will be needed (ENGL, 2015).

3.3. Applicability of the PCR-based strategy targeting the cat gene

With the aim to assess the applicability of the PCR-based strategy targeting the *cat* gene, four commercialized vitamin B2 feed additive matrices, including three presenting a positive signal (2014, 2016 and 2018) and one presenting a negative signal (2017) for the real-time PCR 558 marker amplifying the integration site of the *cat* gene in the EU unauthorized vitamin B2-producing GM *B. subtilis* RASFF2014.1249,

were selected (Fig. 1, Table 3). The matrices collected in 2018 and 2014 have previously led respectively to the RASFF2018.2755 and RASFF2014.1249 notifications (RASFF portal, Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Paracchini et al., 2017).

First, DNA extracted from the four commercialized vitamin B2 feed additive powders were submitted to a first line real-time PCR screening analysis using the cat marker in order to determine the potential presence of the *cat* gene (Turgeon et al., 2008). As expected, the three matrices (2014, 2016 and 2018) with a positive signal for the 558 marker presented a positive signal for the cat marker (Table 3). Second, with the aim to determine the potential presence of the full-length of the detected *cat* gene in these three feed additive samples, the developed nested-PCR method was applied. These three feed additive matrices sampled in 2018, 2016 and 2014 presented a single amplicon at the expected size corresponding at the sequence level to the targeted *cat* gene while no PCR amplification was observed for the feed additive matrix sampled in 2017 that was used as negative control (GenBank: NC_002013.1) (Table 3, Supplementary files 6–7). Based on these results, the applicability of proposed PCR-based strategy was thus demonstrated.

4. Conclusion

Based on the analysis of publicly available patents, a noteworthy part of GMM producing microbial fermentation products used in the food and feed industry was in the present study estimated to harbour a CmR marker conferred essentially by the *cat* gene (GenBank accession: NC_002013.1) (Supplementary file 2). This corroborates the observations from enforcement laboratories that have led to several notifications reporting in microbial fermentation products the unexpected presence of both such GMM, or associated recombinant DNA, carrying this *cat* gene (GenBank accession: NC_002013.1) as well as contaminations of the chloramphenicol antibiotic potentially used in the selection of such GMM (RASFF portal). Moreover, the CmR marker, especially conferred by this *cat* gene, represents an interesting target to detect GMM in microbial fermentation products due to its limited natural widespread. The detection of this *cat* gene highlighted also the potential presence of other AMR genes since the performed patent analysis has indicated the frequent association of other AMR markers to the CmR marker (Supplementary file 2). A first line real-time PCR screening including the cat marker and/or the event-specific 558 method, specific to the GM *B. subtilis* RASFF2014.1249 strain in targeting its junction between the *B. subtilis* recA gene and the *cat* gene, represents thus a relevant indicator for the presence of GMM harbouring the *cat* gene (Tables 1–3) (Paracchini et al., 2017; Turgeon et al., 2008). In term of food and feed safety, to evaluate the level of risks of likely AMR gene acquisition by pathogens and gut microbiota, it is essential to determine if the full-length AMR genes are present. This critical issue was particularly pointed out still very recently by the competent authorities (EFSA, 2019). Therefore, in order to assess the presence of the full-length *cat* gene potentially detected upstream by the real-time PCR cat marker, a nested-PCR method was developed in the present study to amplify a large fragment of its sequence (Fig. 1). This strategy is based on the PCR technology which is cost- and time-effective as well as required a technical expertise acquired by enforcement laboratories from several years. Moreover, in agreement with EFSA (2018a), the proposed strategy allows to test the presence of DNA from the production microbial strains by PCR in amplifying an AMR fragment not exceeding the size of the smallest AMR genes harboured by the production microbial strains. Based on the generated results using the proposed PCR-based strategy, the competent authorities could thus for the first time assess more precisely the potential risks for the human and animal health and the environment in case of unexpected contaminations of unauthorized GMM, or associated recombinant DNA, in the food and feed chain, consequently facilitating their discussions related to the most appropriate measures to be taken. The proposed strategy developed in the present study may also be used in order to target other AMR genes, taking into account their occurrence in both GMM and wild-type strains.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of interests

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.

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Appendix A. Supplementary data

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