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Retrospective survey of unauthorized genetically modified bacteria harbouring antimicrobial resistance genes in feed additive vitamin B2 commercialized in Belgium: Challenges and solutions

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ABSTRACT

In Belgium, an official control plan was established in 2016 to detect the potential presence of an unauthorized genetically modified (GM) *Bacillus subtilis* RASFF2014.1249 strain in commercialized feed additive vitamin B2 products. To this end, two real-time PCR markers specific to this unauthorized genetically modified microorganism (GMM), named UGMVit-B2 and 558, were used. In the present study, the first four-year results from 67 feed additive vitamin B2 samples from the official control are presented. It includes 5 samples positive for real-time PCR methods specific to the unauthorized GM *B. subtilis* RASFF2014.1249 strain and has led to the RASFF2018.2755 and RASFF2019.3216 notifications. Moreover, a retrospective study using the same feed additive vitamin B2 samples was performed, allowing to provide a first picture of GM bacterial contaminations. It consisted in a first-line screening strategy gathering available PCR-based methods targeting both the *B. subtilis* species, frequently used to produce vitamin B2, and a set of antimicrobial resistance (AMR) genes commonly harboured as selection marker by GM bacteria used to produce microbial fermentation products. On this basis, suspicious samples contaminated with additional unknown GM bacterial strains as well as potential health and environmental risks related to the unexpected presence of full-length AMR genes could be highlighted. In addition, the possible complementary use of additional data, like chloramphenicol presence and DNA concentration, as indicators for GMM contaminations was assessed. Based on results generated in the present study, the relevance to use the proposed first-line screening strategy supplemented by indicators in order to strengthen the current control strategy was emphasized.

1. Introduction

In 2014, unexpected genetically modified microorganism (GMM) contaminations in a feed additive vitamin B2 product commercialized on the European (EU) market were notified (RASFF2014.1249; RASFF2014.1360; RASFF2014.1657) for the first time by enforcement laboratories (RASFF portal). These notifications were related to the identification of a living genetically modified (GM) *Bacillus subtilis* strain overproducing vitamin B2.

In the food and feed industry, additives are usually produced through microbial fermentation processes using microorganisms that are often GM microbial strains carrying antimicrobial resistance (AMR) genes as selection marker (Barbau-Piednoir et al., 2015a; Barbau-Piednoir et al., 2015b; Berbers et al., 2020; EFSA, 2019; Fraiture et al., 2020a; Fraiture et al., 2020b; Paracchini et al., 2017). According to the EU legislation, the commercialization of a specific microbial fermentation product,

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including enzymes, additives and flavourings, produced by a GMM requires an authorization by the EU commission. For this purpose, a dossier is confidentially submitted by the applicant to the European Food Safety Authority (EFSA) for safety evaluation. For feed additive vitamin B2, positively evaluated dossiers, mentioning the use of GM bacteria, belonging to *B. subtilis*, and GM fungi, belonging to *Ashbya gossypii*, have currently been evaluated (Barbau-Piednoir et al., 2015; Barbau-Piednoir et al., 2015; Paracchini et al., 2017; Berbers et al., 2020; Regulation EC No 1831/2003, 2003; Regulation EC No 2019/901, 2019; EFSA, 2016; EFSA, 2018a; EFSA, 2018b; EFSA, 2018c; EFSA, 2018d). Among the requirements of EFSA, viable GMM as well as associated recombinant DNA should be absent in commercialized microbial fermentation products. Moreover, none of the GMM used to produce microbial fermentation products have currently been approved for the EU regulation EC/1829/2003 related to the commercialization of GMO in the food and feed chain. Therefore, any GMM identified in feed additives on the EU market is automatically considered as unauthorized and feed additive vitamin B2 products contaminated by the GM *B. subtilis* strain overproducing vitamin B2 or its DNA were assessed as non-conform (Barbau-Piednoir et al., 2015; Barbau-Piednoir et al., 2015; Paracchini et al., 2017; Berbers et al., 2020; Regulation EC No 1829/2003, 2003; EFSA, 2016; EFSA, 2018a; EFSA, 2018b; EFSA, 2018c; EFSA, 2018d). In addition to the respect of the EU legislation allowing to guarantee the tractability of the food and feed chain as well as the freedom of choice for consumers, concerns associated to the safety of the food and feed chain were raised. More precisely, AMR genes, conferring a resistance to chloramphenicol, ampicillin, kanamycin, neomycin, bleomycin, erythromycin and tetracycline, were harboured by the GM *B. subtilis* strain overproducing vitamin B2 previously identified in 2014 on the EU market. The presence of these AMR genes in food and feed represents indeed a health risk due to their potential horizontal transmission to pathogens and microbiota gut. This risk is especially emphasized because AMR genes are frequently utilized as selection marker for GMM used by the food and feed industry to produce microbial fermentation products, such as vitamins (EFSA, 2004; Barbau-Piednoir et al., 2015; Barbau-Piednoir et al., 2015; Paracchini et al., 2017; Berbers et al., 2020; Fraiture et al., 2020; Fraiture et al., 2020; EFSA, 2019; Bacanli & Basacan, 2019; Munita & Arias, 2016; Rozwandowicz et al., 2018; Sharma et al., 2018; von Wrighta & Bruce, 2003; Xiong et al., 2018).

With the aim to both guarantee the respect of the EU legislation regarding GMO and the safety of the food and feed chain, the Belgian Competent Authorities have consequently established since 2016 a control plan for vitamin B2 commercialized as feed additive on the Belgian market. This control was performed using two available real-time PCR methods specific to the vitamin B2-overproducing GM *B. subtilis* RASFF2014.1249 strain. These methods correspond to the 558 marker, covering the junction between the *B. subtilis recA* gene and the *chloramphenicol acetyl-transferase* (*cat*) gene, and the VitB2-UGM marker, targeting the junction between the *B. subtilis* riboflavin biosynthesis operon and the vector used to construct the GM *B. subtilis* RASFF2014.1249 strain (Barbau-Piednoir et al., 2015; Paracchini et al., 2017). These methods were previously developed based on the characterization by whole-genome sequencing (WGS) of the living GM *B. subtilis* RASFF2014.1249 strain isolated from the commercialized feed additive vitamin B2 associated to the RASFF2014.1249 notification (Barbau-Piednoir et al., 2015a, 2015b; Paracchini et al., 2017). Based on this control plan, unexpected contaminations with this specific GM strain were reported in 2018 (RASFF2018.2755) and in 2019 (RASFF2019.3216) (RASFF portal). To perform such control, no additional method was available at that moment. This is in contrast with the testing for GM plant events, where the enforcement laboratories have at their disposal a large set of real-time PCR markers. Indeed, dossiers related to GMM are confidentially submitted to EFSA for a safety assessment and no identification method to trace these GMM is required (Regulation EC No 1829/2003, 2003; EFSA, 2011; EFSA, 2018d; EURL-GMFF, 2011). Consequently, as enforcement laboratories have at their disposal only methods targeting specifically the GM *B. subtilis* RASFF2014.1249 strain, any other unauthorized GMM potentially present in the tested samples could bypass the current control plan.

To overcome such limitation, a first-line PCR-based strategy was recently proposed to target a large spectrum of unauthorized GMM used by the food and feed industry to produce microbial fermentation products, including enzymes, flavourings and additives (Fraiture et al., 2020a, 2020b, 2020c). Based on the analysis of publicly available patents, three key AMR genes commonly used as selection marker in GM bacteria producing food and feed microbial fermentation products were selected to be targeted by the proposed PCR-based strategy. The combination of these key AMR genes, being a *cat* gene (GenBank: NC_002013.1) conferring a resistance to chloramphenicol (CmR), an *aminoglycoside adenyltransferase* (*aadD*) gene (GenBank: M19465.1) conferring a resistance to both kanamycin and neomycin (KanR) and a *tet-L* gene (GenBank: D00946.1) conferring a resistance to tetracycline (TetR), was estimated to cover approximately 90% of the inventoried publicly available patents related to GM bacteria producing food and feed microbial fermentation products (Fraiture et al., 2020a, 2020b, 2020c). Moreover, through this patent analysis, *B. subtilis* was identified as being one of the most frequent bacterial species used to produce GMM (Deckers et al., 2020a; Fraiture et al., 2020). This *Bacillus* species represents therefore an additional key target to investigate in a first-line screening the potential presence of GMM in microbial fermentation products. Even if no real-time PCR marker has previously been developed to target specifically *B. subtilis* especially in the context of GMM detection, several available studies with such *B. subtilis* marker exist (Cangiano et al., 2014; Fernandez-No et al., 2011; Gao et al., 2011; Mortazavi et al., 2014; Nakano, 2020; Xie et al., 2019).

In this study, we present the analysis of 67 feed additive vitamin B2 samples, previously collected by the Belgian Competent Authorities from 2016 to 2019 in the context of the Belgian control plan, using the real-time PCR vitB2-UGM and 558 markers specific to the GM *B. subtilis* RASFF2014.1249 strain (Barbau-Piednoir et al., 2015; Paracchini et al., 2017). Moreover, in the frame of a retrospective study, these feed additive vitamin B2 samples were investigated for the potential presence of additional unauthorized GMM. To this end, similarly to the current GM plant screening analysis combining species and transgenic element markers, a first-line screening strategy was proposed and applied to feed additive vitamin B2 products. More precisely, it consists of a general analysis workflow gathering available methods to target both the *B. subtilis* species (personal communication; Cangiano et al., 2014) as well as three key AMR genes (Fraiture et al., 2020a, 2020b, 2020c). Moreover, the complementary use of additional data, related to the chloramphenicol antibiotic quantification and the DNA extraction yield, was investigated as potential indicators for unauthorized GMM contaminations.

Through the present study, using the real-time PCR vitB2-UGM and 558 markers, we present for the first time a survey of GMM contaminations in commercialized microbial fermentation products, including the RASFF2018.2755 and RASFF2019.3216 notifications. In addition, a retrospective study was performed, using methods targeting a large spectrum of GM bacteria. On this basis, a broader picture of the GMM contamination status in commercialized feed additive vitamin B2 products was established. Moreover, the strengths and weaknesses of the current control using the real-time PCR vitB2-UGM and 558 markers could be evaluated.

2. Materials and methods

2.1. Materials

Feed additives containing vitamin B2 commercialized on the Belgian market were sampled between 2016 and 2019 by the Federal Agency for the Safety of the Food Chain (FASFC) (Table 1). All of these 67 samples

were previously analysed for the presence of the GM *B. subtilis* RASFF2014.1249 strain by the Belgian NRL-GMO enforcement laboratory of the Transversal activities in Applied Genomics (TAG) Service from Sciensano. The expiration date of the samples n°8 and 10 had passed during the analysis time-frame, explaining that no RASFF notification was released for these samples contaminated by GMM. Sample n°45 and its counter-expertise sample (sample n°48) are related to the RASFF2018.2755 notification and sample n°67 is related to the RASFF2019.3216 notification.

2.2. DNA extraction, concentration and purity

DNA from the feed additive vitamin B2 matrices was extracted using an adapted CTAB-based procedure (ISO 21571) (International Standard ISO 21571, 2005; Barbau-Piednoir et al., 2015). Briefly, 1 g of sample was mixed with 15 ml of CTAB extraction buffer (NaCl 1.4 M, EDTA 0.02 M, Tris-HCl 0.1 M, CTAB 2%) and 150 μl of RNase A (Sigma-Aldrich) at 10 mg/ml. After an incubation at 65 °C for 30 min, 300 μl of Proteinase K (Sigma-Aldrich) at 20 mg/ml was added. After an incubation at 65 °C for 30 min and a subsequent centrifugation at 8000 g for 15 min, the supernatant was mixed with 1 volume of chloroform (Merck). Following a centrifugation at 8000 g for 20 min, the supernatant was mixed with 0.6 vol of isopropanol (Merck) and 0.4 vol of ammonium acetate (Merck) at 10 M for an incubation at 4 °C for 30 min. After a centrifugation at 8000 g for 20 min, the pellet was washed with 1 ml of ethanol (Merck) at 70%. Following a centrifugation at 8000 g for 20 min, the dried pellet was resuspended in 200 μl of TE buffer (10 mM Tris-0.2 mM EDTA pH 8.0), incubated at 50 °C for 2 min, and agitated at 4 °C overnight. DNA concentration was measured by spectrophotometry using Nanodrop® 2000 (ThermoFisher) and DNA purity was evaluated using the A260/A280 and A260/A230 ratios.

2.3. Real-time PCR assays

The present study gathered available real-time PCR methods that were initially not all developed for the same purpose and neither especially designed to work together, explaining for instance their difference in the real-time PCR chemistries. The previously described TaqMan® real-time PCR markers for vitB2-UGM (Barbau-Piednoir et al., 2015), 558 (Paracchini et al., 2017), CmR (Fraiture et al., 2020; Turgeon et al., 2008), KanR (Fraiture et al., 2020) and TetR (Fraiture et al., 2020; Turgeon et al., 2008) were tested in duplicates on 25 ng of DNA from samples listed in Table 1 (Supplementary file 1). Each TaqMan® real-time PCR assay was applied on a standard 25 μl reaction volume containing 1X TaqMan® PCR Mastermix (Diagenode), 250 or 400 nM of each primer (Eurogentec), 100 or 200 nM of the probe and 5 μl of DNA. The real-time PCR program consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 45 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C or 64 °C (annealing-extension step). The SYBR®Green real-time PCR marker for spBS (personal communication; adapted from Cangiano et al., 2014) was tested in duplicates on 25 ng of DNA from samples listed in Table 1 (Supplementary file 1). Each SYBR®Green real-time PCR assay was applied on a standard 25 μl reaction volume containing 1X SYBR®Green PCR Mastermix (Diagenode), 400 nM of each primer (Eurogentec) and 5 μl of DNA. The real-time PCR program consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). The program for melting curve analysis was performed gradually increasing the temperature from 60 to 95 °C in 20 min (±0.6_/20 s). For each assay, an NTC (No Template Control) and a positive control, being DNA from the GM *B. subtilis* RASFF2014.1249 strain, were included. All runs were performed on a CFX96 Touch Real-Time PCR Detection System (BioRad) or an ABI 7300 qPCR system (Applied Biosystems).

2.4. Conventional PCR and sanger sequencing assays

The PCR markers for CmR (Fraiture et al., 2020), KanR (Fraiture et al., 2020) and TetR (Fraiture et al., 2020), to assess the presence of full-length AMR genes, were tested in duplicates as previously described on 25 ng of DNA from samples listed in Table 1 (Supplementary file 1). The assessment of the full-length AMR gene is important for the health risk evaluation regarding the likelihood of AMR gene acquisition, via horizontal gene transfer mechanisms, by pathogens and gut microbiota following the ingestion of food and feed contaminated by GMM or associated recombinant DNA (EFSA, 2011). For each assay, an NTC and a positive control, being DNA from the GM *B. subtilis* RASFF2014.1249 strain, were included. 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. 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). The final PCR products were visualized by electrophoresis using the Tapestation 4200 device with the associated D1000 Screen Tape and reagents (Agilent) (Supplementary file 2). Following a purification step using USB ExoSAP-IT PCR Product Cleanup (Affymetrix), final PCR products were sequenced on a Genetic Sequencer 3500 using the Big Dye Terminator Kit v3.1 (Applied Biosystems). The generated sequences were analysed using MUSCLE (Supplementary file 2).

2.5. Microbial viability test

The potential presence of viable GMM was investigated for suspicious samples (n°8, 10, 45, 48 and 67). 1 g of the feed additive vitamin B2 matrix was added to 250 ml of Brain-Heart Infusion broth (Sigma-Aldrich) for an incubation overnight at the adequate temperature and oxygen condition (Barbau-Piednoir et al., 2015). 100 μl of the culture was plated on nutrient agar (Sigma-Aldrich) for an incubation overnight at the adequate temperature and oxygen condition.

2.6. Chloramphenicol measurements

The chloramphenicol content was determined by performing an extraction method on 2.5 g of each vitamin B2 sample by adding 7.5 ml of water, 25 μl of the internal standard solution at 0.1 μg/ml (isotopically labelled standard D5-chloramphenicol) (Dr Ehrenstorfer Gmbh), earlier diluted in methanol (HPLC grade), and 12 ml of ethyl acetate (HPLC grade). The sample was vigorously mixed for 1 min and centrifuged at 20 °C at 2000 g for 5 min. The supernatant was then concentrated under a nitrogen stream until obtaining a viscous phase.

Next, the concentrated extract was purified as follows. First, the extract was mixed with 7 ml of petroleum ether (Biosolve) and 1 ml of the solution earlier prepared with ammonium acetate aqueous solution (Merck) at 10 mM and pH 4.3/acetonitrile (Biosolve) (80:20; v/v). After a centrifugation at 2000 g for 5 min, the lower layer phase was mixed with 3 ml of n-pentane (HPLC grade). After a centrifugation at 2000 g for 5 min, the lower layer phase was mixed with 2 ml of ethyl acetate (HPLC grade). After a centrifugation at 2000 g for 5 min, the upper layer phase was then evaporated until dryness and reconstituted in 200 μl of ammonium acetate aqueous solution (Merck) at 10 mM and pH 9.

The sample was transferred to an injection vial with insert for LC-MS/MS analysis, performed on an UPLC™ system (Waters) coupled to a Xevo-TQ-XS™ mass spectrometer (Waters) equipped with an electrospray ionization (ESI) interface operated in ESI negative mode. The optimized MRM (multiple reaction monitoring) parameters and the monitored precursor and product ions are described in Supplementary file 3. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (1.7 μm, 100 × 2.1 mm) (Waters). The column

Table 1
Analysis performed on all feed additive vitamin B2 samples (n°1–67).

Samples	First-line screening				GMM identification		Potential additional indicators	
	B. subtilis	AMR genes			GM *B. subtilis* RASFF2014.1249 strain		Antibiotics	Extracted DNA
	spBS	CmR	KanR	TetR	VitB2-UGM	558	Chloramphenicol (μg/kg)	DNA concentration (ng/μl)
1	–	–	–	–	–	Not tested	<LOQ	2,7
2	–	–	–	–	–	Not tested	<LOQ	6,3
3	–	–	(+) (35.9)	–	–	Not tested	<LOQ	3,6
4	–	–	–	–	–	Not tested	<LOQ	1,2
5	–	–	–	–	–	Not tested	<LOQ	3,3
6	–	–	–	–	–	Not tested	<LOQ	5,5
7	–	–	–	–	–	Not tested	<LOQ	10,5
8 (Expired product)	+ (18.2)	+ (21.8)	+ (18.7)	(+) (32.8)	+ (16.5)	+ (20.0)	0,023[a]	27,2
9	–	–	–	–	–	Not tested	<LOQ	26,1
10 (Expired product)	+ (32.2)	+ (32.2)	+ (31.9)	–	+ (30.3)	+ (33.1)	0,077	548,8
11	–	–	–	–	–	Not tested	<LOQ	5
12	–	–	–	–	–	Not tested	<LOQ	15926,6
13	–	–	–	–	–	Not tested	<LOQ	12,7
14	–	–	–	–	–	Not tested	<LOQ	18,8
15	–	–	–	–	–	Not tested	<LOQ	0,6
16	–	–	–	–	–	Not tested	<LOQ	12,5
17	–	–	–	–	–	Not tested	<LOQ	22,9
18	–	–	–	–	–	Not tested	<LOQ	12191,1
19	–	–	–	–	–	Not tested	<LOQ	7
20	+ (36.4)	–	–	–	–	Not tested	<LOQ	21,2
21	–	–	–	–	–	Not tested	<LOQ	7,9
22	–	–	–	–	–	Not tested	<LOQ	6,3
23	–	–	–	–	–	Not tested	<LOQ	6410,6
24	–	–	–	–	–	Not tested	<LOQ	5,9
25	–	–	–	–	–	Not tested	<LOQ	18,7
26	–	–	–	–	–	Not tested	<LOQ	2,7
27	–	–	–	–	–	Not tested	<LOQ	2,6
28	–	–	–	–	–	Not tested	<LOQ	20,9
29	–	–	–	–	–	Not tested	<LOQ	14
30	–	–	–	–	–	Not tested	<LOQ	33,8
31	–	–	–	–	–	Not tested	<LOQ	26,6
32	–	–	–	–	–	Not tested	<LOQ	3,8
33	–	–	–	–	–	Not tested	<LOQ	29,5
34	–	–	–	–	–	Not tested	<LOQ	15,3
35	–	–	–	–	–	Not tested	<LOQ	8,1
36	–	–	–	–	–	Not tested	<LOQ	7,3
37	–	–	–	–	–	Not tested	<LOQ	16,2
38	–	–	–	–	–	Not tested	<LOQ	18,1
39	–	–	–	–	–	Not tested	<LOQ	11,8
40	–	–	–	–	–	Not tested	<LOQ	16700,1
41	–	–	–	–	–	–	<LOQ	14,9
42	–	–	–	–	–	Not tested	<LOQ	294,3
43	–	–	–	–	–	Not tested	<LOQ	46,5
44	–	–	–	–	–	Not tested	<LOQ	77,6
45 (RASFF2018.2755)	+ (34.1)	+ (37.7)	+ (34.8)	–	+ (39.2)	+ (39.9)	<LOQ	33,2
46	–	–	–	–	–	–	<LOQ	15,3
47	–	–	–	–	–	–	<LOQ	21,4
48 (RASFF2018.2755)	+ (34.8)	+ (38.3)	+ (34.6)	–	+ (34.5)	+ (35.9)	<LOQ	42
49	–	–	–	–	–	–	<LOQ	14,3
50	–	–	–	–	–	–	<LOQ	98,6
51	–	–	–	–	–	–	<LOQ	37,5
52	–	–	–	–	–	–	<LOQ	31,9
53	–	–	–	–	–	–	<LOQ	23
54	–	–	–	–	–	–	<LOQ	61,7
55	–	–	–	–	–	–	<LOQ	26,3
56	–	–	–	–	–	–	<LOQ	37,8
57	–	–	–	–	–	–	<LOQ	25,8
58	–	–	–	–	–	–	<LOQ	4,5
59	–	–	–	–	–	–	<LOQ	52
60	–	–	–	–	–	–	<LOQ	17,3
61	–	–	–	–	–	–	<LOQ	49,1
62	–	–	–	–	–	–	<LOQ	121,1
63	–	–	–	–	–	–	<LOQ	40,6
64	–	–	–	–	–	–	<LOQ	4,4
65	–	–	–	–	–	–	<LOQ	3,6
66	–	–	–	–	–	–	<LOQ	1,8
67 (RASFF2019.3216)	+ (21.8)	+ (22.5)	+ (21.3)	(+) (37.7)	+ (19.8)	+ (22.5)	<LOQ	150.5

In the frame of the first-line screening and subsequent GMM identification analysis, for each sample, the presence of *B. subtilis* (real-time PCR spBS marker), key AMR genes (real-time and conventional PCR followed by sequencing for CmR, KanR and TetR markers) and the GM *B. subtilis* RASFF2014.1249 strain (real-time PCR VitB2-UGM and 558 markers) was assessed.
As potential additional indicators, the presence of the chloramphenicol antibiotic (μg/kg) was measured as well as the DNA concentration (ng/μl) of DNA extracts was measured.

For real-time and conventional PCR data, a positive and a negative signal is respectively symbolized by + or -.
The detected AMR genes for which the full-length size cannot be confirmed are symbolized by (+). For real-time PCR data, the C_q value are indicated between brackets.
[a] Semi-quantitative result since the result is slightly lower than the reporting LOQ.

temperature was set at 50 °C. A mobile phase consisting of eluents A (water with formic acid (Merck) 0.1%) and B (acetonitrile (HPLC grade) formic acid (Merck) 0.1%) was used at a flow rate of 0.45 mL/min. A gradient elution was applied as follows: 0 min (starting condition), 80% A; 5 min, 0% A; 5.1 min, 80% A; 7 min, 80% A. The injection volume was 7.5 µL.

The calibration curve was prepared from buffer B (ammonium acetate (Merck) at 10 mM) with the chloramphenicol-D5 as internal standard. The linearity range of the method was 0.025–1 µg/kg of matrix. The limit of quantification (LOQ) was fixed at 0.025 µg/kg of matrix, corresponding to the lowest point of the calibration curve. It should be noted that this is the reporting LOQ and not the actual LOQ of the developed method. Each analytical batch contained a procedural blank and a control sample (CS) consisting in a fortified sample at 0.1 µg/kg.

3. Results and discussion

3.1. First-line screening strategy for unauthorized GMM in feed additive vitamin B2

To investigate the potential presence of unauthorized GM bacteria, including the GM *B. subtilis* RASFF2014.1249 strain as well as other unknown GM strains, in vitamin B2 products, a first-line screening strategy was proposed. It consisted of a general analysis workflow in which available methods were gathered in order to analyse DNA extracted from the feed vitamin B2 matrices (Fig. 1). On one hand, the potential presence of the *B. subtilis* species was tested using the real-time PCR spBS marker (personal communication; adapted from Cangiano et al., 2014). This *B. subtilis* marker was selected because this bacterial species is frequently used for the production of feed additive vitamin B2 (Zarour et al., 2017; Thakur et al., 2016). Moreover, *B. subtilis* is currently the only feed additive vitamin B2-producing bacterial species authorized by the EU legislation and the feed additive vitamin B2 dossiers evaluated by EFSA ((Regulation EC No 1831/2003, 2003); EFSA, 2016, EFSA, 2018a; Barbau-Piednoir et al., 2015; Paracchini et al., 2017). On the other hand, the potential presence of key AMR genes harboured by GM bacteria was first determined using real-time PCR. Subsequently, the full-length of the detected key AMR genes was evaluated through conventional PCR followed by sequencing. Regarding the key AMR genes, the *cat* gene (GenBank: NC_002013.1) conferring CmR, the *aadD* gene (GenBank: M19465.1), conferring KanR, and the *tet-L* gene (GenBank: D00946.1), conferring TetR were targeted allowing to cover most of the GM bacteria used to produce microbial fermentation products, including feed additive vitamin B2 (Fraiture et al., 2020a, 2020b, 2020c).

Based on the proposed first-line screening strategy, the presence of unauthorized GMM can be suspected in the following different scenarios. On one hand, if both *B. subtilis* and at least one key AMR gene in its full-length are detected, the presence of the GM *B. subtilis* RASFF2014.1249 strain is tested using the real-time PCR VitB2-UGM and 558 markers (Barbau-Piednoir et al., 2015; Paracchini et al., 2017). In case of positive signals, the presence of the unauthorized GM *B. subtilis* RASFF2014.1249 strain is confirmed (scenario 1a, see Fig. 1). However, in case of negative signals with the real-time PCR VitB2-UGM and 558 markers, the presence of an unknown GMM is therefore suspected (scenario 1b, see Fig. 1). On the other hand, if *B. subtilis* is not

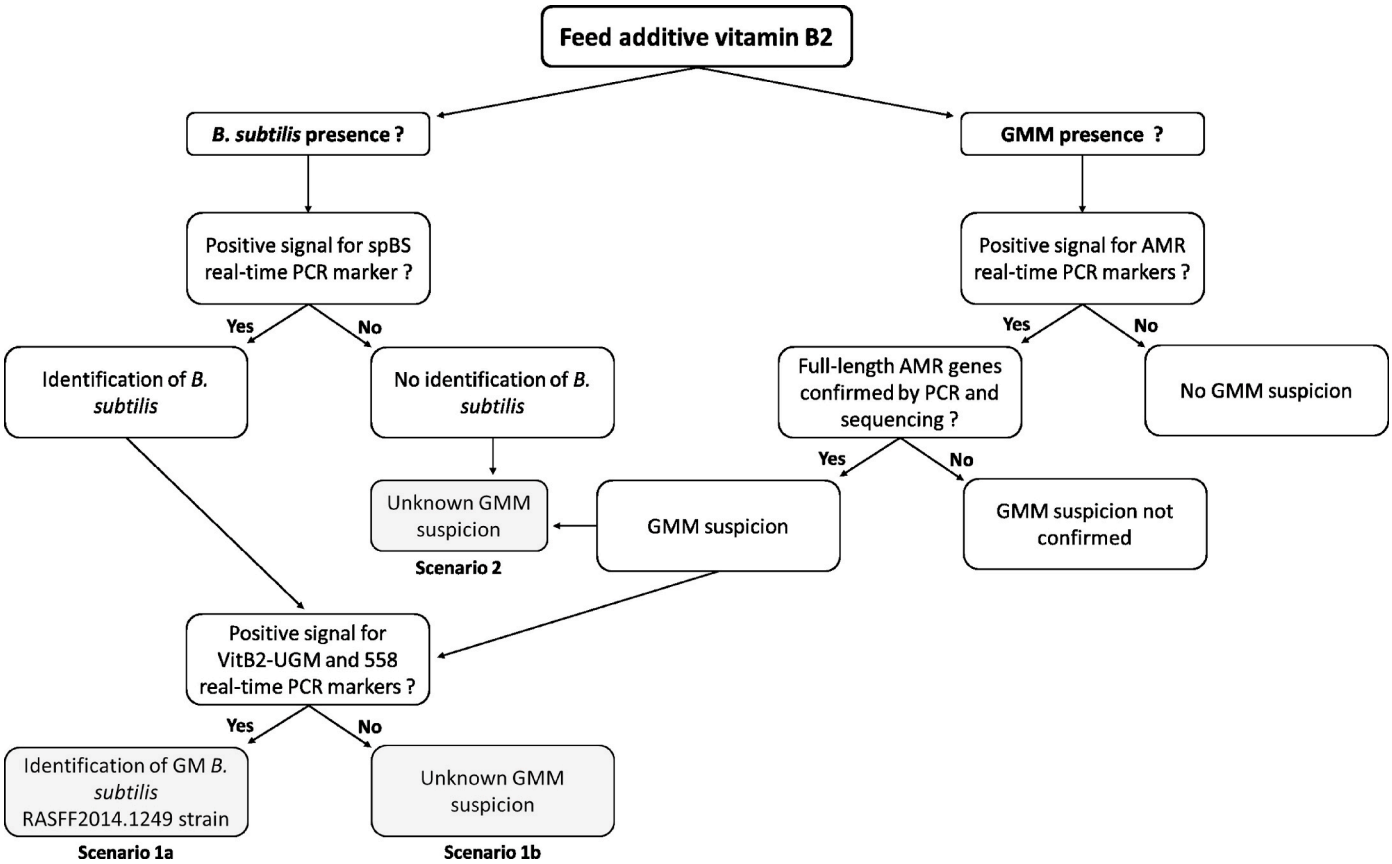

Fig. 1. Schematic representation of the general analysis workflow used as a first-line screening to target unauthorized GMM in feed additive vitamin B2 products.

detected while at least one key AMR gene in its full-length is observed, the potential presence of an unknown GMM, not belonging to the *B. subtilis* species, is suggested (scenario 2, see Fig. 1). Indeed, even if *B. subtilis* is the only feed additive vitamin B2-producing bacterial species currently authorized by the EU legislation and the feed additive vitamin B2 dossiers evaluated by EFSA, the use of other microbial species cannot be excluded by this non-exhaustive list ((Regulation EC No 1831/2003, 2003); EFSA, 2016; EFSA, 2018a).

Compared to the current control strategy using only the real-time PCR vitB2-UGM and 558 markers specific to the GM *B. subtilis* RASFF2014.1249 strain, the proposed first-line screening strategy presents the advantages to cover additional unknown GMM and to indicate potential health and environmental risks related to the unexpected presence of full-length AMR genes.

3.2. Analysis of feed additive vitamin B2 samples for potential unauthorized GMM contaminations

To control potential contaminations of unauthorized GMM in feed additive vitamin B2 products commercialized on the Belgian market, 67 feed additive vitamin B2 matrices were sampled between 2016 and 2019 by the Belgian Competent Authority. These samples were initially analysed by the current control strategy using the real-time PCR VitB2-UGM and/or 558 markers. On this basis, 5 samples (n°8, 10, 45, 48 and 67) were highlighted for GMM contaminations, leading to the RASFF2018.2755 and RASFF2019.3216 notifications (Fig. 1, Table 1).

For the retrospective study, the proposed first-line screening strategy, described in section 3.1, was applied on the same 67 feed additive vitamin B2 matrices (Fig. 1, Table 1). On one hand, six samples (n°8, 10, 20, 45, 48 and 67) presented a positive signal for the real-time PCR marker targeting the *B. subtilis* species (Table 1). On the other hand, the full-length of at least one key AMR gene was observed for 5 samples (n°8, 10, 45, 48 and 67) (Table 1, Supplementary file 2). All these samples contained the full-length of both the *cat* gene and the *aadD* gene. In addition, following to a standard viability test, the presence of culturable GMM in these 5 samples (n° 8, 10, 45, 48 and 67) was not demonstrated. Based on all these results, the following points were established.

First, among all tested feed additive vitamin B2 samples, 5 of them (n°8, 10, 45, 48 and 67) were suspected to be contaminated by GMM using the proposed first-line screening strategy. For these samples, positive signals for *B. subtilis* species and for full-length AMR genes, being the *cat* gene (and the *aadD* gene, were simultaneously observed. Therefore, these samples were automatically suspected for the presence of unauthorized GM *B. subitilis* harbouring AMR genes. Using the real-time PCR VitB2-UGM and/or 558 markers, the presence of the unauthorized GM *B. subtilis* RASFF2014.1249 strain was then detected for all of these samples (Table 1). Such contaminations, associated to potential health risks, corresponded to 7.5% of the tested feed additive vitamin B2 samples and were exclusively originated from the previously characterized GM *B. subtilis* RASFF2014.1249 strain. Therefore, no suspicion for additional presence of unknown GM strains carrying AMR genes could be established in the feed additive vitamin B2 samples collected in the context of the Belgian official control (Table 1, Fig. 1).

Second, the GM *B. subtilis* RASFF2014.1249 strain was previously characterized as harbouring the three targeted AMR genes (the *cat*, *aadD* and *tet-L* genes). However, surprisingly, for the 5 feed additive vitamin B2 samples contaminated with this GMM (n° 8, 10, 45, 48 and 67), only two out of these three full-length AMR genes were detected, being the *cat* and *aadD* genes. This observation could be explained by the different types of insertions of these key AMR genes in the GM *B. subtilis* RASFF2014.1249 strain. More precisely, as previously characterized by Berbers et al. (2020), the *cat* and *aadD* genes were inserted at the chromosomic level while the *tet-L* gene was inserted on a plasmid which could be easily lost without sufficient selection pressure (Berbers et al., 2020; Subbiah et al., 2011; Sultan et al., 2018).

Third, it should be noted that the feed additive vitamin B2 sample n°20 presented a weak positive signal for the real-time PCR *B. subtilis* marker (C_q: 36.4), suggesting a low contamination level (Table 1). However, for this sample, the wild-type or GM status of this *B. subtilis* strain remains undetermined. Indeed, with such low contamination levels, positive signals for key AMR genes and the VitB2-UGM and/or 558 markers may probably be lower than the limit of detection of these methods.

Finally, none of the other 62 tested feed additive vitamin B2 samples presented a positive signal for *B. subtilis* species and for full-length AMR genes (Table 1). Therefore, the presence of GMM carrying full-length AMR genes was not suspected for these samples.

3.3. Assessment of potential indicators for unauthorized GMM contaminations

In addition to the proposed first-line screening strategy, the use of additional data, including the chloramphenicol presence and the DNA extraction yield, as complementary indicators for unauthorized GMM contaminations in feed additive vitamin B2 products was assessed (Table 1).

On one hand, the potential presence of chloramphenicol was investigated for the two following reasons. First, the *cat* gene was recently estimated to be frequently utilized as selection marker in GM bacteria used by the food and feed industry to produce microbial fermentation products. This is for example the case with the unauthorized GM *B. subtilis* RASFF2014.1249 strain identified in 2014 on the EU market (Fraiture et al., 2020). Secondly, although the use of this antibiotic is banned in food-producing animals and other food products on the EU market due to its toxicity for humans at any dose, its prohibited presence in microbial fermentations products has been notified several times by enforcement laboratories (EFSA, 2014; RASFF portal(http); Fraiture et al., 2020; Hanekamp et al., 2003). Among all tested feed additive vitamin B2 samples (n°8, 10, 45, 48 and 67), two of them (n°8 and 10) were identified as containing chloramphenicol (Table 1). These two products were thus considered as non-conform, representing consequently a potential risk for the food and feed safety. Given that these two feed additive vitamin B2 samples contained also the unauthorized GM *B. subtilis* RASFF2014.1249 strain harbouring CmR, the chloramphenicol measurement can therefore be considered as a relevant indicator for the potential presence of unauthorized GMM (Table 1–2). On this basis, the numerous microbial fermentation products previously notified as non-conform for the presence of chloramphenicol represent thus highly suspicious samples for the presence of unauthorized GMM. It would therefore be recommended that feed additive vitamin B2 samples containing prohibited chloramphenicol are analysed with the proposed first-line screening strategy in order to assess for instance the prevalence of unauthorized GMM on the market.

On the second hand, the amount of DNA extracted from feed additive vitamin B2 samples was also investigated as potential indicator for GMM contamination (Table 1). For the majority of the samples, a DNA concentration between 1 and 30 ng/µl was observed. However, irrelevant values were also observed for a few samples (n°12, 18, 23 and 40), which suggest to use DNA yield as indicator for GMM contaminations in feed additive vitamin B2 products with caution. Indeed, feed additive vitamin B2 matrices occur like a fine yellow-orange powder. These colour pigments can be found in the DNA extracts, leading to interferences during the DNA concentration measurements (Dehestani & Kazemi, 2007). As an alternative indicator based on DNA extracts, the presence of bacterial DNA could be investigated using a marker targeting the 16S rDNA region by conventional PCR. This approach has previously been successfully assessed to deal with microbial species used by the food and feed industry to produce microbial fermentation products (Deckers et al., 2020). However, the sensitivity of conventional PCR methods is usually expected to be weaker than the sensitivity of real-time PCR methods. In addition, given the number of the 16S rDNA

region can be different between and even within bacterial species, the sensitivity of this approach can vary. Therefore, this approach is expected to deal preferably with high contamination levels. Moreover, in order to discard potential aspecific PCR amplifications, this conventional PCR approach requires a subsequent sequencing of the generated PCR amplicons, which is not frequently performed by enforcement laboratories responsible of GMO routine analysis (Deckers et al., 2020; Dorn-In et al., 2015).

4. Conclusion

In this study, the results associated to the Belgian control plan for GMM contaminations in feed additive vitamin B2 products, targeting exclusively the GM *B. subtilis* RASFF2014.1249 strain, were presented for all samples collected from 2016 to 2019 by the Belgian Competent Authority. These results indicated GMM contaminations for 5 samples and led to the RASFF2018.2755 and RASFF2019.3216 notifications. In addition, through the retrospective analysis performed on these feed additive vitamin B2 samples, a first-line screening strategy, targeting both AMR genes (GMM markers) and *B. subitilis* (taxon-specific marker), was proposed to strengthen the current Belgian control strategy. In this way, the presence of a larger spectrum of unauthorized GMM could be covered, including both the GM *B. subtilis* RASFF2014.1249 strain as well as additional unknown GM strains.

Among the 67 feed additive vitamin B2 samples tested, all contaminations highlighted by the current control strategy were also identified by the proposed first-line screening strategy, demonstrating its applicability. Moreover, all used real-time PCR markers, targeting the key AMR genes, the *B. subtilis* species and the GM *B. subtilis* RASFF2014.1249 strain, were developed in previous studies and can easily be implemented in GMO routine analysis by enforcement laboratories as the latter are commonly mastered this technology. All these PCR methods, except the *B. subtilis* marker, were developed in the context of GMM detection in order to be compatible with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL, 2015). Regarding the *B. subtilis* marker, further analysis would thus be necessary to verify if this method meets these minimum performance requirements. Moreover, at the practical level, the use of the same real-time PCR chemistry for the first-line screening methods would be easier. However, to this end, additional analysis are required. Regardless these considerations, the observed results suggest that the current control strategy was seemingly sufficient to indicate GMM contaminations in these feed additive vitamin B2 samples. The presence of other GMM than the GM *B. subtilis* RASFF2014.1249 strain was also not suspected. Moreover, based on the detection of chloramphenicol, even at trace level, potential GMM contaminations were indicated.

The proposed first-line screening strategy, in targeting key AMR genes, was previously estimated to cover around 90% of GM bacteria used by the food and feed industry to produce microbial fermentation products (Fraiture et al., 2020a, 2020b, 2020c). However, the presence of unknown GM bacterial strains carrying other AMR genes or selective markers cannot be excluded. To this end, non-a-priori knowledge approaches are needed. Among them, WGS can be applied on viable isolated GM strains. However, the required bacterial isolation step is not always achievable. To bypass such bottleneck, metagenomics, a culture-independent approach, can be directly applied on the whole sample. Nonetheless, these non-a-priori approaches currently present a weak sensitivity, a cumbersome and expensive analysis workflow and/or required specific expertise and capacities, making their implementation complex (Deckers et al., 2020a, 2020b; Dorn-In 2015; Fraiture et al., 2020d). In addition, the proposed first-line screening strategy provides information on the unexpected presence of full-length AMR genes, which is important for the Competent Authorities for the evaluation of potential health and environmental risks. In the present study, 7.5% of the tested feed additive vitamin B2 samples contained full-length AMR genes and no culturable GM strain was observed for these contaminated samples.

Although the proposed first-line screening strategy was in this study applied on feed additive vitamin B2 products, a broader range of products originating from microbial fermentation can also be interesting to analyse. Various other microbial fermentation products are indeed also massively used on the market and consumed by large segments of the human and animal population, often for life. These products are incorporated either in feed and food (additives, enzymes and flavourings) or in individual capsules (food supplements). In analysing all these microbial fermentation products, a global view of their potential contaminations with GMM harbouring AMR genes will be provided. The proposed first-line screening strategy represents thus a key tool for the control of microbial fermentation products, in link with the effectiveness of (inter)national actions combatting AMR spread though the feed and food chain. In addition to the proposed first-line screening strategy, an evaluation of the risk for AMR transmission via horizontal transfer when microbial fermentation products are contaminated by living GMM or its DNA could be pertinent.

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.

CRediT authorship contribution statement

Marie-Alice Fraiture: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Laure Joly:** Formal analysis, Writing - review & editing. **Els Vandermassen:** Formal analysis, Writing - review & editing. **Maud Delvoye:** Formal analysis, Writing - review & editing. **Dirk Van Geel:** Formal analysis, Writing - review & editing. **Jean-Yves Michelet:** Formal analysis, Writing - review & editing. **Els Van Hoeck:** Formal analysis. **Nathalie De Jaeger:** Writing - review & editing. **Nina Papazova:** Writing - review & editing. **Nancy H.C. Roosens:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107476.

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