Food Control 141 (2022) 109170

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Recombinant DNA in fermentation products is of no regulatory relevance

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ARTICLE INFO

Keywords:
Recombinant DNA
Scope of legislation
Regulatory compliance
Analytical capabilities
Safety
Global harmonization

ABSTRACT

A large variety of fermentation products are used in food and feed production, but also in other industries, and many of these products are produced with genetically modified microorganisms (GMMs). In food and feed production, prominent examples are amino acids, vitamins, food and feed enzymes, colorants, non-caloric sweeteners, human milk oligosaccharides, or vegan alternatives of dairy, egg and meat products.

From a regulatory perspective, fermentation products are typically produced under containment. This means that premises, equipment and work processes need to be designed to prevent or at least minimize release of GMMs into the environment. The fermentation products themselves should not contain any live cells of the GMM. Over the past years, there have been concerning developments, particularly in the European Union, stipulating that also absence of recombinant DNA *might* be interpreted as a regulatory requirement for fermentation products produced with GMMs.

In this paper, we (i) attempt to place these developments into the historical context, (ii) sketch the potential negative repercussions for the food and feed industries, (iii) elaborate on the safety of recombinant DNA, and (iv) postulate that recombinant DNA should remain an integral part of the safety assessment of fermentation products but should *not* be misconstrued as a criterion for regulatory classification of products of biotechnology.

1. Introduction

In its strict sense, fermentation is a chemical reaction in which a microorganism extracts energy from the metabolism of organic molecules *under anaerobic conditions*, for instance the anaerobic conversion of sugar to ethanol by yeast. More broadly, however, industrial fermentation is understood as the intentional use of microorganisms such as bacteria or fungi to make products useful to humans, under either anaerobic or aerobic conditions.

As early as 7000 BCE, fermentation was used in food production. Long before microorganisms were actually discovered, fermentation was exploited to preserve milk, fruits and vegetables, and to enhance the quality of life by producing beverages, cheeses, bread, pickled foods and vinegar (Demain, 2010). But it was only in the 19th century that the foundation was laid for industrial fermentation, through three key discoveries by Louis Pasteur: (i) he concluded that fermentation was a living process of yeast; (ii) he established that each type of fermentation was mediated by a specific microorganism; and (iii) he discovered that yeast fermentations turning sour were contaminated by bacteria, and that such contamination could be prevented by a mild heat treatment, which later became known as pasteurization. Thus, Louis Pasteur established two key principles that are still valid for present-day fermentation processes: (i) the use of a single microorganism (or a defined, limited set of synergistic microorganisms) to make the product of interest; and (ii) the concept of process hygiene to secure that the selected microorganism(s) robustly and reproducibly can make the product of interest.

In the early days of fermentation, only wild-type microorganisms

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https://doi.org/10.1016/j.foodcont.2022.109170
Received 20 May 2022; Received in revised form 7 June 2022; Accepted 9 June 2022
Available online 15 June 2022

were available and used. In the mid-20th century, advances in the understanding of fermentation processes, and particularly their deployment for the production of antibiotics (e.g., penicillin), increased interest in improving microorganisms for purposes useful to humans. Initially, the improvements were achieved with methods of random mutagenesis, e.g. by using X-ray or UV irradiation or chemicals to induce mutations in the microbial genome, followed by selection of mutants with improved properties. In the 1970s, methods of targeted genetic modification[1] were introduced and applied initially to fermentative production of pharmaceuticals, most prominently to the microbial production of human insulin. Over time, targeted genetic modification of microorganisms for their use in industrial fermentation processes was increasingly applied in the food and feed sectors. The large variety of such products includes, for instance: (i) essential amino acids, which make livestock diets nutritionally more balanced, thereby reducing feed consumption (Leuchtenberger et al., 2005); (ii) feed enzymes which improve the digestibility and nutritional value of animal feed, or degrade antinutritional factors such as phytic acid, thereby also improving feed efficiency (Adeola & Cowieson, 2011); (iii) food (processing) enzymes which help to degrade gluten or lactose for intolerant people, degrade or prevent the formation of toxins such as acrylamide, and/or improve the efficiency of food production or the properties of final food products (Olempska-Beer et al., 2006); (iv) food ingredients such as vitamins, colorants, omega-3 fatty acids, non-caloric sweeteners, flavors or flavor enhancers which are used to increase the nutritional value or the appeal of foods to consumers (Vandamme & Revuelta, 2016); (v) human milk oligosaccharides (HMOs), which are highly abundant in mother's milk, deliver many health benefits, and can now be produced by fermentation and added to milk replacers for infants (Bych et al., 2019); and (vi) vegan alternatives of dairy and other animal-derived products which are obtained by industrial fermentation, with the ambition to significantly reduce their ecological footprint (Pua et al., 2022). In all these cases, industrial fermentation offers biological solutions for the manufacturing of products of interest to humans, typically under mild, environmentally friendly conditions. Industrial fermentation is poised to be an important component of the toolbox for continuously improving the sustainability of food and feed production. This is facilitated by further advances in the genetic modification of microorganisms, including the recent advent of genome editing technologies (Javed et al., 2018). The current political climate calling for transformative change towards more sustainable food production is reflected, for instance, in the European Green Deal and its Farm to Fork Strategy (Purnhagen et al., 2021).

It seems pertinent to emphasize the differences between a genetically modified organism (GMO; which could be a genetically modified plant, animal or microorganism) released into the environment vs. a genetically modified microorganism (GMM) used in an industrial fermentation process under containment. Genetically modified plants are typically grown on open fields and need to cope with differences in soil and weather conditions, nutrient availability, and pest pressure. In addition, they directly interact with the environment and potentially bear the risk of unintentional spreading. Since they need to maintain their "fitness" to deal with their environment, the potential to optimize plants for efficient production of molecules of interest is often limited.

In sharp contrast, industrial fermentations are typically run using axenic cultures (i.e., containing a single strain of a microorganism) under strictly controlled process conditions optimized for the particular production microorganism as well as for optimal production of the molecule of interest. Contaminating microorganisms are excluded through the contained growth of the microorganism in closed fermenters and by appropriate process hygiene. As a result of these measures, the microorganism can be "tuned" for the high-level synthesis of the product of interest. Compared to genetically modified plants, such improvement is further facilitated by the much smaller genome size and the ease of genetic modification of the commonly used safe microorganisms. Typically, the tuning of a microorganism is accompanied by a decrease in its fitness to survive in the natural environment (De Leij et al., 1998; Lenski, 1993). Next to the containment measures applied, this will further reduce the risk of unintended environmental spreading.

It is against this background that the European Union has drafted its regulatory framework on GMOs. It covers contained use, deliberate release and placing of products on the market. Right from the beginning in 1990, fermentation using GMMs in closed fermenters has been governed by the rules on contained use. The resultant fermentation products, on the other hand, are not captured by the EU's GMO framework unless they contain live GMMs. Instead, they are ruled under sectoral food laws such as those on food enzymes, food flavorings and food or feed additives.

This holds true until today, also with a view to other regulations enacted in 2003. Fermentation products – e.g. nutritional ingredients obtained through the contained use of GMMs – are outside the scope of Regulation (EC) No 1829/2003 as well as Regulation (EC) No 1830/2003[2] and, thus, not subject to the GMO and GM food and feed market authorization procedures. As a result, contained-use fermentation products are also not subject to GMO labelling obligations in the EU. This explains why the general public in the EU is largely unaware of the widespread commercial use of products produced with GMMs by fermentation.

To secure legal certainty, it is crucial to apply clear, unequivocal criteria for distinguishing between deliberate-release and contained-use products. Next to the different production set-ups (open fields vs. closed, contained installations), fermentation products need to be free of live cells of the production microorganism; otherwise, these products would result in the deliberate release of the production microorganism into the environment and, consequently, would fall under Directive 2001/18/EC and Regulation (EC) No 1829/2003.

A common misunderstanding is that recombinant DNA can be used as a more sensitive indication of the presence of the GMO. A GMO can contain recombinant DNA, but lysis of the cells can release recombinant DNA that is no more capable of replicating outside of the organism. In spite of that, over the past years, absence of recombinant DNA has emerged as an additional requirement for fermentation products in the European Union, causing legal uncertainty in the food and feed industries. As the DNA analysis techniques can basically detect one molecule, the presence of recombinant DNA as a regulatory criterion for fermentation products threatens to move all the products produced with GMMs in the scope of Directive 2001/18/EC and Regulation (EC) No 1829/2003, with serious implications for the whole fermentation industry.

In the following sections, we (i) describe the historical developments, which have led to the current, unfortunate situation, (ii) outline the challenges and potential implications of these developments for the fermentation industry and the enforcement authorities in the EU, as well as for international trade, and (iii) explore ways of resolving the current impasse.

Although the main focus of this paper is on the European Union, this paper has implications for other regions as well. On the one hand, due to its elaborate food safety system, the European Union often serves as a point of reference for other countries when developing their national food safety systems. Therefore, a requirement for absence of recombinant DNA in fermentation products may spill over to other countries. For

[1] Throughout this text, we refer to the term "genetic modification" as used in the EU regulatory context; "genetic engineering" could be used synonymously.

[2] Regulation (EC) No 1829/2003 on genetically modified food and feed and Regulation (EC) No 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC.

instance, absence of recombinant DNA has been listed as a specification for food ingredients produced by industrial fermentation under contained conditions in Brazil (ANVISA, 2020). On the other hand, absence of recombinant DNA is the determining criterion whether "bio-engineered" labelling is required for final food products in the USA, as stipulated in the National Bioengineered Food Disclosure Standard (USA, 2018). Absence of recombinant DNA needs to be demonstrated with a DNA-based method (e.g. PCR) having *appropriate* sensitivity.

2. How absence of recombinant DNA has evolved to become a quasi-requirement for food and feed fermentation products in the EU

The source and still main reason for the confusion is a simple recital in Regulation (EC) No 1829/2003 which has not been drafted with sufficient care (see Fig. 1). A few points are worth emphasizing: (i) Recitals are meant to provide the frame and context and to inform about the intent of the Union legislature. They are not meant to be legally binding. The legally binding constraints and obligations are addressed explicitly in the main text (i.e., in the articles) of a regulation. (ii) It is defined nowhere in the relevant EU legislation what is meant by "material derived from the genetically modified source material". Also the target product of a fermentation process could reasonably be interpreted as a material derived from the GMM; thus, if "material" meant any molecule from the GMM, the entire recital would be meaningless and obsolete. (iii) If, in 2003, the intention had been to interpret "material" as infinitesimally small amounts of impurities or residues from the source material, it is very likely that the recital would have been worded differently. (iv) And finally, processing aids and food and feed which are manufactured with the help of a genetically modified processing aid are explicitly excluded from the scope of Regulation (EC) No 1829/2003.

Still, over the years, this cryptic second sentence of recital No 16 has raised a lot of questions and discussions among the EU Member States and operators regarding the 'material' and its analysis, as well as the legal status of the fermentation products. In a meeting of the Commission Standing Committee on the Food Chain and Animal Health in September 2004, consensus was reached that "Food and feed (including food and feed ingredients such as additives, flavourings and vitamins) produced by fermentation using a GMM which is kept under contained conditions and is not present in the final product are not included in the scope of Regulation (EC) No 1829/2003. Such food and feed have to be considered as having been produced with the GMM, rather than from the GMM" (European Commission, 2004). However, the apparent clarity generated with this statement was (again) compromised with vague wording in the following paragraph: "Food or feed (including food and feed ingredients such as additives, flavourings and vitamins) produced by fermentation using a GMM which is present in the final product, totally or *partially*, whether alive or not, is included in the scope of Regulation (EC) No 1829/2003, in regard of both authorization and labelling."

In 2006, the European Commission released a report on the implementation of Regulation (EC) No 1829/2003 which provided, inter alia, additional clarification on the status of food or feed produced by fermentation using GMMs (European Commission, 2006). The report concluded that "Food or feed produced using genetically modified micro-organisms as processing aids are not falling under the scope of the Regulation" (i.e., Regulation (EC) No 1829/2003), whereby food [feed] processing aids were defined as "any substance not consumed as a food [feedingstuff] by itself, intentionally used in the processing of raw materials, foods or their ingredients [feedingstuffs or feed materials], to fulfil a certain technological purpose during treatment or processing and *which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product*, provided that these residues do not present any health risk [do not have an adverse effect on animal health, human health or the environment] and do not have any technological effect in the finished product [on the finished feed]". The definition of processing aid may be valid, e.g., when the microorganism is removed after the fermentation and the produced food or feed is further purified in the production process, or when the microorganism is attached/fixed to a support ("matrix") in such a way that it is used during the treatment or processing of the food or feed, but is not transferred into the final product as such or under an altered form. However, when the GMM is not removed during the production process, it is not used as a processing aid. In such a case, the produced food, feed, food ingredient or feed additive falls under the scope of Regulation (EC) No 1829/2003. The report reminded that EFSA's guidance on the risk assessment of GMMs and their derived products intended for food and feed use (i.e., at that time, EFSA, 2006) applies irrespective of whether the GMM is used as a processing aid or not, and whether the product falls under the scope of Regulation (EC) No 1829/2003 or not. To conclude,

1 This Regulation should cover food and feed produced "from" a GMO but not food and feed "with" a GMO.

2 The determining criterion is whether or not material derived from the genetically modified source material is present in the food or in the feed.

3 Processing aids which are only used during the food or feed production process are not covered by the definition of food or feed and, therefore, are not included in the scope of this Regulation.

4 Nor are food and feed which are manufactured with the help of a genetically modified processing aid included in the scope of this Regulation.

5 Thus, products obtained from animals fed with genetically modified feed or treated with genetically modified medicinal products will be subject neither to the authorisation requirements nor to the labelling requirements referred to in this Regulation.

Fig. 1. Recital 16 of Regulation (EC) No 1829/2003. Sentences are numbered (in red) for easier referencing.

this report made it entirely clear that residues of GMMs in a fermentation product are not *per se* a decision criterion to bring them into the scope of Regulation (EC) No 1829/2003.

For the next 10 years, the Commission seemed to pay little attention to the issue. Therefore, let us have a look how residual recombinant DNA was addressed in the relevant EFSA guidance documents (EFSA, 2006, 2011, 2018, 2019). In its guidance from 2006, EFSA states that "Not all requirements of the guidance document may be applicable for all products" and that "There may be circumstances in which the DNA as such introduced into a GMM gives cause for concern and *in this case* it needs to be subjected to risk assessment. Data on the absence of DNA need to be very robust in such instances". In addition, EFSA acknowledged that "no method will give absolute proof that DNA is absent".

According to both the 2006 and 2011 guidance documents (EFSA, 2006, 2011), absence of recombinant DNA was assessed on a case-by-case basis. No threshold nor target sensitivity for the analytical tests was considered or prescribed by EFSA for demonstrating absence of recombinant DNA. In addition, it is not within the remit of EFSA to make any legal interpretations concerning the presence or absence of recombinant DNA. Instead, the potential need for absence of DNA was linked to safety in terms of horizontal gene transfer (HGT) and transfer of antimicrobial resistance genes in particular, independently from any legal interpretation of the existing regulatory framework. Over the years it became clear, however, that the limit of detection varied a lot among different application dossiers, and the Commission again started paying attention to it, also reopening the discussion whether recombinant DNA should be seen as a regulatory criterion for distinguishing between deliberate-release and contained-use products, and thereby for triggering the application of Regulation (EC) No 1829/2003; this discussion is still ongoing (see, e.g., European Commission, 2022). Over the past few years, industry – through its industry associations EuropaBio (https://www.europabio.org/), AMFEP (https://amfep.org/) and FEFANA (https://fefana.org/) – has attempted repeatedly to get clarity on the issue, emphasizing (i) the importance of legal certainty for both industry and competent EU and national authorities, and (ii) that – if absence of recombinant DNA were to be considered as a binding requirement for fermentation products – clear performance criteria for the analytical methods would be required, as well as a legally binding threshold under which recombinant DNA would be acceptable for contained-use fermentation products. The European Commission's Directorate General for Health and Food Safety (DG SANTE) pushed back and repeatedly affirmed that they have no mandate, under the current EU GMO regulatory framework, to establish such a legally binding threshold.

The most recent EFSA guidance documents (EFSA, 2018, 2019; 2021a) seem to offer a practicable and pragmatic compromise, by requesting a minimal sensitivity (Limit of Detection, LOD) that needs to be met by the analytical method used to demonstrate absence of recombinant DNA. This minimal LOD was set at 10 ng of genomic DNA per gram of product. In other words, EFSA provides a basis for consistency of requirements over time and does not expect that analytical sensitivities are pushed to the limit and continuously adapted to further advances in methodological developments. Still, industry repeatedly cautioned that this compromise is not a sustainable, long-term solution, and that it may someday be challenged should an analytical lab, wherever on this globe, detect recombinant DNA in fermentation products when using ultrasensitive methods. This suggested risk turned into reality when Sciensano, the Belgian research institution for public health and food safety, published a series of papers, using ultrasensitive DNA analytics to detect traces of recombinant DNA in fermentation products and postulated that, due to purported zero tolerance for recombinant DNA in the EU, these products would not be in compliance with the EU GMO regulations (Berbers et al., 2020; Deckers et al., 2020a,b; Fraiture et al., 2020a,b, 2021a,b,c,d).

3. Assessment of the legal basis for "absence of DNA" as a requirement under the current EU food and feed regulatory framework

Accordingly, these developments raised the legal question whether food or feed products obtained through fermentation using GMMs are subject to Regulation (EC) No 1829/2003 if recombinant DNA is present in a given product. This question has to be answered in the negative, though: Regulation (EC) No 1829/2003 does not apply to fermentation products if the microorganisms have been removed during downstream processing and the products do not contain any living cells originating from the microorganisms. The presence of recombinant DNA in the fermentation products in whatever amount does not have any significance and, therefore, does not lead to any other conclusion for the following reasons (for a full-fledged legal analysis see Dederer, 2021):

Whether Regulation (EC) No 1829/2003 applies depends on (i) whether the fermentation product contains or consists of GMOs or (ii) whether the product is produced from GMOs or (iii) whether the product contains ingredients produced from GMOs (cf. Articles 3(1), 12(1), 15 (1), 24(1) of Regulation (EC) No 1829/2003).[3] However, neither of the aforementioned three alternatives (i)-(iii) is satisfied, i.e. all three questions have to be answered in the negative.

Ad (i): Even if recombinant DNA is present in fermentation products, such a product does not contain or consist of 'GMOs'. Recombinant DNA as such is not an 'organism', i.e. a "biological entity capable of replication or of transferring genetic material" (Article 2(5) of Regulation (EC) No 1829/2003 in conjunction with Article 2(1) of Directive 2001/18/EC). It is well-established that 'naked' DNA does not fit under this definition (European Commission, 1992). Therefore, if recombinant DNA is not an 'organism', it is, a fortiori, not a 'GMO' either.

Ad (ii): The all-dominant issue with a view to the regulatory status of fermentation products containing recombinant DNA originating from GMMs is whether the food or feed product is 'produced from' GMOs. For sure, the GMMs themselves, which are used for fermentation purposes, are GMOs. Food or feed products are produced 'from' such GMOs if they are "derived, in whole or in part, from GMOs, but [do] not contain[..] or consist[..] of GMOs" (Art. 2(10) of Regulation (EC) No 1829/2003). According to the wording of this provision, the presence of recombinant DNA is immaterial. It all depends on whether the relevant food or feed product is 'derived from' GMOs. Unfortunately, this term is not further defined and, thus, requires interpretation. A food or feed product is a derivative of GMOs if the GMOs themselves are processed into the relevant product. This means that, through processing, the GMOs as such are transformed into the food or feed product (such as, e.g., soybeans are transformed into soy oil). This is not the case if the GMOs form production units only, which manufacture a particular separate or separable substance that can be extracted as such from the organism without need of converting the organisms themselves into the relevant food or feed product. In this case, the substance is produced 'with' or 'with the help of' or 'by' the organism. For instance, fermentation products are not produced 'from' but rather 'with' or 'with the help of' or 'by' the GMMs used for fermentation purposes. It is not the microorganisms which, through a series of processing steps, are themselves converted into the fermentation product. This interpretation of Articles 2(10), 3(1)(c), 12(1)(b), 15(1)(c), 24(1) of Regulation (EC) No 1829/2003 is confirmed by its Recital No 16 which is provided in full in Fig. 1.

The GMMs used for fermentation purposes are 'processing aids' within the meaning of sentence 4 of the recital. Therefore, the fermentation products are not covered by the Regulation since they are

[3] It is clear that Regulation (EC) No 1829/2003 is inapplicable anyway if the fermentation product is neither a 'food' nor 'feed'. However, many, if not most, products obtained through fermentation using GMMs, such as enzymes, vitamins or flavorings, may constitute food or feed within the meaning of EU food and feed law.

manufactured with the help of the GMMs. Consequently, the fermentation products can also not be captured by sentence 2 no matter whether they contain traces of recombinant DNA or not – which is why it is immaterial whether recombinant DNA might be 'material derived from the genetically modified source material' within the meaning of sentence 2 of the recital. This follows from the recital's structure: sentence 1 lays down that there is an essential distinction to be drawn between food or feed 'produced from' and 'produced with' GMOs. The general criterion for making this distinction is laid down in sentence 2. However, sentences 3 and 4 stipulate special criteria for the purposes of the decisive distinction between food or feed 'produced from' and 'produced with' GMOs. Thus, if the special criteria are met, the general criterion becomes irrelevant (i.e. the well-known conflict rule 'lex specialis derogat legi generali' applies by analogy).[4]

Ad (iii): The recombinant DNA originating from the GMMs used for fermentation purposes is also not an 'ingredient produced from GMOs'. The term 'ingredient' is legally defined as "any substance […] used in the manufacture […] of a food and still present in the finished product" (Art. 2(13) of Regulation (EC) No 1829/2003 in conjunction with Art. 2 (2)(f) of Regulation (EU) No 1169/2011). This definition continues by clarifying that "residues shall not be considered as 'ingredients'". It follows from the foregoing reasoning that fermentation products are 'produced with' the GMMs which, in turn, are mere 'processing aids'. Accordingly, what is 'used in the manufacture' of the fermentation product is not the recombinant DNA but are the GMMs. Consequently, traces of recombinant DNA stemming from the GMMs have to be considered 'residues' only left over after downstream processing.

Thus, the overall conclusion is that Regulation (EC) No 1829/2003 does not apply to food or feed products obtained by fermentation of GMMs if the microorganisms have been removed during downstream processing and no living cells are remaining in the fermentation products. The presence of traces of recombinant DNA originating from the GMMs is of no legal relevance as regards the applicability of the regulation. Such recombinant DNA traces are residues only. If such residues should raise any safety concerns for human health, they can be addressed adequately within the existing legal framework applying to, e.g., food additives, enzymes or flavorings (cf. Regulations (EC) No 1331/2008, 1332/2008, 1333/2008 and 1334/2008), or additives for use in animal nutrition (cf. Regulation (EC) No 1831/2003).

This overall conclusion is fully consistent with earlier assessments by the European Commission and the Standing Committee on the Food Chain and Animal Health. E.g., in 2014, the European Commission confirmed that "when the GM micro-organism is used as a processing aid (the GMM is removed during the downstream processing), the food and feed resulting from such production process [is] not to be considered as falling under the scope of the Regulation [(EC) No 1829/2003]" (European Commission, 2014). Much earlier, already in 2006, the European Commission had held that "[f]ood or feed produced using genetically modified micro-organisms as processing aids [is] not falling under the scope of the Regulation [(EC) No 1829/2003]" (European Commission, 2006). What is more, the Standing Committee had concluded in 2004 that the travaux préparatoires of Regulation (EC) No 1829/2003 "clearly suggested that Council did not intend the scope of Regulation No 1829/2003 to include food produced by fermentation using GMMs" (European Commission, 2004). As early as 2004, legal literature had arrived at the very same conclusion (Girnau, 2004).

[4] Sentence 5 (see Fig. 1) only provides one particular (albeit important) example illustrating the distinction made in sentence 1 of the recital.

4. Technical challenges of eliminating DNA of the production microorganism

4.1. Purification processes for fermentation products: what is feasible in terms of residual DNA?

Microorganisms intended for use as such or as production organisms are cultivated in contained systems, in which fermentation takes place under controlled conditions. This "upstream" process is followed by "downstream" processes (DSP) to recover the product (Fig. 2).

The cultivation of the microbial strain in the fermentation tank is enabled by carbon and nitrogen sources and micronutrients. At the end of fermentation, the broth consists of the microorganism, the product to be recovered, as well as substances derived from the fermentation medium and from the microorganism. The fermentation ends with the inactivation of the microorganism (e.g., by acidification or heat treatment) or by stopping biomass production (e.g., by reducing the temperature or shutting down the nutrient supply).

The complexity of the DSP is determined by the required purity of the product, which depends on the application and authorization. Performance criteria to be considered in developing an optimized purification process are speed, recovery, capacity, and resolution (Milne, 2017). A process developed in a laboratory (e.g., in 0.5–10 L fermenters) must be translated into a full manufacturing scale process (e.g., 20.000 to 2.000.000 L fermenters; Crater & Lievense, 2018). In general, the following steps are included in DSP:

- Biomass separation: The product is separated from the production microorganism by filtration or centrifugation, resulting in a cell-free broth.
- Product recovery/purification: The aim is to recover the product efficiently, reproducibly, and safely to its required specification, while achieving maximum product yield at minimum recovery costs. This is done by, e.g., precipitation/flocculation, filtration, centrifugation, chromatography, or crystallization. To achieve optimum economic viability, a primary objective is to keep the number of steps as low as technically feasible.
- Formulation: To increase stability (shelf-life), decrease dusting and/or improve mixability and bioavailability, the product may be formulated by mixing with stabilizers or carriers, and by eventually applying granulation, spray-drying or freeze-drying. The final formulation can be liquid or solid.

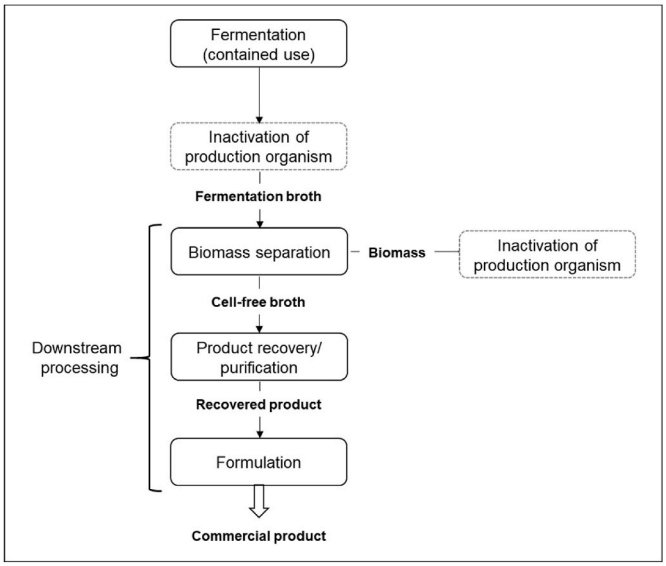

Fig. 2. Generic manufacturing process for fermentation products.

What is the fate of DNA in the DSP? A major part of the DNA will be removed as it (i) remains inside the intact cells or (ii) sticks to cellular debris, which both are separated from the fermentation medium to obtain the cell-free broth. In addition, DNA is partially degraded and is removed during the DSP by different measures like filtration, flocculation or chromatography.

What would be needed to get further reductions in DNA? One possibility would be to degrade DNA by heat and/or acid treatment. DNA is degraded into smaller fragments at temperatures above 100 °C. At low pH, i.e. acidic conditions, a depurination of DNA takes place, which is based on the hydrolysis of glycosidic bonds. At very low pH (<2) the phosphodiester bonding of the DNA is disrupted. Acid-catalysed reactions are accelerated by simultaneous heat treatment. Bitskinashvili et al. (2018) have shown a degradation of wheat and maize DNA to fragments ≤200 bp by a combination of 100 °C and pH 2. Many biological molecules would not survive these very harsh conditions without the loss of their structure and thus functionality. In addition, such harsh conditions may increase side product formation and thereby compromise product quality.

Another possibility would be dedicated DSP steps tailored to remove or degrade DNA (e.g. through DNases). The implementation of additional DSP steps would add process costs and complexity and would need to be optimized/validated for every single production process. When designing their processes, industrial producers of fermentation products must focus on those aspects that are most important from a product quality and safety perspective, which are most notably the purity level of the active ingredients (which should be optimized), and the relevant side products (which typically should be kept below certain limits).

Demonstrating absence of recombinant DNA at the 10 ng/g level, as currently stipulated in the relevant EFSA guidance documents (2018, 2019) is already a significant challenge. Aiming to reach even lower levels of recombinant DNA is not sensible from a scientific, environmental or economic perspective since – recognizing it is not a safety issue (see section 5 below) – it would add a lot of additional complexity to process design, would question the economic feasibility of fermentation products in general due to higher DSP costs and losses in yields, and will compromise the environmental footprint of fermentation products.

4.2. Implementation and enforcement

The Belgian research institution for public health and food safety, Sciensano, has proposed that there should be zero tolerance for recombinant DNA in fermentation products (see section 2 above). The meaning of "zero'', however, is ambiguous and its application is problematic at best (Wilson & Worosz, 2014). Zero tolerance rules have been taken up most prominently in the context of deviance, which describes a behavior that violates a cultural rule. In the practice of feed/food analysis, the legal concept of "zero" means analytical limits of detection using the best available techniques (Hanekamp et al., 2003). The availability and sensitivity of analysis methods are continuously improving. These improvements result in the detection of increasingly lower amounts of residues, which would previously have gone undetected.

The relevant EFSA guidance documents from 2018 (EFSA, 2018 for feed additives) and 2021 (EFSA, 2021 for food enzymes) stipulate that residual DNA from the microbial production strain in regulated feed/-food products obtained by fermentation should be assessed with a PCR test targeting a DNA sequence specific for the production strain. If the strain does not contain acquired[5] antimicrobial resistance (AMR) genes, the recombinant DNA is not a safety concern. In this case the size of the amplified DNA sequence should not exceed 1 kb, reflecting the size of a small/medium-sized gene. If the strain contains acquired AMR genes, the complete, functional gene is considered a safety hazard. In this case, the size of the amplified DNA sequence must not exceed the size of the smallest AMR gene present. In both cases, the limit of detection (LOD) should be equal to or lower than 10 ng DNA per g or ml of product, which is considered by operators as a practical and technically feasible level to demonstrate absence of recombinant DNA.

There are DNA analysis methods that are much more sensitive than requested by EFSA; for instance, Sciensano has described an experimental set-up that is approx. 5000-fold more sensitive (Berbers et al., 2020; Deckers et al., 2020a,b; Fraiture et al., 2020a,b). Application of such methods for demonstrating absence of recombinant DNA would create major challenges for industry. It would also represent a huge burden for Member States' control authorities and create marked enforcement issues. The PCR strategies and LODs vary a lot depending on the fermentation product and the production strain. For a successful PCR, a key factor is the DNA quality (and quantity), which strongly depends on the food/feed sample and the DNA extraction method used. Inhibitory substances or proteases in the sample lead to a shift of the LOD to higher values. A reliable PCR analysis of residues of recombinant DNA requires a specific, validated test for each product/production strain combination.

It should be noted that a zero-tolerance policy for residual DNA, if eventually considered to be enacted and enforced, would result in disproportionate costs and administrative burden. It will be a challenge for each national control laboratory to be able to perform the hundreds of product-specific optimized DNA extraction and PCR methods for commercial product formulations.

5. Safety considerations on (residual) DNA from the production microorganism

Regulatory processes are in place to assess the safety of feed and food products obtained by fermentation. The regulated product(s) and/or production organism(s) are authorized for example under Regulation (EC) No 1831/2003 only if considered safe for humans, animals and the environment. Key safety requirements include the non-pathogenicity and non-toxigenicity of the organisms. This type of information is readily available for most species used for fermentation purposes. For microorganisms that do not have a history of use in the fermentation industry yet, the genome sequence provides valuable information for the unequivocal taxonomic identification of the strain, as well as for the characterization of potential functional traits of concern (e.g. virulence factors, production of or resistance to antimicrobials of clinical relevance, production of known toxic metabolites). Microbial strains belonging to a taxonomic group that includes members known to be capable of producing toxins or other virulence factors are subject to appropriate tests to demonstrate at a molecular and, if necessary, cellular level the absence of any cause for concern. Microbial strains intended for use as feed/food shall not contribute further to the reservoir of antibiotic resistance genes already present in the gut flora of animals and the environment. Consequently, all strains of bacteria are analysed for resistance to antibiotics in use in human and veterinary medicine. Where resistance is detected, the risk of transfer of resistance to other gut-inhabiting organisms shall be assessed. If there is no safety concern, the genome, i.e., the DNA, is not considered a safety risk. Moreover, the general properties of DNA, including the fact that DNA as such is generally not a cause of concern, also apply to DNA from genetically modified (micro)-organisms, consisting of the same building blocks as any other DNA present in nature. With our traditional diet we normally ingest gram quantities of DNA daily. Animal tissues comprise a higher content of DNA (about 15–20 g/kg dry matter in the liver of calf, beef and pig) compared to plant storage tissues, as in grains or potatoes (about 1–3 g/kg dry matter; Jonas et al., 2001). The DNA contents in

[5] In the context of this paper, acquired antimicrobial resistance genes mean antibiotic resistance marker genes introduced intentionally for selection reasons into the GMM.

bacteria, yeasts and mushrooms vary from 1 to 6 g per kg of dry matter.

The traces of DNA present in fermentation products (see section 4 above) will not be intact. The degradation of DNA starts already during the DSP steps, continues in food/feed processing and further in the gastrointestinal (GI) tract. A few hours after feed/food intake, most of the ingested DNA (95% or more) is broken down beyond detection (Rizzi et al., 2012). The GI tract is constantly exposed to foreign DNA by the flow of partly or completely digested nutrients (Jonas et al., 2001). Action by pancreatic nucleases in the intestine cleaves nucleic acids into nucleotides and the latter are cleaved into nucleosides and phosphoric acid by enzymes found on the luminal surfaces of the mucosal cells. Nucleosides are subsequently cleaved to produce sugars and purine and pyrimidine bases, most of which are re-used for the organism's needs.

The reason for the all-encompassing analysis of the genetic basis of detected resistance to antimicrobials in the safety assessment of microbial strains is the possibility that antimicrobial resistance genes present in the microorganism are spread via horizontal gene transfer (HGT). There is no doubt that antimicrobial resistance is one of the most formidable threats to global human health. However, this is a result of the past century during which antimicrobial therapies were discovered and put into extensive use to treat human infections, and for animal husbandry and agriculture. Today the consumption of raw products such as leafy greens is increasingly recognized as a source of pathogenic bacteria, antibiotic-resistant bacteria and antibiotic resistance genes. A recent study showed that an impressive diversity of self-transmissible multiple resistance plasmids is present in bacteria associated with raw products, and exogenous capturing into *Escherichia coli* suggests that they could transfer to gut bacteria as well via HGT (Wallace et al., 2020). However, it is important to point out the differences in relative risks of HGT between (i) residual levels of 'naked' recombinant DNA without genetic elements facilitating DNA transfer, and (ii) intact DNA from live organisms containing AMR genes on plasmids and/or flanked by mobilizing elements.

HGT is a natural process and an integral part of microbial life in terms of its contribution to natural evolution (de Santis et al., 2018). The probability that a specific gene will be successfully acquired by a new host depends on the specific mechanism of HGT (transformation, transduction, conjugation), on the relationship of these mechanisms to the types of nucleic acids (e.g., single-stranded, double-stranded, linear, circular) as well as the environmental conditions. If DNA has been transferred but does not provide a selective advantage, it is likely to be lost in the population.

The breakdown of DNA during feed/food processing and passage through the gastrointestinal tract reduces the likelihood that intact genes will be transferred to the gut microflora via HGT. The available residual DNA in fermentation products is comprised of short linear DNA fragments in naked/free form, not organism associated. These DNA fragments can only be acquired by transformation via recombination to a resident prokaryotic genome (van den Eede et al., 2004). A prerequisite for transformation is the availability of DNA, which must obey some minimal specifications related to structure, sequence, and length. Due to the previously described DNA degradation such minimum requirements are in most cases not met, and such transfer can thus be virtually ruled out. Native, intact DNA is present exclusively in living cells or resting forms (such as bacterial spores). As soon as a cell dies, any repair of the inevitably ongoing damage stops, and enzymatic degradation starts. This implies that the acquisition of new genes, such as antibiotic resistance genes from feed/food is – most likely – an infinitesimally rare event. Moreover, the transfer event by itself, the integration of a gene and its expression, is not sufficient for the spread of genes, but it is the selective pressure that drives the spread of genes in a particular environment. Once a bacterium acquires a functional gene conferring antibiotic resistance and selective pressure is exerted, usually by therapeutic use of the agent, this functional gene is likely to spread and accumulate in the emerging population.

Residual levels of recombinant DNA from a microorganism used as production strain in feed/food production are a highly unlikely source of antimicrobial resistance (EFSA, 2021b; Founou et al., 2016; Woegerbauer et al., 2020). And their contribution to the reservoir of AMRs and thereby to the spread of antibiotic resistance, if any, is negligible.

6. Conclusions and proposed way forward

The arguments presented in this article lead us to the conclusion that there is no meaningful scientific, safety, regulatory or political rationale for using residual levels of recombinant DNA *generically* as a criterion for regulatory classification, and specifically as a regulatory trigger for Regulation (EC) No 1829/2003. In particular, both a historical survey on how this discussion has evolved, as well as a detailed legal analysis clearly demonstrate that a poorly framed and logically hardly comprehensible recital (i.e., recital No 16 in Regulation (EC) No 1829/2003) cannot be misconstrued as a legal basis for a zero tolerance for recombinant DNA in fermentation products.

To be meaningful and effective, to secure the safety of food and feed products, and to create the required legal certainty for both producers and competent authorities, legislation should be to the point, proportionate, and preferably harmonized across the globe. An organization building international consensus is the Joint Expert Committee on Food Additives (JECFA) which is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). In its guidelines on food enzymes, JECFA (2020) has stated the following: "For enzymes produced by strains of microorganisms not previously evaluated by JECFA, information is required about the taxonomy, genetic background and other aspects related to the safety of the strain, and commercial use in foods (if any). Enzyme preparations produced by such microorganisms should not contain either antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment or transformable DNA that could potentially contribute to the spread of antibiotic resistance." In keeping with this JECFA position, we would propose the following as a best practice and as basis for global harmonization: (1) The legally binding criteria for regulation of fermentation products should be: (a) the method of production, i.e. on open fields vs. in closed, contained facilities; and (b) the absence of live cells of the production microorganism in contained-use products. (2) Absence vs. presence of recombinant DNA shall not be used as a regulatory criterion, but shall be an integral part of the safety assessment required under product-specific legislation. (3) Absence of recombinant DNA shall only be required for strains containing full-length acquired antibiotic resistance genes for antibiotics that are (critically) important for human health (WHO, 2019). (4) Even for recombinant DNA encoding acquired antibiotic resistance genes, harmonized performance criteria for the analytical methods should be established, together with a legally binding threshold. At the same time, though, the fermentation industry is well advised to phase out all remaining production strains containing acquired antibiotic resistance marker genes.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The views and opinions expressed in this document are those of the authors in their personal capacity. None of the observations, opinions or conclusions expressed herein are to be attributed to or construed as the views or positions of any organization, association or company. RC is employed by a research institute that performs research on microorganisms used in fermentation. AL, ED and MW are employed by companies which commercially produce products of interest by industrial fermentation. AB is employed by an international industry

association representing the biotechnology industry in Europe.

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