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Safety Evaluation of Fermotein: Allergenicity, Mycotoxin Production, Biochemical Analyses and Microbiology of a Fungal Single-cell Protein Product

Marjolein van der Spiegel[1], José J. van den Driessche[1], Elisa Leune[2], Lucie Pařenicová[2] and Wim de Laat[2*]

[1]Schuttelaar & Partners, Wageningen, Netherlands.
[2]The Protein Brewery, Breda, Netherlands.

Authors' contributions

This work was carried out in collaboration among all authors. Authors MS and JJD wrote the first draft of the manuscript and supported in the strategy of analyses and studies. Authors MS and EL performed the literature review. Authors EL, LP and WL reviewed the manuscript and managed the analyses of the studies and study design. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Single-cell proteins (SCPs) are considered as innovative and sustainable alternatives to animal-based products. Fermotein is an innovative SCP obtained from fermentation of the filamentous fungus *Rhizomucor pusillus*. The toxicity, capability to produce secondary metabolites and allergenic potential of this fungus has never been assessed before. Like other filamentous fungi, there is a lack of information on this species to assess its safety for human consumption. The objective of the current study was to investigate the safety of Fermotein and its source *Rhizomucor pusillus* regarding toxicity, capability to produce secondary metabolites and allergenicity. In addition, possible contaminants were also examined.
Methodology: The genome of *Rhizomucor pusillus* was sequenced and annotated in order to screen for production of common mycotoxins, antibiotic synthesis pathways, mucormycosis-related virulence factors and *in silico* potential cross-reactivity with known food allergens. The presence of

**Corresponding author: Email: wimdelaat@theproteinbrewery.nl;*

mycotoxins and allergens were validated by laboratory analysis. The level of RNA, heavy metals and microbiological contaminants were also determined.
Results: No mycotoxin production-related genes were identified in the genome of *Rhizomucor pusillus* nor were mycotoxins found in Fermotein. Six proteins present in Fermotein showed high homology with five known food allergens. No gene clusters were found that corresponded with antibiotic synthesis pathways. Although 10 proteins in the genome of *Rhizomucor pusillus* may represent mucormycosis-related virulence factors, no cases of mucormycosis after oral intake are reported. The level of heavy metals and microbiological contaminants were below legislative limits, whereas RNA content was 4.9 ± 0.2% of dry matter.
Conclusion: No safety concerns were identified for Fermotein or its source *Rhizomucor pusillus*, except the potential for cross-reactivity with five known food allergens. This should be taken into account for communication with consumers. Information from the current study contributes to the body of evidence for determination of Qualified Presumption of Safety status of *Rhizomucor pusillus.*

Keywords: Single-cell protein; safety; filamentous fungi; Rhizomucor pusillus; mycotoxins; allergenicity; antibiotics; contaminants.

1. INTRODUCTION

Globally, it is expected that the population will reach 9 billion individuals by 2042, which may result in challenges to provide food [1]. Insufficient amounts of animal-based proteins will be available for the high number of people, whereas more consumption will have negative effects on climate change [2]. Therefore, introduction of sustainable alternative protein sources is of major importance [3-6]. Examples of these protein sources are legumes, duckweed, insects and single-cell proteins [7,8]. Single-cell protein (SCP) refers to protein biomass from microbial sources, including microalgae, bacteria and fungi [9]. More specifically, mycoprotein is the term used for a fungal SCP.

Considering human consumption, not all fungal species are suitable for SCP production [9]. A safety assessment is therefore needed when a new SCP product is placed on the market. In the European Union (EU), a pre-market safety assessment or a history of safe use before 1997 is required under the Novel Food Regulation for novel food products [10]. In the USA, all substances that will be added to food are subject to pre-market approval by the FDA unless such substance is generally recognized as safe (GRAS) among qualified experts under the conditions of its intended use [11].

A well-known market example of mycoprotein is Quorn, obtained from the filamentous fungus *Fusarium venenatum* [9]. Quorn is considered to be GRAS in the USA for use as food in general except meat products, poultry products and infant formula [12]. It also has a history of safe use as a meat replacer in the EU [9]. History of safe use in the EU before 1997 has been established for other fungal species, including *Rhizopus oryzae, Aspergillus sojae* and *Aspergillus oryzae* for production of tempeh (products), soy sauce and as an alternative mineral source in foods and food supplements respectively [13-15]. These three fungi are consumed in low amounts in Western countries.

The filamentous fungus *Rhizomucor pusillus* is a promising microorganism that produces a new food protein source called Fermotein. The fungus has no history of use as a SCP but it has been used for the production of food enzymes [16-21]. Despite the safe use as enzyme-producing fungi, *Rhizomucor* spp. and other filamentous fungi could not granted a Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) due to insufficient literature information on toxicity, capability to produce secondary metabolites and allergenicity [22-24]. Therefore, the safety of *Rhizomucor pusillus* needs to be assessed in more detail before the biomass Fermotein could be used as a food ingredient in both compressed and powder forms, for broad food applications like bakery products, meat replacers, pasta and fermented milk products.

The objective of the current study is to investigate the potential toxicity, the capability to produce secondary metabolites and the allergenic potential of Fermotein and its source *Rhizomucor pusillus*. In addition, the levels of chemical and microbial contaminants in Fermotein were investigated. This information contributes to the body of knowledge regarding

the safety of *Rhizomucor pusillus* and its derived products.

2. METHODOLOGY

2.1 Fermotein Production

Fermotein is a SCP product obtained from a wild type filamentous fungus *Rhizomucor pusillus*. *Rhizomucor pusillus* cells were plated onto PDA plates and incubated at 46°C for at least 16 hours. A shake flask was inoculated with *Rhizomucor pusillus* spores from the PDA plate and incubated at 46°C, 180 rpm for at least 16 hours. The shake flask medium was composed of 20 g/L glucose, minerals, tartaric acid as buffer and ammonium sulphate.

For the aerobic, submerged, temperature and pH-controlled fermentation process, 95 DE glucose syrup from maize (C*Sweet™ D 027R3 from Cargill), glucose syrup from maize (Sirodex 321 from Tereos Starch & Sweeteners Europe), dextrose from wheat (Meritose 200 from Tereos Starch & Sweeteners Europe) or cane sugar (Western ruwe rietsuiker) were used as nutrients together with nitrogen sources (Ammonium salts, aqueous NH_3) and minerals, while olive oil was used to prevent foaming. The biomass was harvested using a solid liquid separation and further processed by adding antioxidants to prevent oxidation of unsaturated lipids, followed by pasteurisation and dewatering by compression. No solvents, pesticides, anti-microbials or anti-parasitic agents were used during the production process. The biomass was compressed to obtain Fermotein Wet (27 - 30% dry weight) and dried to obtain Fermotein Dry (93 - 97% dry weight). Fermotein Wet was frozen and stored at -18°C, whereas Fermotein Dry was stored at 20°C. Products were stored under these conditions until analysis.

Raw materials were processed and handled according to general food safety principles, food contaminant requirements and microbiological requirements as laid down in EU regulations. Processing occurred based on ISO standards and quality control checks were performed on the final product.

Five independently produced batches of Fermotein were used for analyses. All analyses were conducted by accredited laboratories (Nutri Control, Veghel, the Netherlands; NutriLab, Giessen, the Netherlands; SYNLAB Analytics & Services Oosterhout B.V., Oosterhout, the Netherlands) and according to validated methods. All analyses were performed with both Fermotein Wet and Dry, except for RNA levels and allergenicity analysis, which were only performed with Fermotein Dry.

2.2 Whole-Genome Sequencing (WGS)

The whole genome of *Rhizomucor pusillus* was de novo sequenced using a combination of PacBio and Illumina technology (Baseclear, Leiden, The Netherlands) and annotated (Biomax Informatics AG, Planegg, Germany). Sequence data were used to screen for the presence of genes encoding proteins (enzymes, transporters etc.) of mycotoxin production pathways, mucormycosis-related virulence factors and antibiotic synthesis pathways. A Blast search was performed in 2020 according to Altschul et al. [25] to investigate the presence of mycotoxin production-related genes in the genome. A keyword algorithm was used to search sequence data (Pedant Pro platform, Biomax AG) for mucormycosis-related virulence factors. The keywords 'virulence' and 'mucormycosis' or 'mucormycosi' were used. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg) was used to investigate the presence of biosynthetic pathways of 12 main classes of antibiotics in *Rhizomucor pusillus*.

2.3 Allergenicity Testing

The translated predicted open reading frames (ORFs) of *R. pusillus* genome, obtained from the WGS, were used to screen for amino acid sequence homology with known food allergens registered in the Allergen Online database (http://www.allergenonline.org) to predict *in silico* potential cross-reactivity. In case of a high sequence homology, a mRNA analysis was performed on Fermotein Wet using reverse transcriptase polymerase chain reaction (RT-PCR) to confirm the presence of the corresponding gene transcript in the end product. Subsequently, the presence of the protein was confirmed using liquid chromatography-mass spectrometry (LC-MS/MS; Proteome Factory AG, Berlin, Germany). The protein extraction of Fermotein was performed using a sequential extraction method as described by Broekman et al. [26].

2.4 Biochemical Analyses

Ribonucleic acid (RNA) content of Fermotein Dry was measured using high-performance liquid

chromatography-ultraviolet (HPLC-UV) [27]. Concentrations of the mycotoxins aflatoxins (B1, B2, G1, G2), fumonisins (B1, B2), deoxynivalenol, diacetoxyscirpenol, nivalenol, ochratoxin A, H2T-toxin, T2-toxin and zearalenone were measured by LC-MS/MS (validated in house method; NutriControl) in Fermotein Wet and Dry. Concentrations of heavy metals as referred to in EU legislation (arsenic, cadmium, lead and mercury) [28] were determined using inductively coupled plasma mass spectrometry (ICP-MS; validated in house method, NutriControl).

2.5 Microbial Analyses

To determine if there was microbiological contamination in Fermotein Wet and Dry, total aerobic count was measured by the reference method NEN-EN-ISO 4833. *Bacillus cereus* concentrations was determined on a 30°C counting plate following NEN-EN-ISO 7932. Concentrations of *Clostridium perfringens*, coagulase-positive Staphylococci and Enterobacteriaceae were measured on a 37°C counting plate according to ISO 7937, NEN-EN-ISO 6888-2 and NEN-ISO 21528-2, respectively. *Escherichia coli* was determined on a 44°C counting plate following ISO 16649-2 and yeasts and moulds on a 25°C counting plate following to ISO 7954:1987. The absence of *Listeria monocytogenes* and *Salmonella* was investigated using PCR according to ISO 11290-1 and NEN-EN-ISO 6579-1, respectively.

2.6 Data Analysis

All data are presented as means ± SDs (standard deviations) of five representative batches of either Fermotein Wet or Dry. Microbial data are provided for each batch of Fermotein Wet and Dry. SDs are not provided when values were below the detection limit. Measured concentrations of contaminants and other components were compared to advisory or legislative limits for human food from the USA and EU if available. It should be noted that limits are occasionally general and not specific for a single-cell protein product. Therefore, the strictest limits for food products with similar food applications were used, except those specifically intended for infants.

3. RESULTS AND DISCUSSION

3.1 Mycotoxin Production

No mycotoxin production-related genes were identified in the genome of *Rhizomucor pusillus* in the Blast search. Results were confirmed by the analyses of common mycotoxins in Fermotein (Table 1).

Table 1. Average concentrations of mycotoxins in Fermotein Wet (n = 5) and dry (n = 5) and advisory levels or legislative limits in the USA and EU

Mycotoxins (µg/kg)	Fermotein Wet	Fermotein Dry	Advisory levels USA [29]	Legislative limits EU [28]
Sum of aflatoxins (B1+B2+G1+G2)	< 4	<4	≤ 20	≤ 4[c]
Aflatoxin B1	< 1.0	< 1.0	-	-
Aflatoxin B2	< 1.0	< 1.0	-	-
Aflatoxin G1	< 1.0	< 1.0	-	-
Aflatoxin G2	< 1.0	< 1.0	-	-
Sum of fumonisins (B1+B2)	< 200	< 200	2000 – 4000[a]	≤ 800[d]
Fumonisin B1	< 100	< 100	-	-
Fumonisin B2	< 100	< 100	-	-
Deoxynivalenol (DON)	< 150	< 150	1000[b]	≤ 500[e]
Diacetoxyscirpenol	< 20	< 20	-	-
Nivalenol	< 150	< 150	-	-
Ochratoxin A	< 1.0	< 1.0	-	≤ 3[c]
HT2-toxin	< 200	< 200	-	-
T2 toxin	< 20	< 20	-	-
Zearalenone	< 20	< 20	-	≤ 50

[a] For maize and maize products; limits depend on processing and product; [b] For wheat products; [c] For cereals and derived products; [d] For maize-based breakfast cereals and maize-based snacks; [e] For bread and fine bakery products

Concentrations of all mycotoxins were below the detection limit, and well below advisory levels and legislative limits in the USA and EU, respectively. The levels of diacetoxyscirpenol, nivalenol, HT2-toxin, T2-toxin comply with the EU Tolerable Daily Intake (TDI values) when the anticipated intake is applied.

The analysis of mycotoxins in Fermotein confirms the *in silico* prediction that *Rhizomucor pusillus* does not produce mycotoxins. This is in line with previous studies investigating the production of mycotoxins and other secondary metabolites by micromycetes growing on food raw materials and plant-based products. Paterson et al [30] and Lugauskas [31] reported no mycotoxin production by *Rhizomucor pusillus*. Mycotoxin production is therefore not considered a safety concern for Fermotein.

3.2 Allergenicity

In silico analysis revealed that six proteins from Fermotein showed high homology with known food allergens. mRNA analysis showed that all six genes, encoding the potential allergenic proteins, are actively expressed during the fermentation of the fungus and are present in the final product. Through proteomic analysis, six proteins were actually identified to be present in Fermotein, meaning that there is a chance of cross-reactivity with salmon, tuna, chicken, pistachio nut, carrot and some shrimp and crab species. Chicken and carrot are not seen as food allergens in the EU as well the USA.

Fungi are known to elicit allergenic responses through inhalation of spores. Among eight phyla of fungi, three phyla are associated with the production of known allergens, including *Zygomycota* [32]. However, only allergens from *Rhizopus* species among the *Zygomycota* have been officially characterized [32,33]. Spore formation and consequently allergic reaction is not considered of concern during Fermotein production, since spores are generally not produced at the fermentation conditions applied in the production process and laboratory analysis showed that the fungus is not viable due to inactivation during the production process and no spores are present at the end of the production process. In addition, to our knowledge, no case reports of allergenic reactions or sensitization to *Rhizomucor pusillus* have been described. Results of the *in silico* analysis, mRNA analysis and proteomics did show potential cross-reactivity and presence in Fermotein for five known food allergens. The potential risk of cross-reactivity should be clearly communicated to consumers via labelling. Furthermore, post-market monitoring is necessary to follow the introduction of *de novo* sensitizations due to the novelty of the food product.

3.3 Mucormycosis-related Virulence Factors and Antibiotic Synthesis Pathways

Invasive infections in humans, known as mucormycosis, can be induced by different fungal species. WGS data and its annotated proteins were used to assess the potential of *Rhizomucor pusillus* to induce mucormycosis. The keyword 'virulence' resulted in 189 entries, of which 10 contained the words 'mucormycosis' or 'mucormycosi'. These 10 proteins may represent mucormycosis-related virulence factors encoded by the genome of *Rhizomucor pusillus*.

In literature, less than 40 cases of human infections caused by *Rhizomucor pusillus* have been reported [34,35]. Most of the cases were associated with profound neutropenia and leukemia in the host. Indeed, mucormycosis primarily occurs in immunocompromised subjects via inhalation of spores [36,37]. There is no evidence of mucormycosis caused by ingestion of foods containing fungi, which makes it highly unlikely that invasive infections can occur via consumption of Fermotein. Since cases of mucormycosis caused by *Rhizomucor pusillus* were identified, it cannot be excluded that the 10 proteins which were identified in the genome, may represent mucormycosis-related virulence factors. However, due to the validated pasteurization step in the production process of Fermotein, *Rhizomucor pusillus* is not viable at the end of the production process and spores are inactivated. Therefore, mucormycosis is not considered a safety issue for Fermotein.

None of the 12 major antibiotic biosynthetic pathway gene clusters were found in the genome of *Rhizomucor pusillus*. It has been reported that other fungi used for food production, such as *Aspergillus oryzae*, may produce antibacterial metabolites [38]. However, *Zygomycetes* are not capable of antibiotic production according to literature [39]. Results of the genome screening support this statement and antibiotics production is therefore not considered a safety concern for Fermotein.

3.4 Heavy Metals and RNA Content

Concentrations of arsenic, cadmium, lead, and mercury were low and well below EU legislative limits (Table 2). No limits for human food categories with similar food applications compared to Fermotein were identified for the USA.

Filamentous fungi are known for their capability to absorb heavy metals and minerals [40]. Although raw materials used in the production of Fermotein are processed according to international standards and requirements, traces of heavy metals could be introduced. Results show that concentrations of the analysed heavy metals are below limits and therefore do not pose a safety threat for the consumption of Fermotein. The accumulation of minerals (data not shown) was also not considered to be a safety issue.

The average concentration (n = 5) of RNA in Fermotein is 4.9 ± 0.2% of dry matter. RNA content is expected to be high in fungal SCP (7 - 10%), which is of concern when used for human consumption since high purine intake can affect health negatively [9]. Compared to those numbers, RNA content of Fermotein is relatively low.

No legislative limits or limits for daily intake for nucleic acids (i.e. DNA and RNA) have been set by authorities. In 1975, the Protein-Calorie Advisory Group of the United Nations suggested a safe maximum intake of 2 g nucleic acids per day via SCPs, based on the review of Kihlberg [41]. The studies underlying the conclusion of this review are not available. However, more recent animal studies did show no adverse effects with diets including 50% mycoprotein containing up to 10% RNA (dry weight) in the GRAS notification of *Fusarium* mycoprotein [12]. It has also been mentioned that daily intake of nucleic acids from the regular diet is about 1 to 2 g/day in the American adult population [42]. Although RNA content of Fermotein seems to be relatively low compared to other fungal SCPs and nucleic acids are already part of the background diet (mainly via animal-based products), levels should be taken into account and used as a limiting factor for the maximum use levels of Fermotein. Replacement of animal-based protein ingredients gives opportunities for the addition of Fermotein to the regular diet.

Table 2. Average concentrations (mg/kg wet weight) of arsenic, cadmium, lead and mercury in Fermotein Wet (n = 5) and legislative limits in the USA and EU

Heavy metals (mg/kg)	Fermotein Wet	Legislative limits USA	Legislative limits EU [28]
Arsenic	< 0.05	-	≤ 0.30[a]
Cadmium	< 0.01	-	≤ 1.0[b]
Lead	< 0.05	-	≤ 0.30[c]
Mercury	< 0.010	-	≤ 0.50[d]

[a] For rice waffles, rice wafers, rice crackers and rice cakes; [b] For fungi; [c] For leafy brassica, salsify, leaf vegetables excluding fresh herbs and the following fungi Agaricus bisporus (common mushroom), Pleurotus ostreatus (Oyster mushroom), Lentinula edodes (Shiitake mushroom); [d] For fishery products and muscle meat of fish

Table 3. Concentrations of microorganisms in Fermotein Wet (n = 5) and Fermotein Dry (n = 5) per batch

Batch	Fermotein Wet					Fermotein Dry				
	#1	#2	#3	#4	#5	#1	#2	#3	#4	#5
Total aerobic colony count (cfu/g)	280	< 40	140	220	30	170	< 100	200	260	130
Bacillus cereus (cfu/g)	< 100	< 40	< 100	< 40	< 50	140	< 100	< 100	140	< 50
Clostridium perfringens (cfu/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Coagulase positive Staphylococci (cfu/g)	< 10	< 10	< 10	< 10	< 50	< 10	< 10	< 10	< 10	< 50
Enterobacteriaceae (cfu/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 40	< 40	< 10	< 10
Escherichia coli (cfu/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Listeria monocytogenes (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Yeasts and moulds (cfu/g)	< 10	< 10	< 10	< 10	< 10	< 40	< 10	< 10	< 10	< 10

3.5 Microbiological Contamination

Concentrations of microorganisms that could cause a food safety hazard or adversely affect shelf life, were low in all batches of Fermotein Wet and Dry (Table 3), indicating that the pasteurisation processing step and the storage conditions of Fermotein assure that microbial contamination is not a concern.

Legislative limits in the EU are set for specific food and foodstuffs but SCP products are not included in any food category [43]. The microorganisms were either absent or their levels were within generally accepted standards for food ingredients. Some colony forming units of the spore forming *Bacillus cereus* were detected. In the EU, limits are only set for strictly controlled food products, such as baby formulae and foods for special medical purposes, where maximum allowed concentrations are 500 cfu/g for 1 out of 5 batches [43]. EFSA has suggested that producers of new products should ensure that 10^3-10^5 cfu/g are not reached at the stage of consumption [44]. Levels of *Bacillus cereus* are well below this threshold after production and the handling and storage have to be controlled. Therefore, microbiological contamination is no safety risk for human consumption of Fermotein with the current production process and quality control checks in place.

4. CONCLUSIONS

Fermotein is a SCP product obtained from the filamentous fungus *Rhizomucor pusillus*, for which no safety data or QPS status are currently available. Our studies show that no safety concerns for Fermotein and *Rhizomucor pusillus* as a source were identified, except for a potential cross-reactivity with a few known food allergens. However, the risk of cross-reactivity can be communicated to consumers via labelling. RNA content should be taken into account when determining maximum use levels of Fermotein due to absence of legislative limits. When labelling for potential cross-reactivity is not preferred or to ensure that a certain group of consumers is excluded, allergenicity testing is an option to investigate the actual potential of cross-reactivity of Fermotein. Post-market monitoring should follow *de novo* sensitisations. Based on the current data on Fermotein and the controlled production process, there is no necessity to perform toxicity studies.

Addition to the QPS list is preferred but it is not a limitation for market authorization. The fungi with a history of safe use (*Aspergillus oryzae*, *Aspergillus sojae* and *Rhizopus oryzae*) are also not added to the QPS list. The information from this study contributes to the body of knowledge that can be used to assess the QPS status of *Rhizomucor pusillus* and derived products.

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COMPETING INTERESTS

Schuttelaar & Partners was hired as a consultancy agency to support the novel food application and to write the current manuscript. Lucie Pařenicová is contracted as a consultant by The Protein Brewery. She performed the studies on mycotoxins and antibiotics and supported the RNA analysis and LC-MS/MS analysis for the allergenicity study.

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