Original Research Article

FERMOTEIN DOES NOT EXERT GENOTOXIC EFFECTS IN BACTERIAL REVERSE MUTATION AND *IN VITRO* MAMMALIAN CELL MICRONUCLEUS TESTS

ABSTRACT

Aim: Fermotein is an innovative single-cell protein obtained from fermentation of the filamentous fungus *Rhizomucor pusillus*. Like other filamentous fungi, a lack of information on this species exists to assess its safety for human consumption. The capability to induce gene mutations or structural and numerical chromosomal aberrations of this fungus and derived products has never been studied before. The objective of the current study was to investigate the genotoxic effects of Fermotein using a bacterial reverse mutation test and an in vitro mammalian cell micronucleus test.

Methodology: The bacterial reverse mutation test and in vitro mammalian cell micronucleus test were performed in accordance with GLP and concurrent OECD guidelines. Dose-range finding tests were used to select appropriate doses of Fermotein Dry. The highest doses in the genotoxicity experiments were determined by the solubility of the mycoprotein.

Results: The bacterial reverse mutation test and *in vitro* mammalian cell micronucleus test were performed in accordance with GLP and concurrent OECD guidelines. Dose-range finding tests were used to select appropriate doses of Fermotein Dry. The highest doses in the genotoxicity experiments were determined by the solubility of the mycoprotein.

Conclusion: No safety concerns regarding genotoxicity were identified for Fermotein and no further *in vivo* genotoxicity testing is required. Information from the current study contributes to the body of evidence for a novel food authorisation of Fermotein in the EU and a GRAS notification in the US.

Keywords : Single-cell protein; safety; filamentous fungi; Rhizomucor pusillus; genotoxicity; bacterial reverse mutation (Ames) test; in vitro mammalian cell micronucleus test.

1. INTRODUCTION

Fermentation has a long history of use in the preservation and production of foods like soy sauce and yoghurt [1]. With the current advances in technology, cultivation of microorganisms can be used to produce protein-rich biomasses for human consumption. Protein-rich biomasses obtained via fermentation are valuable alternatives to animal-based proteins and, as replacers of meat, dairy, and egg proteins, can contribute to the protein transition towards a more sustainable and plant-based diet. Several single-cell organisms can be used to produce fermented foods, such as algae, bacteria or fungi [2]. The term mycoprotein specifically refers to biomasses sourced from fungi. A well-known example of mycoprotein used as a meat replacer is Quorn, which has been on the international market for decades.

New mycoproteins as food ingredients for human consumption must proceed through a pre-market safety assessment procedure in the European Union (EU) [3] or United Kingdom and should obtain a Generally Recognized as Safe (GRAS) status to enter the market in the United States (US) [4]. Such a new mycoprotein product is Fermotein, a single-cell protein biomass produced by fermentation of the filamentous fungus *Rhizomucor pusillus*. Like other filamentous fungi, there is a lack of information on this species to assess its safety for human consumption [5-7]. We have reported earlier that no safety concerns were identified regarding the production of virulence factors, mycotoxins and antibiotics of *Rhizomucor pusillus*, and the chemical and microbial contamination of Fermotein [8].

Due to the potential serious health effects of genetic alterations, genotoxicity testing is found to be essential within safety assessment procedures [9]. Recommendations have been made by regulatory bodies for strategies to test for genotoxic effects of substances [10]. In general, genotoxicity assessment is based on three endpoints since they are implicated in carcinogenesis and heritable diseases: gene mutations, structural chromosomal aberrations (clastogenicity) and numerical chromosomal aberrations (aneuploidy) [9]. These endpoints are covered by a combination of an *in vitro* bacterial reverse mutation test and an i*n vitro* mammalian cell micronucleus test. The substance of interest is considered not to have genotoxic potential if no effects are found on all endpoints.

Besides investigating contaminants and capability of the fungus to produce secondary metabolites Fermotein should also be examined for its genotoxicity before the biomass could enter the market as a food ingredient for broad food applications like bakery products, meat replacers, pasta and fermented milk products. The objective of the current study was therefore to investigate the genotoxic potential of Fermotein using an *in vitro* bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test.

2. METHODOLOGY

2.1 Test Material

Fermotein is a single-cell protein product obtained through fermentation by the wild-type filamentous fungus *Rhizomucor pusillus*. A detailed description of the production process has been published before [6]. In short, after the fermentation process using the fungus *Rhizomucor pusillus* and a medium containing common nutrients and minerals, the biomass is harvested, pasteurized, dewatered, and dried to obtain Fermotein Dry (93 – 97% dry weight). Analyses of five representative batches of Fermotein Dry showed limited signs of mycotoxins, heavy metals, or microbiological contamination (Table 1) [8]. The product was supplied by The Protein Brewery (Breda, the Netherlands) and stored at room temperature protected from light until use in the genotoxicity tests.

Table 1: Average concentrations of mycotoxins, heavy metals, and microbiological contaminants in 5 batches of Fermotein Dry

Analyte	Unit	Concentration
Mycotoxins		
Sum of aflatoxins	ug/kg	< 4
Sum of fumonisins	ug/kg	< 200
Deoxynivalenol	ug/kg	< 150
Ochratoxin A	ug/kg	< 1.0
Zearalenone	ug/kg	< 20
Heavy metals		
Arsenic	mg/kg	< 0.05
Cadmium	mg/kg	< 0.01
Lead	mg/kg	< 0.05
Mercury	mg/kg	< 0.010
Microbiology		
Total aerobic colony count*	cfu/g	172
*Bacillus cereus**	cfu/g	106
Clostridium perfringens	cfu/g	< 10
Coagulase positive Staphylococci	cfu/g	< 50
Enterobacteriaceae	cfu/g	< 40
Escherichia coli	cfu/g	< 10
Listeria monocytogenes	in 25 g	Absent
Salmonella	in 25 g	Absent
Yeasts and moulds	cfu/g	< 40

cfu: colony forming units.
* Upper-bound values used for calculation of the average concentration

2.2 Genotoxicity Tests

All genotoxicity tests were performed at Charles River Laboratories (Den Bosch, the Netherlands) in accordance with GLP and concurrent OECD guidelines, as requested for regulatory approval procedures. Prior to the bacterial reverse mutation test and the *in vitro* mammalian micronucleus test, the solubility of Fermotein Dry was examined. A homogenous suspension was formed at a concentration of 2.5 mg/mL in dimethyl sulfoxide (DMSO). DMSO was therefore used as a vehicle for Fermotein Dry in both tests.

2.2.1 Bacterial Reverse Mutation (Ames) Test

One batch of Fermotein Dry (batch code FGB6) was used for the bacterial reverse mutation test with *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 uvrA (pKM101) in the absence and presence of metabolic activation. All experiments were conducted in triplicate and according to OECD guideline No. 471 [11], EC guideline No. 440/2008 [12] and ICH Harmonised Tripartite Guideline S2(R1) [13].

Cultures of each bacterial strain were freshly grown for each test by culturing frozen stock samples until optical density of 1.0 at 700 nm (109 cells/mL) was reached. Metabolic activation occurred with rat liver microsomal enzyme S9 from Sprague Dawley rats (Trinova Biochem GmbH, Giessen, Germany) and the S9-mix was prepared freshly before use. The vehicle of the test material (DMSO) was used as the negative or solvent control. Positive controls were used according to the OECD guideline. Agar plates were incubated with 0.1 mL fresh bacterial culture, 0.1 – 1.0 mL of the test material dilution or controls, and 0.5 mL S9-mix (with metabolic activation) or phosphate buffer (without metabolic activation). After incubation of 48 hours at 37˚C, revertant colonies were counted using a semi-automated scoring system (Sorcerer, Instem UK). Plates with precipitation were counted manually.

The dose-range finding test was performed with tester strains TA100 and WP2 uvrA with and without the S9-mix. Eight concentrations ranging from 1.7 to 1250 ug/plate were tested in triplicate. The test material exhibited limited solubility. Therefore, a second dose-range finding study based on the treat and wash method was performed with concentrations ranging from 50 to 2500 ug/plate with the tester strains TA100 and WP2 uvrA (pKM101). According to OECD guidelines, the levels at which the test material exhibited limited solubility in the dose-range finding tests were used as the highest concentrations in both a direct plate assay and a treat and wash assay.
Based on the dose-range finding tests, the direct plate assay and the treat and wash assay were conducted with five concentrations (5.4, 17, 52, 100 and 250 ug/plate) of the test material with the tester strains TA1535, TA1537 and TA98 with and without metabolic activation. Since no dose level with cytotoxicity was observed, an additional experiment was executed with 500 ug/plate test material with strains TA1535, TA1537 and TA98 with and without metabolic activation using the treat and wash assay.

2.2.2 *In Vitro* Mammalian Cell Micronucleus Test

One batch of Fermotein Dry (batch code FGB6) was used for the *in vitro* mammalian cell micronucleus experiments using cultured human lymphocytes in the absence and presence of metabolic activation. All assays were performed in duplicate and in accordance with OECD guideline No. 487 [14]. Possible clastogenicity and aneugenicity of Fermotein were tested in two independent cytogenetic assays including a dose-range finding test.
Vehicle control (DMSO) and positive controls according to OECD guideline were included. Metabolic activation occurred with rat liver microsomal enzyme S9 from Sprague Dawley rats (Trinova Biochem GmbH, Giessen, Germany) and the S9-mix was prepared freshly before use. Heparin-treated blood samples were obtained from healthy (non-smoking) adult volunteers via venipuncture and cultured for 48 hours in a culture medium and phytohaemagglutin (PHA, Remel Europe, Dartford, UK).
For the first cytogenetic assay, lymphocytes were exposed to selected doses of the test material or controls for 3 hours in the absence and presence of metabolic activation. After the 3-hour exposure, cells were centrifuged, rinsed after removal of the supernatant, and resuspended in culture medium containing 5 ug/mL Cytochalasine B (Sigma) for an incubation period of 24 hours. Cells were then harvested, fixed onto slides, and stained. For the second cytogenetic assay, lymphocytes were exposed to the test material or controls for 24 hours with 5 ug/mL Cytochalasine B in the absence of the S9-mix. After incubation, cells were not rinsed but immediately fixed onto slides and stained. For each condition or dose tested, two slides were used. After preparation of slides, the number of mononucleate, binucleate and multinucleate cells were counted from a minimum of 500 cells per culture and the cytokinesis-block proliferation index (CBPI) was used as a measure of cytotoxic and cytostatic activity according to OECD guidelines [14]. The selected doses of the test material and controls were scored for micronuclei.
A dose-range finding test with six concentrations ranging from 1.6 to 50 ug/mL culture medium was performed. Based on the findings of this test, appropriate dose levels were chosen for the cytogenic assays, and the highest dose level was determined by the solubility of Fermotein in the culture medium. The first and second cytogenetic assays were conducted with concentrations of 12.5, 25 and 50 ug/mL culture medium in the absence and presence of S9-mix.

2.3 Data Analysis

All data is presented as mean ± SD, unless indicated otherwise. Criteria for positive results are defined according to OECD guidelines.
In the bacterial reverse mutation test, positive results were defined as a reproducible 2-fold increase in the total number of revertants in the tester strains TA100 or WP2 uvrA and a 3-fold increase in the tester strains TA1535, TA1537 or TA98 compared to the concurrent negative controls.
For the *in vitro* micronucleus test, statistical analysis was performed using GraphPad Prism (version 8.4.2, GraphPad Software, San Diego, USA). Differences between Fermotein Dry and the concurrent negative control were tested using a one-sided Chi-square test. In case of a statistically significant difference, a Cochran Armitage trend test was conducted to examine whether a dose-response trend was present. The test material was deemed positive for clastogenic or aneugenic effects if it differed significantly from the negative control, there was a significant dose-response trend, and the values were outside the 95% control limits of historical control data ranges. P-values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Bacterial Reverse Mutation (Ames) Test

Negative and positive control values with and without metabolic activation were within historical control ranges showing adequate test conditions. Results of the dose-range finding tests are described as part of the mutation tests.

Direct plate assay

The first dose-range finding test and direct plate assay showed that precipitation of Fermotein occurred at concentrations of 164 ug/plate and above in tester strains TA100 and WP2 uvrA and 100 ug/plate and above in tester strains TA1535, TA1537 and TA98. Fermotein in concentrations up to 1250 ug/plate did not exert toxic effects as no reduction in bacterial background lawn and no relevant decrease in the number of revertants were observed.

No relevant increase in the number of revertants in any of the tester strains was observed after treatment with Fermotein under all conditions tested (Table 2). In tester strain TA1537 with metabolic activation, a 3.7-fold increase in the number of revertant colonies was observed at 5.4 ug/plate compared to the solvent control. This increase was, however, not deemed biologically relevant, since the increase was well within historical control data ranges and the absence of a dose-related effect. Metabolic activation had no effect on the number of revertants.

Table 2: Mean ± SD (n = 3) number of revertant colonies in the direct assays dose-range finding test (*Salmonella typhimurium* TA100 and *E. coli* WP2uvrA) and first experiment (*Salmonella typhimurium* TA1535, TA1537, TA98*)*

Concentration (ug/plate)	**Without S9-mix**					**With S9-mix**				
	TA100	**WP2 uvrA**	**TA 1535**	**TA 1537**	**TA98**	**TA100**	**WP2 uvrA**	**TA 1535**	**TA 1537**	**TA98**
Solvent control	98 ± 12	16 ± 5	7 ± 5	5 ± 2	8 ± 4	74 ± 8	16 ± 4	12 ± 2	3 ± 3	16 ± 3
1.7	78 ± 4	15 ± 4	-	-	-	57 ± 4	21 ± 3	-	-	-
5.4	90 ± 6	20 ± 5	4 ± 1	3 ± 1	12 ± 2	73 ± 2	13 ± 9	7 ± 4	11 ± 4*	22 ± 5
17	80 ± 3	14 ± 1	5 ± 2	4 ± 1	17 ± 4	59 ± 7	15 ± 1	7 ± 2	2 ± 1	17 ± 4
52	90 ± 10	14 ± 6	6 ± 3	5 ± 2	15 ± 1	63 ± 7	17 ± 2	12 ± 6	4 ± 5	14 ± 7
100	-	-	8 ± 3[A]	4 ± 2[A]	16 ± 6[A]	-	-	6 ± 5[A]	5 ± 2[A]	20 ± 13[A]
164	70 ± 17[A]	20 ± 5[A]	-	-	-	64 ± 9[A]	14 ± 5[A]	-	-	-
250	73 ± 8[A]	15 ± 1[A]	6 ± 3[A]	8 ± 2[A]	12 ± 4[A]	60 ± 2[A]	23 ± 6[A]	12 ± 7[A]	5 ± 1[A]	18 ± 8[A]
500	72 ± 7[A]	16 ± 3[A]	-	-	-	66 ± 24[A]	15 ± 4[A]	-	-	-
1250	64 ± 10[A]	21 ± 5[A]	-	-	-	64 ± 3[A]	26 ± 3[A]	-	-	-
Positive control	706 ± 51	1366 ± 35	883 ± 80	1282 ± 54	1530 ± 65	1353 ± 420	356 ± 74	323 ± 31	321 ± 37	1079 ± 47

[A]: slight precipitation; *: more than 2- or 3-fold increase

Treat and wash assay

In the second dose-range finding test and the treat and wash assay, Fermotein precipitated at concentrations of 100 ug/plate, 250 ug/plate and 500 ug/plate and above in tester strains TA100, WP2 uvrA (pKM101) and TA1535, TA1537 and TA98 respectively.

A reduction of the bacterial background lawn was observed at Fermotein concentrations of 1250 and 2500 ug/plate in tester strains TA100 and WP2 uvrA (pKM101). This was, however, attributed to an increase in solvent used at these concentrations. Moderate reduction of the background lawn was observed when higher concentrations of solvent control (0.5 or 1.0 mL) were added to the tester strains TA100 and WP2uvrA-pKM101 (Table 3). It can therefore be concluded that the test material itself did not induce cytotoxicity. No signs of cytotoxicity could be detected in the tester strains TA1535, TA1537 and TA98 in the treat and wash assay. Therefore, an additional treat and wash assay was conducted with 500 ug/plate test material, and no cytotoxicity was detected here as well.

Table 3: Mean ± SD (n = 3) number of revertant colonies in the second dose range-finding test (*Salmonella typhimurium* TA100 and *E. coli* WP2uvrA) and treat and wash assays (*Salmonella typhimurium* TA1535, TA1537, TA98*)*

Concentration (µg/plate)	Without S9-mix					With S9-mix				
	TA100	WP2 uvrA pkM101	TA1535	TA1537	TA98	TA100	WP2 uvrA pkM101	TA1535	TA1537	TA98
Treat and wash assay										
Solvent control	81 ± 2	31 ± 2	8 ± 3	3 ± 2	9 ± 2	72 ± 12	41 ± 8	9 ± 5	3 ± 2	10 ± 4
0.5 mL	71 ± 12[B]	9 ± 3[B]	-	-	-	54 ± 19[A]	61 ± 25[B]	-	-	-
1.0 mL	39 ± 3[B]	3 ± 2[B]	-	-	-	97 ± 32[B]	76 ± 13[B]	-	-	-
5.4	-	-	4 ± 1	3 ± 2	10 ± 1	-	-	6 ± 4	4 ± 2	14 ± 4
17	-	-	4 ± 1	3 ± 2	12 ± 8	-	-	10 ± 5	6 ± 3	16 ± 5
50 or 52	77 ± 5	34 ± 7	10 ± 2	2 ± 2	13 ± 4	83 ± 17	57 ± 7	4 ± 1	8 ± 1*	15 ± 1
100	76 ± 11[A]	43 ± 17	5 ± 1	4 ± 2	10 ± 2	70 ± 7[A]	50 ± 10	7 ± 4	3 ± 1	17 ± 4
250	63 ± 12[A]	29 ± 1[A]	10 ± 4	3 ± 3	13 ± 5	74 ± 6[A]	53 ± 9[A]	13 ± 5	4 ± 2	22 ± 4
1250	61 ± 9[AB]	9 ± 6[AB]	-	-	-	46 ± 6[AB]	29 ± 12[AB]	-	-	-
2500	49 ± 6[AB]	17 ± 13[AB]	-	-	-	72 ± 88[AB]	3 ± 2[AB]	-	-	-
Positive control	160 ± 9	430 ± 116	163 ± 15	99 ± 32	980 ± 44	613 ± 768	254 ± 38	126 ± 12	114 ± 12	376 ± 34
Additional treat and wash assay with 500 µg/plate test material										
Solvent control	-	-	5 ± 3	11 ± 1	4 ± 1	-	-	6 ± 3	3 ± 2	10 ± 4
500	-	-	6 ± 4[A]	8 ± 5[A]	10 ± 5[A]	-	-	7 ± 2[A]	10 ± 4[A]	14 ± 9[A]
Positive control	-	-	133 ± 2	61 ± 6	707 ± 38	-	-	119 ± 10	116 ± 15	328 ± 30

[A]: slight or moderate precipitation; [B]: bacterial background lawn moderately reduced; *: more than 2 or 3-fold increase

No increase in the number of revertant colonies were observed after incubation with Fermotein, except in the tester strain TA1537 with the S9-mix. A 3.3-fold increase in number of revertant colonies compared to the solvent control was found, but this increase is still within historical control ranges and might be related to a relatively low solvent control measurement. The increase is therefore not considered biologically relevant.

Based on the OECD guideline [11], it was decided in advance that the test material was deemed positive for mutagenic effects if a reproducible increase in the total number of revertants was found. In the direct plate and first treat and wash assays, a more than 3-fold increase in the revertant colonies were found, but at concentrations of 5.4 and 52 ug/plate respectively. In the additional treat and wash assay, no increase was found, altogether indicating that no reproducible increase was present and that the increases found were not biologically relevant. All positive controls induced an increase in revertants and were within historical control ranges, just like the negative controls, indicating adequate testing conditions.

Precipitation as well as minor microbiological contamination may interfere with automatic colony counting [15]. Although microbiological contamination was very low, it may have contributed to the occasional increase in revertants. Precipitation occurred at the highest concentrations tested, but those plates were counted manually, and is therefore not expected to have interfered with results [15].

Fermotein is considered a high-protein food, also containing substantial amounts of histidine and tryptophan. The *Salmonella* and *E. coli* strains rely on histidine and tryptophan, respectively, for growth. It has therefore been suggested that test materials high in protein may interfere with the

results [16]. Especially for poorly soluble test materials, amino acid release from the test material may be localized on the plate, which may lead to incidental positive findings. Modification of the test procedure by using the treat and wash assay instead of the direct plate assay was found effective in avoiding false positive results [16]. Therefore, the treat and wash assay was used in the current study and it is therefore not expected that the high-protein nature of Fermotein interfered with the results.
To the best of our knowledge, the potential of foods produced with *Rhizomucor pusillus* to induce gene mutations has not been investigated before. The only already consumed mycoprotein that has been tested for its genotoxicity is Quorn. This mycoprotein was evaluated using a modified *Salmonella* reverse mutation assay [17]. No genotoxic effects were found. Both Quorn and Fermotein are mycoproteins obtained via fermentation with a fungal species. However, Quorn is obtained from the filamentous fungus Fusarium venenatum, whereas Fermotein results from fermentation with the filamentous fungus *Rhizomucor pusillus*.
Based on the results of the bacterial reverse mutation test, it can be concluded that Fermotein is not mutagenic in the tested species.

3.2 *In Vitro* Mammalian Cell Micronucleus Test

Precipitation of the test material in the culture medium occurred at the highest concentration tested (50 ug/mL).
In the first experiment, no effects of Fermotein on the cytokinesis-block proliferation index or cytostasis could be detected (Table 4). The total number of cells with micronuclei did not differ between the different concentrations of Fermotein and the solvent control without or with metabolic activation (Table 5).

Table 4: Mean and range (n = 2) of cytokinesis-block proliferation index and percentage of cytostasis after 3-hour exposure with and without S9-mix and 24-hour exposure without S9-mix with different concentrations of Fermotein.

Concentration	*First experiment*				*Second experiment*	
	3-hour exposure without S9-mix		**3-hour exposure with S9-mix**		**24-hour exposure without S9-mix**	
	CBPI (mean and range)	**% Cytostasis**	**CBPI (mean and range)**	**% Cytostasis**	**CBPI (mean and range)**	**% Cytostasis**
Solvent control	1.84 (1.84 – 1.84)	0	1.84 (1.83 – 1.85)	0	1.76 (1.75 – 1.76)	0
12.5	1.80 (1.78 – 1.82)	5	1.78 (1.77 – 1.79)	7	1.74 (1.74 – 1.75)	2
25	1.76 (1.74 – 1.78)	10	1.79 (1.78 – 1.80)	6	1.74 (1.74 – 1.75)	2
50[A]	1.85 (1.85 – 1.86)	-1	1.81 (1.78 – 1.83)	4	1.59 (1.58 – 1.60)	22
Positive controls[B]	1.57 (1.56 – 1.57) 1.45 (1.43 – 1.47) 1.14 (1.11 – 1.16)	33 47 84	1.41 (1.41 – 1.42) 1.36 (1.36 – 1.36)	51 57	1.39 (1.38 – 1.41) 1.33 (1.33 – 1.34) 1.01 (1.01 – 1.01)	48 56 99

[A]: Precipitation in the culture medium occurred.
[B]: Positive controls: 0.25 µg/mL mitomycin C, 0.38 µg/mL mitomycin C and 0.1 µg/mL colchicine for 3-hour exposure without S9-mix; 15 and 17.5 µg/mL cyclophosphamide for 3-hour exposure with S9-mix; 0.15 µg/mL mitomycin C, 0.23 µg/mL mitomycin C and 0.05 µg/mL colchicine for 24-hour exposure without S9-mix.

The second experiment was conducted to obtain more information about possible clastogenic and aneugenic effects by 24-hour exposure without S9-mix to different concentrations of Fermotein (Table 4 and 5). An increased number of micronuclei (3 micronuclei per 2000 mononucleated cells) compared to the solvent control (0 micronuclei per 2000 cells; $P < 0.05$) was found at the highest concentration of 50 ug/mL. The increase was found to be dose-related ($P = 0.0201$ for the Cochran Armitage trend test). However, the number of cells with micronuclei in the highest concentration was found to be within the 95% control range of historical data for solvent controls (4 micronuclei per 2000 cells). To verify the results, an additional 2000 cells were scored (1000 per duplicate) for the highest concentration and merged with the previous scoring. No difference or dose-related trend in the number of micronuclei was found between the solvent control (1 micronuclei per 4000 mononucleated cells) and the 50 ug/mL concentration of Fermotein (4 micronuclei per 4000 mononucleated cells).

Table 5: Total number (n = 2) of mononuclear (MN) or binuclear (BN) cells with micronuclei per 2000 cells (1000 per duplicate) after 3-hour exposure with and without S9-mix and 24-hour exposure without S9-mix with different concentrations of Fermotein.

Concentration	*First experiment*				*Second experiment*	
	3-hour exposure without S9-mix		3-hour exposure with S9-mix		24-hour exposure without S9-mix	
	Number of MN cells with micronuclei[C]	Number of BN cells with micronuclei[C]	Number of MN cells with micronuclei[C]	Number of BN cells with micronuclei[C]	Number of MN cells with micronuclei[C]	Number of BN cells with micronuclei[C]
Solvent control	2 (1, 1)	5 (2, 3)	0 (0, 0)	6 (4, 2)	0 (0, 0)	10 (6, 4)
12.5	0 (0, 0)	2 (2, 0)	0 (0, 0)	1 (1, 0)	0 (0, 0)	9 (4, 5)
25	0 (0, 0)	2 (1, 1)	1 (1, 0)	1 (1, 0)	0 (0, 0)	6 (3, 3)
50[A]	0 (0, 0)	4 (3, 1)	0 (0, 0)	6 (4, 2)	3* (2, 1)	13 (8, 5)
Positive controls[B]	0 (0, 0) 67** (35, 32)	73** (35, 38) 1 (1, 0)	0 (0, 0)	44** (22, 22)	1 (0, 1) 132** (61, 71)	103** (53, 50) 54** (37, 17)

MN: mononucleated; BN: binucleated; * P-value < 0.05; **: P-value < 0.0001
[A]: Precipitation in the culture medium.
[B]: Positive controls: 0.25 µg/mL mitomycin C and 0.1 µg/mL colchicine for 3-hour exposure without S9-mix; 15 µg/mL cyclophosphamide for 3-hour exposure with S9-mix; 0.15 µg/mL mitomycin C and 0.05 µg/mL colchicine for 24-hour exposure without S9-mix.
[C]: Total number of cells with micronuclei is the sum of two duplicates (counts per duplicate indicated between brackets)

Micronuclei are detected within the *in vitro* mammalian cell micronucleus test and result from chromosomal damage, either chromosomal fragments or whole chromosomes unable to migrate to the poles during the anaphase stage of cell division [14]. The assay can thereby detect both structural aberrations (clastogenicity) and numerical aberrations (aneuploidy) in chromosomes [9]. The positive controls used in the experiments induced an increase in either the number of mono- or binucleated cells with micronuclei compared to the solvent control, indicating adequate testing conditions. It was decided beforehand, in line with OECD guidelines, that the test material was positive for clastogenicity or aneuginicity if the number of micronuclei differed significantly from the negative control, there was a significant dose-response trend, and the values were outside the 95% control limits of historical control data ranges. Only in the second experiment, using 24-hour exposure to Fermotein, two of these conditions were met, but the values were still within the distribution of historical negative control data. Precipitation of the test material in the culture medium may lead to artefactual positive results with staining or scoring [14] and since precipitation occurred at the highest concentration tested, this may have contributed to the increase in micronuclei. When additional scoring was applied, there was no statistically significant dose-related increase.
Therefore, it can be concluded that Fermotein does not have clastogenic or aneugenic effects in human lymphocytes. No other studies investigating the effects of foods produced with *Rhizomucor pusillus* on structural or numerical chromosomal aberrations were identified.

4. CONCLUSIONS

Fermotein, a mycoprotein product obtained from fermentation with the filamentous fungus *Rhizomucor pusillus*, did not exert genotoxic effects in the bacterial reverse mutation tests or *in vitro* mammalian cell micronucleus test. The test item did not induce gene mutations or structural and numerical chromosomal aberrations, which are considered main genotoxic endpoints and are implicated in carcinogenesis and heritable diseases. No further in vivo genotoxicity testing is therefore deemed required. These results compliment previous compositional data on Fermotein, where no safety concerns regarding potential toxicity and secondary metabolites for Fermotein and *Rhizomucor pusillus* as a source were identified. Results from the current study contribute to the body of evidence for a novel food authorisation of Fermotein in the EU and a GRAS notification of Fermotein in the US.

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