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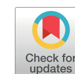
Mutagenicity and genotoxicity of ClearTaste



B.K. Soni*, J.P. Langan

MycoTechnology, Inc., 3155 N Chambers Rd, Suite E, Aurora, CO, 80011, United States

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ABSTRACT

The present study investigates whether ClearTaste is mutagenic/genotoxic by employing it as a test article in bacterial reverse mutation (Ames test) and in vitro human peripheral blood lymphocyte micronucleus assays conducted by a Good Laboratory Practice certified third party as parameterized by the United States Food and Drug Administration. ClearTaste is a taste modulator derived from the filtrate of submerged Cordyceps sinensis and is typically processed into a powder. It functions as a bitter, sour, astringency, metallic and lingering aftertaste mitigator/blocker. The Ames test includes revertant colony counts almost exclusively less than 100/plate and significantly fewer ClearTaste counts as opposed to known mutagen counts. The micronucleus assay reported cytotoxicity exclusively < 25% for doses up to 2,000 μg/L with Cytokinesis Block Proliferation Indices less than water and statistically significant differences between micronucelated cells post dosing compared to cyclophosphamide and vinblastine controls. The conclusion of these data is that ClearTaste is neither muta- nor carcinogenic.

1. Introduction

The commercialization of any novel ingredient/foodstuff is requisitely accompanied by safety tests. The present journal article discusses bacterial reverse mutation (Ames test) and *in vitro* human peripheral blood lymphocyte (HPBL) micronucleus assays utilizing ClearTaste, a novel taste modulating powder made through the culturing of *Cordyceps sinensis*, as a test article. ClearTaste was discovered at MycoTechnology, Inc. in July 2014.

Taste modulation has been the subject of much interest over the decades in part due to the discipline's important economic implications in driving consumer preference. While the perception and modulation of all five conventional tastes have been intensely investigated and better understood over the last 2–3 decades, food science has taken particularly extensive measures to identify novel bitter blockers, an effort perhaps only matched by the investigation of sweetness intensifiers [1–8]. ClearTaste is unique as a bitter blocker being that it is derived through the culturing of a fungus. When used at proper concentrations (typically < 50 ppm) ClearTaste can also mitigate sour, metallic and lingering off tastes. ClearTaste's functionality makes it highly alluring to the food and flavor industry, heightening the pertinence of this journal article.

The purpose of reverse mutation and micronucleus assays are, respectively, to investigate the extent to which a test article is mutagenic or genotoxic/induces chromosome instability. Reverse mutation assays analyze frameshift and basepair substitution mutations in *Salmonella typherium* and *Eschericia coli*. Micronucleus assays monitor the extent that micronuclei, small cytoplasmic membrane bodies carrying pieces of or an entire chromosome due to a malfunctioning anaphase, form when exposed to a test article. Known mutagens and micronuclei inducers are used as control articles in each test, respectively. These tests determine an important aspect of food safety and are essential to informing potential consumers about the nature of novel food. Some physicochemical properties and the proximate analysis of ClearTaste are shown in Tables 1 and 2.

2. Materials and methods

2.1. Statement of GLP validation

The bacterial reverse mutation and *in vitro* HBPL micronucleus assays were conducted by a third party according to Good Laboratory Practice as parameterized by the United States Food and Drug Administration. Detailed methods for the execution of these procedures and be found in the List of References, with certain references discussing the bacterial reverse mutation assay [9–11] and others discussing the micronucleus assay [12–14].

2.2. Bacterial reverse mutation assay

2.2.1. Test system

The tester strains used were the *Salmonella typhimurium* histidine

* Corresponding author.
E-mail addresses: bsoni@mycotechcorp.com (B.K. Soni), jim@mycotechcorp.com (J.P. Langan).

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Table 1
Physicochemical Properties of ClearTaste.

Solubility	~99.5% soluble at up to 6% ClearTaste m/v
Density	0.5 g/L
pH[a]	4.3
Melting Point	193–205 °C
Ignitability	Not ignitable

[a] Done according to EPA method SW9045C.

Table 2
ClearTaste Proximate Composition.

Property	Concentration (%)
Moisture (vacuum oven)	1.8
Protein	1.3
Fat (acid hydrolysis)	0.7
Ash	2.6
Carbohydrates (by difference)	93.6

All values not done by difference conducted according to AOAC methods at Certified Labs, Inc.

auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames et al. [9] and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel [10].

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations rather than frameshift mutations. *Salmonella* tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. Historical data for the test system is provided in Table 3. Historical data are more important in micronucleus assays for determining outcomes of the assay but are included herein for the Ames assay for those interested.

2.2.2. Preparation of tester strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel containing 30–50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125–175 rpm and incubating at 37 ± 2 °C for approximately 12 h before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells/mL. The actual titers were determined by viable count assays on agar plates.

Table 4
Historical Negative and Positive Control Values for Non-S9 Activated Micronucleus Assay, 2013–2015.

Micronucleated Binucleated Cells (%)	Negative Control		Positive Control[a]	
	4 h	24 h	4 h	24 h
Mean	0.36	0.39	3.77	1.76
Standard Deviation	0.23	0.31	1.66	0.86
95% Control Limits	0.00–0.82	0.00–1.01	0.46–7.08	0.04–3.48
Range[b]	0.05–1.43	0.10–2.00	1.00–10.10	0.50–5.70

[a] Positive control for non-activated 4 h studies is Mitomycin C, Positive control for activated 24 hour study is Vinblastine.
[b] Range is from minimum to maximum.

Table 5
Historical Negative and Positive Control Values for S9 Activated Micronucleus Assay, 2013–2015.

	Micronucleated Binucleated Cells (%)	
	Negative Control	Positive Control[a]
Mean	0.33	1.51
Standard Deviation	0.23	0.50
95% Control Limits	0.00–0.78	0.50–2.51
Range[b]	0.10–1.50	0.40–3.30

[a] Positive control for S9 activated studies is cyclophosphamide.
[b] Range is from minimum to maximum.

Table 6
Reverse Mutation Assay Tester Strain Titer Results.

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 uvrA
	Titer Value (x10^{9} cells/mL)				
Mutagenicity Assay	11.5	11.1	8.7	11.2	12.4
Confirmatory Mutagenicity Assay	3.0	4.0	2.4	6.5	2.6

2.2.3. Exogenous metabolic activation

Aroclor™ 1254-induced rat liver S9 was used as the metabolic

Table 3
Historical Negative and Positive Control Values for Reverse Mutation Assay, 2014.

Strain	Control	Activation: None					Rat Liver				
		Mean	SD	Min	Max	95% CL[a]	Mean	SD	Min	Max	95% CL*
TA98	Neg	16	5	5	42	6–26	24	7	5	53	10–38
	Pos	232	258	57	2691		400	165	109	1382	
TA100	Neg	94	14	66	152	66–122	102	18	63	164	66–138
	Pos	681	176	213	1767		681	259	186	2793	
TA1535	Neg	11	4	2	31	3–19	13	5	2	36	3–23
	Pos	586	226	16	2509		117	99	23	1060	
TA1537	Neg	7	3	1	19	1–13	9	4	1	23	1–17
	Pos	411	355	32	2921		72	52	10	562	
WP2 uvrA	Neg	25	7	7	62	11–39	28	8	10	55	12–44
	Pos	376	123	99	1026		302	102	91	687	

[a] 95% CL = mean ± 2 SD (but not less than zero).

Table 7
Preliminary Toxicity Assay without S9 Activation.[a]

Strain	Article	Dose (µg/ plate)	Revertants (mean/ plate)	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	22	1.4	22^A 1 NP
		3333	9	0.6	9^A 1 NP
		1000	13	0.9	13^A
		667	11	0.7	11^A
		333	13	0.8	13^A
		100	27	1.7	27^A
		66.7	10	0.6	10^A
		33.3	19	1.2	19^A
		10.0	10	0.6	10^A
		6.67	14	0.9	14^A
	Water	100	16		16^A
TA100	ClearTaste	5000	113	1.4	113^A 1 NP
		3333	80	1.0	80^A 1 NP
		1000	89	1.1	89^A
		667	93	1.1	93^A
		333	82	1.0	82^A
		100	104	1.3	104^A
		66.7	99	1.2	99^A
		33.3	97	1.2	97^A
		10.0	88	1.1	88^A
		6.67	73	0.9	73^A
	Water	100	83		83^A
TA1535	ClearTaste	5000	13	0.9	13^A 1 NP
		3333	11	0.8	11^A 1 NP
		1000	10	0.7	10^A
		667	11	0.8	11^A
		333	17	1.2	17^A
		100	17	1.2	17^A
		66.7	11	0.8	11^A
		33.3	11	0.8	11^A
		10.0	8	0.6	8^A
		6.67	9	0.6	9^A
	Water	100	14		14^A
TA1537	ClearTaste	5000	6	1.0	6^A 1 NP
		3333	1	0.2	1^A 1 NP
		1000	5	0.8	5^A
		667	3	0.5	3^A
		333	6	1.0	6^A
		100	6	1.0	6^A
		66.7	8	1.3	8^A
		33.3	8	1.3	8^A
		10.0	7	1.2	7^A
		6.67	8	1.3	8^A
	Water	100	6		6^A
WP2uvrA	ClearTaste	5000	27	1.1	27^A 1 NP
		3333	24	1.0	24^A 1 NP
		1000	21	0.9	21^A
		667	22	0.9	22^A
		333	22	0.9	22^A
		100	26	1.1	26^A
		66.7	19	0.8	19^A
		33.3	16	0.7	16^A
		10.0	14	0.6	14^A
		6.67	11	0.5	11^A
	Water	100	24		24^A

The numerical marking ‘1’ indicates normal background.
The abbreviation ‘NP’ indicates non-interfering particulate.
[a] The superscript marking ‘A’ indicates an automatic count.

Table 8
Preliminary Toxicity Assay with S9 Activation.[a]

Strain	Article	Dose (µg/ plate)	Revertants (mean/ plate)	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	16	0.6	16^A 1 NP
		3333	30	1.1	30^A 1 NP
		1000	18	0.7	18^A
		667	34	1.3	34^A
		333	19	0.7	19^A
		100	31	1.1	31^A
		66.7	22	0.8	22^A
		33.3	23	0.9	23^A
		10.0	24	0.9	24^A
		6.67	25	0.9	25^A
	Water	100	27		27^A
TA100	ClearTaste	5000	76	1.0	76^A 1 NP
		3333	88	1.1	88^A 1 NP
		1000	72	0.9	72^A
		667	74	0.9	74^A
		333	95	1.2	95^A
		100	71	0.9	71^A
		66.7	105	1.3	105^A
		33.3	80	1.0	80^A
		10.0	95	1.2	95^A
		6.67	82	1.0	82^A
	Water	100	80		80^A
TA1535	ClearTaste	5000	11	0.8	11^A 1 NP
		3333	7	0.5	7^A 1 NP
		1000	13	0.9	13^A
		667	8	0.6	8^A
		333	7	0.5	7^A
		100	17	1.2	17^A
		66.7	14	1.0	14^A
		33.3	18	1.3	18^A
		10.0	15	1.1	15^A
		6.67	15	1.1	15^A
	Water	100	14		14^A
TA1537	ClearTaste	5000	3	0.4	3^A 1 NP
		3333	7	1.0	7^A 1 NP
		1000	8	1.1	8^A
		667	7	1.0	7^A
		333	9	1.3	9^A
		100	6	0.9	6^A
		66.7	6	0.9	6^A
		33.3	9	1.3	9^A
		10.0	2	0.3	2^A
		6.67	13	1.9	13^A
	Water	100	7		7^A
WP2uvrA	ClearTaste	5000	21	1.2	21^A 1 NP
		3333	25	1.4	25^A 1 NP
		1000	21	1.2	21^A
		667	19	1.1	19^A
		333	16	0.9	16^A
		100	21	1.2	21^A
		66.7	24	1.3	24^A
		33.3	15	0.8	15^A
		10.0	22	1.2	22^A
		6.67	26	1.4	26^A
	Water	100	18		18^A

The numerical marking ‘1’ indicates normal background.
The abbreviation ‘NP’ indicates non-interfering particulate.
[a] The superscript marking ‘A’ indicates an automatic count.

activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3586, Exp. Date: 09 February 2018) was purchased commercially from MolTox (Boone, NC). Upon receipt the S9 was stored at −60 °C or colder until use. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to Salmonella typhimurium TA100. The S9 mix was prepared on the day of use with 4 mM β-nicotinamide-adenine dinucleotide phosphate, 5 mM glucose-6-phosphate, 33 mM potassium chloride, 8 mM magnesium chloride, 100 mM pH 7.4 phosphate buffer and 10% (v/v) S9 homogenate. The Sham mix, containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

2.2.4. Frequency and route of administration

The test system was exposed to ClearTaste via the plate incorporation methodology originally described by Ames et al. [9] and updated by Maron and Ames [11]. Water was the vehicle of choice. ClearTaste

Table 9
Mutagenicity Assay without S9 Activation.[a]

Strain	Article	Dose (µg/ plate)	Revertants (mean/ plate)	Standard Deviation	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	14	3	1.2	15[A] 1 NP, 17[A] 1 NP, 11[A] 1 NP
		1500	12	3	1.0	9[A], 14[A], 14[A]
		500	10	3	0.8	7[A], 9[A], 13[A]
		150	9	1	0.8	9[A], 10[A], 8[A]
		50	13	3	1.1	14[A], 10[A], 16[A]
	Water	100	12	1		11[A], 13[A], 11[A]
TA1535	ClearTaste	5000	9	2	1.0	9[A] 1 NP, 7[A] 1 NP
		1500	11	4	1.2	11[A], 7[A], 14[A]
		500	10	6	1.1	3[A], 15[A], 13[A]
		150	12	2	1.3	14[A], 10[A], 13[A]
		50	9	1	1.0	9[A], 8[A], 10[A]
	Water	100	9	2		9[A], 8[A], 10[A]
TA1537	ClearTaste	5000	6	1	1.2	6[A] 1 NP, 5[A] 1 NP, 7[A] 1 NP
		1500	4	1	0.8	5[A], 3[A], 3[A]
		500	6	1	1.2	6[A], 6[A], 6[A]
		50	6	2	1.2	8[A], 5[A], 6[A]
	Water	100	5	3		3[A], 8[A], 3[A]
WP2uvrA	ClearTaste	5000	18	6	0.9	15[A] 1 NP, 25[A] 1 NP, 15A 1 NP
		1500	22	10	1.0	11[A], 24[A], 30[A]
		500	28	9	1.3	18[A], 33[A], 34[A]
		150	19	4	0.9	22[A], 15[A], 19[A]
		50	22	2	1.0	23[A], 23[A], 19[A]
	Water	100	21	4		17[A], 21[A], 24[A]
TA98	2NF	1	111	24	9.3	84[A], 128[A], 121[A]
TA100	SA	1	555	31	6.6	524[A], 556[A], 586[A]
TA1535	SA	1	435	24	48.3	462[A], 415[A], 428[A]
TA1537	9AAD	75	388	75	77.6	382[A], 317[A], 466[A]
WP2uvrA	MMS	1000	296	82	14.1	209[A], 308[A], 372[A]

The numerical marking '1' indicates normal background.
The abbreviation 'NP' indicates non-interfering particulate.
2NF is 2-nitrofluorene.
SA is sodium azide.
9AAD is 9-aminoacridine.
MMS is methyl methanesulfonate.
[a] The superscript marking 'A' indicates an automatic count.

formed workable suspensions in water at concentrations of approximately 1–50 mg/mL with sonication at 37 °C for 70 min.

2.2.5. Preliminary toxicity assay

The preliminary toxicity assay was used to establish the dose-range over which ClearTaste would be assayed. TA98, TA100, TA1535, TA1537 (*Salmonella typherium*) and WP2 *uvr*A (*Escherichia coli*) were exposed to the vehicle alone and ten dose levels of ClearTaste, with a single plate/condition, on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the mutagenicity assay were based upon the absence of post-treatment toxicity.

2.2.6. Mutagenicity assay

TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to water alone, the positive controls 2-nitrofluorene, sodium azide, 9-aminoacridine, methyl methanesulfonate and five dose levels of ClearTaste, in triplicate, in the absence of Aroclor-induced rat liver S9 and in its presence was identically treated but for the control only having been 2-aminoanthracene.

2.2.7. Confirmatory mutagenicity assay

TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to water alone, positive controls 2-nitrofluorene, sodium azide, 9-aminoacridine, methyl methanesulfonate and 2-aminoanthracene and five dose levels of ClearTaste, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

2.2.8. Treatment of test system

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of ClearTaste and the water, all ClearTaste dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or ClearTaste dilution were added to 2 mL of Petri plates with 0.8% m/v BBL select agar, 0.5% m/w sodium chloride, 50 mM each of L-histidine, D-biotin and L-tryptophan at 45 ± 2 °C. When plating the positive controls, the ClearTaste aliquot was replaced by a 50 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar containing 0.8% m/v BBL select agar and 1.5% mv Vogel-Bonner minimal medium E. After the overlay had solidified, the plates were inverted and incubated for 48–72 h at 37 ± 2 °C. Plates that were not counted immediately following the incubation period were stored at 2–8 °C until colony counting could be conducted.

2.2.9. Criteria for determination of a valid test

The following criteria must be met for the mutagenicity and confirmatory mutagenicity assays to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the

Table 10
Mutagenicity Assay with S9 Activation.

Strain	Article	Dose (µg/ plate)	Revertants (mean/ plate)	Standard Deviation	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	24	2	1.1	23^{A} 1 NP, 22^{A} 1 NP, 26^{A} 1 NP
		1500	20	1	1.0	21^{A}, 19^{A}, 21^{A}
		500	18	2	0.9	19^{A}, 15^{A}, 19^{A}
		150	20	4	1.0	15^{A}, 22^{A}, 23^{A}
		50	19	3	0.9	16^{A}, 21^{A}, 19^{A}
	Water	100	21	2		19^{A}, 21^{A}, 23^{A}
TA100	ClearTaste	5000	91	4	1.0	95^{A} 1 NP, 89^{A} 1 NP, 88^{A} 1 NP
		1500	99	13	1.1	104^{A}, 100^{A}, 100^{A}
		500	7	2	0.8	96^{A}, 100^{A}, 105^{A}
		150	8	2	0.9	71^{A}, 101^{A}, 82^{A}
	Water	100	9	1		88^{A}, 86^{A}, 89^{A}
TA1535	ClearTaste	5000	8	3	0.9	10^{A} 1 NP, 5^{A} 1 NP, 9^{A} 1 NP
		1500	9	6	1.0	3^{A}, 8^{A}, 15^{A}
		500	7	2	0.8	6^{A}, 6^{A}, 9^{A}
		150	8	2	0.9	6^{A}, 9^{A}, 8^{A}
		50	9	2	1.0	7^{A}, 9^{A}, 10^{A}
	Water	100	9	1		9^{A}, 8^{A}, 9^{A}
TA1537	ClearTaste	5000	11	6	0.9	17^{A} 1 NP, 6^{A} 1 NP, 11^{A} 1 NP
		1500	13	4	1.1	15^{A}, 16^{A}, 8^{A}
		500	11	3	0.9	15^{A}, 10^{A}, 9^{A}
		150	14	1	1.2	14^{A}, 15^{A}, 13^{A}
		50	11	4	0.9	14^{A}, 6^{A}, 13^{A}
	Water	100	12	4		10^{A}, 9^{A}, 16^{A}
WP2uvrA	ClearTaste	5000	24	12	1.3	22^{A} 1 NP, 13^{A} 1 NP
		1500	20	4	1.1	19^{A}, 17^{A}, 24^{A}
		500	20	5	1.1	16^{A}, 19^{A}, 26^{A}
		150	25	2	1.3	25^{A}, 23^{A}, 27^{A}
		50	28	6	1.5	23^{A}, 26^{A}, 35^{A}
	Water	100	19	4		21^{A}, 15^{A}, 22^{A}
TA98	2AA	1	239	42	11.4	277^{A}, 246^{A}, 194^{A}
TA100	2AA	2	313	22	3.6	293^{A}, 310^{A}, 336^{A}
TA1535	2AA	1	81	10	9.0	75^{A}, 93^{A}, 75^{A}
TA1537	2AA	2	40	11	3.3	36^{A}, 31^{A}, 52^{A}
WP2uvrA	2AA	15	304	109	16.0	206^{A}, 286^{A}, 421^{A}

*The superscript marking 'A' indicates an automatic count.
The numerical marking '1' shown after the automatic count indicates normal background.
The abbreviation 'NP' indicates non-interfering particulate.
2AA is 2-aminoanthracene.

presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10–50; TA100, 80–240; TA1535, 5–45; TA1537, 3–21; WP2 *uvr*A, 10–60.

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 0.3×10^9 cells/mL.

The mean of each positive control must exhibit at least a 3 fold increase in the number of revertants over the mean value of the respective vehicle control.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A $>$ 50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

2.2.10. Evaluation of test results

For ClearTaste to be mutagenic it must cause a dose-related increase in the mean revertants/plate of at least one tester strain over a minimum of two increasing concentrations of ClearTaste.

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2 times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

2.3. In vitro human peripheral blood lymphocyte micronucleus assay

2.3.1. Characterization of test and control articles

The vehicle used to deliver ClearTaste to the test system was water supplied by Gibco, CAS # 7732-18-5. Dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light. Controls besides water were cyclophosphamide and vinblastine.

Vinblastine was dissolved in sterile distilled water to stock concentration of 0.0005, 0.00075, and 0.001 mg/mL (final concentrations of 5, 7.5, and 10 ng/mL, respectively) as the positive control in the non-activated test system. Cyclophosphamide was dissolved and diluted in sterile distilled water to stock concentrations of 0.25, 0.5 and 0.75 mg/mL (final concentrations of 2.5, 5 and 7.5 µg/mL, respectively) for use

Table 11
Confirmatory Mutagenicity Assay without S9 Activation.[a]

Strain	Article	Dose (µg/ plate)	Revertants (mean/ plate)	Standard Deviation	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	17	1	1.2	17^A 1 NP, 17^A 1 NP, 18^A 1 NP
		1500	10	5	0.7	7^A, 7^A, 16^A
		500	10	3	0.7	7^A, 10^A, 13^A
		150	12	2	0.9	13^A, 10^A, 13^A
		50	12	5	0.9	8^A, 11^A, 18^A
	Water	100	14	4		16^A, 16^A, 9^A
TA100	ClearTaste	5000	88	2	1.0	90^A 1 NP, 87^A 1 NP, 87^A 1 NP
		1500	94	16	1.0	113^A, 79^A, 86^A
		500	104	13	1.1	98^A, 75^A, 80^A
		150	97	8	1.0	83^A, 95^A, 92^A
		50	98	16	1.0	95^A, 80^A, 96^A
	Water	100	84	9		
TA1535	ClearTaste	5000	10	4	0.8	6^A 1 NP, 9^A 1 NP, 14^A 1 NP
		1500	9	4	0.7	7^A, 6^A, 14^A
		500	13	4	1.0	10^A, 17^A, 11^A
		150	18	4	1.5	14^A, 10^A, 16^A
		50	13	3	1.0	15^A, 15^A, 14^A
	Water	100	13	4		9^A, 14^A, 17^A
TA1537	ClearTaste	5000	10	1	1.1	10^A 1 NP, 10^A 1 NP, 9^A 1 NP
		1500	10	3	1.1	10^A, 13^A, 7^A
		500	9	1	1.0	8^A, 10^A, 8^A
		150	7	2	0.8	5^A, 7^A, 8^A
		50	9	2	1.0	7^A, 11^A, 8^A
	Water	100	9	5		14^A, 5^A, 8^A
WP2uvrA	ClearTaste	5000	36	8	1.3	27^A 1 NP, 43^A 1 NP, 38^A 1 NP
		1500	31	12	1.1	21^A, 29^A, 44^A
		500	32	6	1.1	38^A, 26^A, 31^A
		150	33	5	1.2	29^A, 39^A, 32^A
		50	31	6	1.1	25^A, 34^A, 35^A
	Water	100	9	5		14^A, 5^A, 8^A
TA98	Water	100	28	6		30^A, 21^A, 32^A
	2NF	1	106	36	7.6	70^A, 141^A, 106^A
TA100	SA	1	715	77	8.5	779^A, 736^A, 630^A
TA1535	SA	1	615	56	47.3	593^A, 678^A, 573^A
TA1537	9AAD	75	348	53	38.7	384^A, 288^A, 373^A
WP2uvrA	MMS	1000	405	99	14.5	294^A, 439^A, 483^A

The numerical marking '1′ indicates normal background.
The abbreviation 'NP' indicates non-interfering particulate.
2NF is 2-nitrofluorene.
SA is sodium azide.
9AAD is 9-aminoacridine.
MMS is methyl methanesulfonate.
[a] The superscript marking 'A' indicates an automatic count.

as the positive control article in the S9-activated test system. Since the non-activated and S9-activated treatments were tested concurrently, the positive control for the non-activated 4 h exposure groups was eliminated. For each positive control article, one dose level exhibiting a sufficient number of scorable cells was selected for analysis. The vehicle for ClearTaste was used as the vehicle control for each treatment group.

Cytochalasin B was dissolved in DMSO to a stock concentration of 2 mg/mL. It was used at 6 µg/mL concentration to block cytokinesis.

2.3.2. Test system

HPBLs were obtained from healthy, non-smoking individuals. For the preliminary toxicity work a 22 year old female had HPBLs collected on April 4th, 2016. For the micronucleus assay a 29 year old female donated HPBLs on April 19th, 2016.

The donors had no recent history of radiotherapy, viral infection or the administration of drugs. This system has been demonstrated to be sensitive to the genotoxicity test for detection of micronuclei of a variety of chemicals according to Clare et al. [12].

2.3.3. Preparation of target cells

HPBLs were cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin, 100 µg/mL streptomycin) by adding 0.5 mL heparinized blood to a centrifuge tube containing 5 mL of complete medium with 2% phytohemagglutinin. The cultures were incubated under standard conditions (37 ± 1 °C in a humidified atmosphere of 5 ± 1% CO_2 in air) for 44–48 h.

2.3.4. Exogenous metabolic activation system

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3563, Exp. Date: 15 Dec 2017) was purchased commercially from MolTox (Boone, NC). Upon receipt the S9 was stored at −60 °C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Table 12
Confirmatory Mutagenicity Assay with S9 Activation.

Strain	Article	Dose (μg/ plate)	Revertants (mean/ plate)	Standard Deviation	Revertant Ratio (dose/ control)	Individual Revertant Colony Counts and Background Codes
TA98	ClearTaste	5000	15	3	1.2	19^{A} 1 NP, 13^{A} 1 NP, 14^{A} 1 NP
		1500	10	4	0.8	14^{A}, 6^{A}, 11^{A}
		500	10	3	0.7	18^{A}, 15^{A}, 13^{A}
		150	12	2	0.9	14^{A}, 16^{A}, 23^{A}
		50	12	5	0.9	16^{A}, 17^{A}, 14^{A}
	Water	100	13	2		14^{A}, 11^{A}, 15^{A}
TA100	ClearTaste	5000	109	19	1.1	131^{A} 1 NP, 97^{A} 1 NP, 99^{A} 1 NP
		1500	94	16	1.0	113^{A}, 79^{A}, 86^{A}
		500	104	13	1.1	98^{A}, 75^{A}, 80^{A}
		150	97	8	1.0	83^{A}, 95^{A}, 92^{A}
		50	98	16	1.0	95^{A}, 80^{A}, 96^{A}
	Water	100	95	5		98^{A}, 89^{A}, 98^{A}
TA1535	ClearTaste	5000	16	3	0.8	19^{A} 1 NP, 16^{A} 1 NP
		1500	9	2	0.9	11^{A}, 13^{A}, 10^{A}
		500	14	4	1.2	16^{A}, 9^{A}, 17^{A}
		150	18	4	1.5	14^{A}, 22^{A}, 19^{A}
		50	9	2	0.8	10^{A}, 11^{A}, 7^{A}
	Water	100	12	4		16^{A}, 9^{A}, 11^{A}
TA1537	ClearTaste	5000	6	3	0.5	2^{A} 1 NP, 8^{A} 1 NP, 8^{A} 1 NP
		1500	9	2	0.8	11^{A}, 8^{A}, 8^{A}
		500	9	5	0.8	5^{A}, 15^{A}, 8^{A}
		150	11	3	0.9	10^{A}, 15^{A}, 9^{A}
		50	13	7	1.1	10^{A}, 9^{A}, 21^{A}
	Water	100	12	5		17^{A}, 11^{A}, 7^{A}
WP2uvrA	ClearTaste	5000	16	2	1.0	18^{A} 1 NP, 15^{A} 1 NP, 15^{A} 1 NP
		1500	19	3	1.2	16^{A}, 21^{A}, 19^{A}
		500	19	6	1.2	23^{A}, 22^{A}, 13^{A}
		150	16	4	1.0	19^{A}, 11^{A}, 17^{A}
		50	17	3	1.1	18^{A}, 19^{A}, 13^{A}
	Water	100	16	3		13^{A}, 17^{A}, 18^{A}
TA98	2AA	1	472	375	36.3	214^{A}, 300^{A}, 902^{A}
TA100	2AA	2	498	77	5.2	553^{A}, 556^{A}, 386^{A}
TA1535	2AA	1	89	22	7.4	106^{A}, 97^{A}, 65^{A}
TA1537	2AA	2	51	14	4.3	66^{A}, 39^{A}, 47^{A}
WP2uvrA	MMS	1000	405	99	14.5	294^{A}, 439^{A}, 483^{A}

*The superscript marking ‘A’ indicates an automatic count.
The numerical marking ‘1’ indicates normal background.
The abbreviation ‘NP’ indicates non-interfering particulate.
2NF is 2-nitrofluorene.
SA is sodium azide.
9AAD is 9-aminoacridine.
MMS is methyl methanesulfonate.

The S9 mix was prepared on the day of use and contained 1 mM β-nicotinamide-adenine dinucleotide phosphate, 1 mM glucose-6-phosphate, 6 mM potassium chloride, 2 mM magnesium chloride and 20 μL/mL S9 homogenate.

2.3.5. Preliminary cytotoxicity test

HPBLs were exposed to water alone and nine dose levels of ClearTaste with half-log dose spacing using single cultures. Precipitation of test article dosing solution in the treatment medium was determined using the unaided eye at the beginning and conclusion of treatment. Dose levels for the micronucleus assay were based upon visible precipitate in the treatment medium at the conclusion of the treatment period. In treatment groups with lack of cytotoxicity or visible precipitate in the treatment medium, the highest dose tested was 2000 μg/mL.

2.3.6. Micronucleus assay

Based on the results of the preliminary toxicity test, the doses selected for testing in the micronucleus assay were 100, 250, 500, 1000 and 2000 μg/mL in a non-activated treatment condition for 4 and 24 h (with 4 and 0 h recovery times, respectively) in the presence of Aroclor-induced rat liver S9 for 4 h with 20 h recovery time.

Precipitation of the test article dosing solution in the treatment medium was determined using the unaided eye at the beginning and conclusion of treatment. The highest dose evaluated for the micronuclei was selected based on visible precipitate at the end of the treatment period in the 4 h (-S9) and 4 h (+S9) treatments and by the highest dose tested in the micronucleus assay (2000 μg/mL) in the 24 h (-S9) treatment. Two additional doses were included in the evaluation of micronuclei.

2.3.7. Treatment of target cells (Preliminary toxicity test and micronucleus assay)

After the 4 h treatment in the non-activated and the S9-activated studies, the cells were centrifuged, the treatment medium was aspirated, washed with calcium and magnesium free phosphate buffered saline (CMF-PBS), re-fed with complete medium containing cytochalasin B at 6.0 μg/mL and returned to the incubator under standard conditions. For the 24 h treatment in the non-activated study, cytochalasin B (6.0 μg/mL) was added at the beginning of the treatment.

Table 13
Preliminary Cytotoxicity Assay Using ClearTaste in the Absence of Exogenous Metabolic Activation, 4 h Treatment and 24 h Harvest.

Test Article	Treatment Condition (μg/mL)	Total # Cells Counted	Count/Total Cells # Nuclei/Cell (1	2	> 2)	CBPI[a]	Cytotoxicity[a] (%)
Water		500	125	335	40	1.830	
ClearTaste	0.2	500	165	320	15	1.700	16
	0.6	500	150	308	42	1.784	6
	2	500	181	273	46	1.730	12
	6	500	207	272	21	1.628	24
	20	500	248	240	12	1.528	36
	60	500	218	245	37	1.638	23
	200	500	247	234	19	1.544	34
	600	500	267	215	18	1.502	40
	2000[b]	495	223	245	27	1.604	27

[a]CBPI (Cell Block Proliferation Index) and cytotoxicity are calculated by the following equations:

$$CBPI = \frac{(1)(mononucleatedcells) + (2)(binucleatedcells) + (3)(multinucleatedcells)}{Totalcellsscored}.$$

$$Cytotoxicity = 100 - 100\left(\frac{CBPI_{water} - 1}{CBPI_{ClearTaste} - 1}\right).$$

[b]Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.

Table 14
Preliminary Cytotoxicity Assay Using ClearTaste in the Presence of Exogenous Metabolic Activation, 4 h Treatment and 24 h Harvest.

Test Article	Treatment Condition (μg/mL)	Total # Cells Counted	Count/Total Cells # Nuclei/ Cell (1	2	> 2)	CBPI[a]	Cytotoxicity[a] (%)
Water		500	195	293	12	1.634	
ClearTaste	0.2	500	221	260	19	1.596	6
	0.6	500	195	290	15	1.640	−1
	2	500	200	280	20	1.640	−1
	6	500	226	264	10	1.568	10
	20	500	200	283	17	1.634	0
	60	500	238	250	12	1.548	14
	200	500	220	270	10	1.580	9
	600	500	202	280	18	1.632	0
	2000[b]	500	237	255	8	1.542	15

[a] See Table 7 for CPBI and cytotoxicity equations.
[b] Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.

Table 15
Preliminary Cytotoxicity Assay Using ClearTaste in the Absence of Exogenous Metabolic Activation, 24 h Treatment and 24 h Harvest.

Test Article	Treatment Condition (μg/mL)	Total # Cells Counted	Count/Total Cells # Nuclei/ Cell (1	2	> 2)	CBPI[a]	Cytotoxicity[a] (%)
Water		500	150	270	80	1.860	
ClearTaste	0.2	500	145	258	97	1.904	−5
	0.6	500	178	248	74	1.792	8
	2	500	193	235	72	1.758	12
	6	500	198	218	84	1.772	10
	20	500	188	223	89	1.802	7
	60	500	212	217	71	1.718	17
	200	500	203	240	57	1.708	18
	600	500	217	240	43	1.652	24
	2000[b]	500	238	213	49	1.622	28

[a] See Table 7 for CPBI and cytotoxicity equations.
[b] Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.

2.3.8. Collection of cells (Preliminary toxicity test and micronucleus assay)

Cells were collected after being exposed to cytochalasin B for 24 h (± 30 min), 1.5–2 normal cell cycles, to ensure identification and selective analysis of micronucleus frequency in cells that have completed one mitosis evidenced by binucleated cells as according to Fenech and Morley [13]. The cytochalasin B exposure time for the 4 h treatment in the non-activated and the S9-activated studies was 20 h (± 30 min).

Cells were collected by centrifugation, swollen with 0.075 M KCl, washed with fixative (methanol: glacial acetic acid, 25:1 v/v), capped and may be stored overnight or longer at 2–8 °C. To prepare slides, the cells were collected by centrifugation and the cells were resuspended in fresh fixative. The suspension of fixed cells was applied to glass microscope slides and air-dried.

2.3.9. Statistical analysis

Statistical analysis was performed using the Fisher's exact test ($p \leq 0.05$) for a pairwise comparison of the percentage of micronucleated cells in each treatment group with that of the vehicle control. The Cochran-Armitage trend test was used to assess dose-responsiveness.

2.3.10. Criteria for determination of a valid test

2.3.10.1. Vehicle controls. The frequency of cells with micronuclei should ideally be within the 95% control limits of the distribution of the historical negative control database, taken in 2014 and shown in Table 3.If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error). Historical data for non-S9 activated and S9 activated systems are shown in Tables 4 and 5.

2.3.10.2. Positive controls. The percentage of micronucleated cells must be significantly greater than the concurrent vehicle control ($p \leq 0.05$). In addition, the cytotoxicity response must not exceed the upper limit for the assay (55%). According to the methods of its calculation as shown in Table 7, cytotoxicity is considered substantial at 55 ± 5%, any test article yielding lower values being considered non-cytotoxic [13].

2.3.10.3. Cell proliferation. The CBPI of the vehicle control at harvest must be ≥1.4.

2.3.11. Evaluation of test results

The test article was considered to have induced a positive response if at least one of the test concentrations exhibited a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), the increase was concentration-related ($p \leq 0.05$) and results were outside the 95% control limit of the historical negative control data.

Table 16
Micronucleus Analysis of HPBLs Treated with ClearTaste in the Absence of Exogenous Metabolic Activation, Definite Assay: 4 h Treatment and 24 h Harvest.

Test Article	Treatment Conditions (μg/mL)	Replicate Culture Identifier	Total # of Cells/ Culture (%)	Micronucleated Binucleated Cells/ Culture (%)	Micronucleated Binucleated Cells/Dose (average%)
Water		A	1000	0.3	0.3
		B	1000	0.3	
ClearTaste	250	A	1000	0.3	0.4
		B	1000	0.4	
	500	A	1000	0.2	0.3
		B	1000	0.3	
	1000[a]	A	1000	0.3	0.3
		B	1000	0.3	

[a] Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.

Table 17
Micronucleus Analysis of HPBLs Treated with ClearTaste in the Presence of Exogenous Metabolic Activation, Definitive Assay: 4 h Treatment and 24 h Harvest.

Test Article	Treatment Conditions (μg/mL)	Replicate Culture Identifier	Total # of Cells/ Culture (%)	Micronucleated Binucleated Cells/ Culture (%)	Micronucleated Binucleated Cells/Dose (average%)
Water		A	1000	0.3	0.3
		B	1000	0.2	
ClearTaste	250	A	1000	0.3	0.3
		B	1000	0.3	
	500	A	1000	0.3	0.4
		B	1000	0.4	
	1000[a]	A	1000	0.4	0.4
		B	1000	0.3	
Cyclophosphamide	5	A	1000	1.3	1.7[b]
		B	1000	2.0	

[a] Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.
[b] $p \leq 0.01$, Fisher's exact test, relative to water.

Table 18
Micronucleus Analysis of HPBLs Treated with ClearTaste in the Absence of Exogenous Metabolic Activation, Definitive Assay: 24 h Treatment and 24 h Harvest.

Test Article	Treatment Conditions (μg/mL)	Replicate Culture Identifier	Total # of Cells/ Culture (%)	Micronucleated Binucleated Cells/ Culture (%)	Micronucleated BinucleatedCells/Dose (average%)
Water		A	1000	0.5	0.5
		B	1000	0.5	
ClearTaste	500	A	1000	0.4	0.3
		B	1000	0.2	
	1000	A	1000	0.2	0.3
		B	1000	0.3	
	2000	A	1000	0.2	0.3
		B	1000	0.3	
Vinblastine	7.5×10^{-3}	A	1000	1.1	1.6[a]
		B	1000	2.1	

[a] Visible precipitate was observed in the treatment medium at the conclusion of the treatment period.

ClearTaste was considered to have induced a clear negative response if none of the criteria for a positive response were met.

3. Results

3.1. Bacterial reverse mutation assay

3.1.1. Sterility and tester strain titer results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test article dilutions or the S9 and Sham mixes. Data for the tester strain titer results are shown in Table 6.

3.1.2. Preliminary toxicity assay

The results of the preliminary toxicity assays without and with S9 activation are presented in Tables 7 and 8, respectively. The tables show what ClearTaste and water concentrations were applied to each strain, the average revertant count/plate, the ratio of each ClearTaste dose to that of the water control and the background codes of each revertant count. The greatest ratio of any ClearTaste dose revertant counts to those of the water control was 1.7 for any strain in either table.

3.1.3. Mutagenicity assay

The results of the mutagenicity assays without and with S9 activation are presented in Tables 9 and 10, respectively. The tables show similar information to Tables 7 and 8 but for the last section which provides data for the mutagenic controls 2-nitrofluorene, sodium azide, 9-aminoacridine and methyl methanesulfonate in Table 5 and 2-aminoanthracene in Table 6. The greatest ratio of any ClearTaste dose revertant counts to those of the water control was 1.5. The lowest and greatest revertant ratios for any of the mutagenic controls were 10 (2-aminoanthracene) and 555 (sodium azide).

3.1.4. Confirmatory mutagenicity assay

The results of the confirmatory mutagenicity assay are presented in Tables 11 and 12 which show data structured identically to Tables

7–10. The greatest revertant ratio for any ClearTaste dose in either table was 1.5.

3.2. Micronucleus assay

3.2.1. Preliminary cytotoxicity test

Results from the preliminary cytotoxicity assay are presented in Tables 13–15. The results include mono-, bi- and trinucleated cell counts for various ClearTatse doses, Cytokinesis Block Proliferation Index (CBPI) and cytotoxicity data. The greatest cytotoxicity for any ClearTaste dose was 28%. Cyclophosphamide and vinblastine provide maximum cytotoxicity values of 59% and 71%, respectively. Doses having visible precipitate are indicated.

3.2.2. Micronucleus assay

Results from the micronucleus assay for individual exposure groups are shown in Tables 16–18. These tables show the average percent of micronucleated cells per dose under varying conditions of exogenous metabolic activation and treatment/harvest times. The data show ClearTaste's ability to induce micronuclei formation was not statistically significant though was for each positive control.

4. Discussion

The results of the bacterial reverse mutation assay indicate that under any of the conditions analyzed ClearTaste did not cause a positive mutagenic response. The results are clear on the matter based on the evaluation criteria. A deeper look at the data shows that ClearTaste does not broach mutagenicity under any experimental circumstance with any average revertant count developed from the data being much lower than the threshold required to confirm mutagenicity.

The results of the micronucleus assay indicate that ClearTaste does not induce micronuclei formation when exposed to HPBLs *in vitro* according to cytotoxicity and statistical comparisons of mononucleated cell development. It can be concluded that ClearTaste poses neither mutagenic nor genotoxic safety issues.

The results displayed and discussed herein indicate that ClearTaste as manufactured by MycoTechnology is safe for incorporation into the food supply according to its intended use, typically at < 50 and up to 1000 ppm, in view of the qualities tested. These results are not necessarily to be expected given that some mushrooms are mutagenic and others not [15].While *C. sinensis* is not discussed in the referenced study, the study implies that fungal material should be assessed for mutagenic and genotoxic potential to be sure of these safety considerations.

Positive results in either assay could indicate the presence of aflatoxin [16], though not all mycotoxins register as mutagens in such assays, as some will only register as mutagenic under certain exogenous metabolic activation systems [16,17]. It is not surprising that no sign of mycotoxin was found as *C. sinensis* has never been reported to create any mycotoxin but given the complexities involved in mutagenesis and genotoxicity it should still be required to conduct such tests to decisively conclude that a novel foodstuff, even in view of literature generally conferring safety to related items, isn't mutagenic or genotoxic according to the parameters of GLP reverse mutation and micronucleus assays [18–22]. The present study continues to confer safety to products derived from *C. sinensis*.

The authors submit that to fully understand the nature of ClearTaste's safety, studies regarding non-genotoxic mechanisms of carcinogenesis should be conducted to finalize comprehension of ClearTaste's full carcinogenic potential [23–25]. With that consideration it is understood that most carcinogenic compounds are mutagenic/genotoxic. According to the literature the work herein addresses ~90% of possible carcinogens with the Ames test alone, constituting an important contribution in confirming important aspects of ClearTaste's safety [19].

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