JHeimbach LLC

November 21, 2018

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Paulette Gaynor, Ph.D.
Senior Regulatory Project Manager
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Gaynor:

Pursuant to 21 CFR Part 170, Subpart E, Cura Global Health, Inc., through me as its agent, hereby provides notice of a claim that the addition of Koji Mineral products to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Cura Global Health has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the conclusion from each member of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the signed statements of the Expert Panel.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5543 or jh@jheimbach.com.

Sincerely,

[redacted]

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

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Generally Recognized As Safe (GRAS) Determination for the Intended Use of Koji Fermented Mineral Products (Koji Mineral)

Prepared by:
JHeimbach LLC
Port Royal Virginia

November, 2018

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Part 1: Signed Statements and Certification

1.1. GRAS Notice Submission

Cura Global Health, Inc., submits this GRAS notification through its agent James T. Heimbach, president of JHeimbach LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2. Name and Address of Notifier

Cura Global Health, Inc.

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1.3. Name of Notified Substance

The subject of this Generally Recognized as Safe (GRAS) notification is Koji Mineral products, comprising a number of products based on Koji Mineral, the biomass of the Koji culture of *Aspergillus oryzae* fermented on a medium containing specified concentrations of target minerals.

1.4. Intended Conditions of Use

Koji Mineral is intended to be added at a maximum concentration of 250 mg/serving to conventional foods, excluding infant and baby foods and foods regulated by the U.S. Department of Agriculture. The food categories to which Koji Mineral products may be added include nonalcoholic beverages excluding carbonated beverages; breakfast cereals and bars; rice and pastas, powdered milk and yogurts; condiments; processed fruits and fruit juices; processed vegetables and vegetable juices; soups and soup mixes; and nutritional drinks. The technical effect of the addition of Koji Mineral is to serve as a nutrient supplement as defined in 21 CFR §170.3(o)(20), providing essential minerals.

1.5 Statutory Basis for GRAS Status

The GRAS determination by Cura Global Health for the intended use of Koji Mineral is based on scientific procedures in accordance with 21 CFR §170.30(b).

1.6. Premarket Exempt Status

The intended use of Koji Mineral is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on the determination by Cura Global Health that it is GRAS.

1.7. Data Availability

The data and information that serve as the basis for the conclusion that Koji Mineral products are GRAS for their intended use will be made available to the FDA upon request. At FDA's option, a complete copy of the information will be sent to FDA in either paper or electronic format, or the information will be available for review at the home office of JHeimbach LLC, located at 923 Water Street, Port Royal VA 22535, during normal business hours.

1.8. Freedom of Information Act Statement

None of the information in the GRAS notice is exempt from disclosure under the Freedom of Information Act, USC 552.

1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of Koji Mineral products.

1.10 FSIS Statement

Not applicable.

1.11. Name, Position and Signature of Notifier

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Cura Global Health

Part 2: Identity, Methods of Manufacture, Specifications, and Physical and Technical Effect

2.1. Name of the GRAS Substance

The notified substances are a variety of Koji Mineral products, the word mineral referring to minerals (calcium, iron, zinc, chromium, selenium, copper, magnesium, manganese, molybdenum or combinations of these) taken up by *Aspergillus oryzae*, Koji culture, during fermentation. The concentration of the minerals in the *A. oryzae* biomass depends on the amount added to the fermentation media as well as the fermentation time and conditions. Koji Mineral products are intended to be marketed as Koji Mineral Multi 7, Koji Mineral Multi 6, Koji Mineral Fe-S, Koji Mineral Fe-P, Koji Mineral Zn, Koji Mineral Se, and so on, with the specific name depending on the mineral source and content. For example, Koji Mineral Fe-S designates a culture grown in a medium enriched with ferrous sulfate, Koji Mineral Fe-P designates a culture grown in the presence of ferric pyrophosphate, Koji Mineral Zn denotes a culture grown in a medium enriched in zinc.

2.2. Source, Mineral Uptake, and Method of Manufacture of the GRAS Substance

2.2.1. Source

The source of Koji Mineral is the Koji culture of *Aspergillus oryzae* allowed to ferment in media to which food-grade mineral compounds have been added, resulting in these minerals being taken up by the fungus. The specific food-grade *A. oryzae* strain used in the preparation of Koji Mineral is maintained at 1-8°C in the China General Microbiological Culture Collection Center. New cultures, in the form of freeze-dried spores, are purchased in glass ampoules every two years or after 100 generations have been grown from the previous purchase, whichever comes sooner.

A DNA barcoding test was conducted by DNA Gensee (France) to compare the genetic sequences from the *A. oryzae* strain in Koji Mineral to those available in public databases as well as in DNA Gensee's databases. The results showed the presence of two markers: Marker 11, which assigned Cura's strain to the genus *Aspergillus* (100% identity), and Marker 14. which assigned it to the species *Aspergillus oryzae* (100% identity). These findings are illustrated in Figure 1.

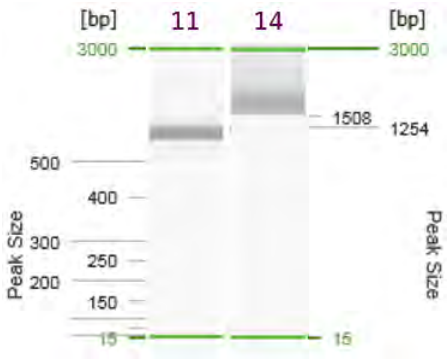


Figure 1. Capillary Electrophoresis Image of Amplified DNA from Koji Mineral.

Carbohydrase and protease from *Aspergillus oryzae* are listed by FDA as "substances derived from microorganisms recognized by FDA as Generally Recognized as Safe in Opinion Letters." The Code of Federal Regulations (21 CFR §137.105) authorizes the presence of alpha-amylase obtained from *A. oryzae* in flour.

2.2.2. Mineral Uptake

Absorption of minerals involves movement of ions by diffusion and by active uptake of nutrient ions in complex processes with numerous membrane proteins that variously function as pumps, carriers, and channels (Chrispeels et al. 1999, Pallardy 2008). Pallardy (2008) noted that, "Elements essential for growth include macronutrients such as N, P, Ca, Mg, S, and micronutrients such as Fe, Mn, Zn, Cu, B, Mo, Ni, and Cl. Mineral nutrients are important as constituents of plant tissues, catalysts, osmotic regulators, constituents of buffer systems, and regulators of membrane permeability."

A. oryzae takes up minerals from the environment and stores them in the vacuoles. The mineral concentrations in Koji Mineral products vary depending on the amounts of individual minerals—calcium, iron, zinc, chromium, selenium, copper, magnesium, manganese, molybdenum, or combinations thereof—added to the fermentation media as well as fermentation conditions and times. The mineral compounds to be added to the fermentation media are listed in Section 2.2.3.7.

The uptake of mineral ions by fungi, including *Aspergillus* species, has been extensively described in the literature, with iron the best studied. Three different mechanisms are used by *Aspergillus* (as well as *Saccharomyces* and *Candida*), depending principally on the availability of iron in the environment (Haas 2014, Brandon et al. 2015): siderophore-mediated ferric iron uptake, reductive iron assimilation through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by ferric reductase, and low-affinity ferrous iron uptake (effective only when iron concentrations are high). It has been hypothesized that the low affinity iron uptake in fungi is a specific adaptation to chronic high iron conditions, similar to the gene upregulation mechanism found in strains adapted to chronic high copper environments (Kosman 2003).

Since fungi lack mechanisms for iron excretion (Brandon et al. 2015), iron storage in the vacuoles is critical for avoiding iron-induced toxicity. Potassium, calcium, magnesium, manganese, and zinc are accumulated in the vacuoles by a similar mechanism, again because no mechanism is available for excretion (Carlile et al 2007). Vacuoles sequester, store, and mobilize iron and other minerals as needed by the cell (Park et al. 2014). Kosman (2003) explained the mechanisms used by *Aspergillus* and other fungi to accumulate iron and other minerals:

> "Successful adaptation to this environmental context [i.e., that trace minerals are relatively bio-unavailable and cytotoxic] has provided fungi with an iron uptake strategy that has three features: it relies on redox cycling to enhance iron bio-availability and reduce iron cytotoxicity; it includes both high- and low-affinity pathways that are mechanistically distinct; and it is autoregulating so as to maintain intracellular iron homeostasis" (Kosman 2003).

In an unpublished study conducted by Genaxxon Bioscience GmbH, samples of freeze-dried *A. oryzae* were grown on media containing 200 mg iron/kg or enriched to 45,700 mg ferrous sulfate/kg or 70,000 mg ferric pyrophosphate/kg. *A. oryzae* did not produce siderophores under any of these conditions, confirming that the conditions by which Koji Mineral products are produced do not promote the production of siderophores—a finding in alignment with current knowledge of iron uptake and metabolism in fungi.

The effect of adding ferrous sulfate to the fermentation medium of a Koji culture is shown in Table 1, in which 2 cultures grown in iron-rich media were compared with a control culture grown on rice; the iron uptake is shown by the higher iron concentration and the increased ash content, while the other proximates (protein, fat, and moisture) were relatively unchanged. The concentration of sulfur did not change with the addition of $FeSO_4$ into the fermentation media, indicating that the sulfur present in ferrous sulfate was not utilized by the fungi.

Table 1. Comparison Between Control and Koji Mineral Fe-S.

Parameter	Koji Control	Koji Mineral Fe-S Sample 1	Koji Mineral Fe-S Sample 2
Protein (%)	40.7	39.0	39.0
Fat (%)	4.00	2.13	3.81
Moisture (%)	9.08	7.68	6.32
Ash (%)	6.78	25.8	20.8
Iron (mg/kg)	263	82,900	60,500
Sulfur (%)	0.43	0.41	0.36

Singh et al. (2007) reported that similar mechanisms underlie handling of other minerals, with homeostasis relying primarily on control of uptake rather than excretion:

> "Transition metal ion homeostasis is strongly dependent on the regulation of nutrient accumulation rather than on excess nutrient excretion. Metal ion storage appears to be a complement of this regulatory pattern, storage that is illustrated by ferritin, the metallo-thioneins, and compartmentalization into cell organelles such as the mitochondria and vacuole. Although in some cases, this storage provides a detoxification mechanism, it is as likely to play a dynamic role in cell and/or organismal metal ion homeostasis. The vacuole in fungi and plants is a dynamic organelle that plays a significant role in the overall nutritional status of the organism. Vacuoles provide a storage depot for newly arrived nutrients as well as being the site of macromolecular degradation and nutrient recycling. In regards to metal metabolism the vacuoles in plants and fungi have been associated with the handling of copper, iron, manganese, and zinc, in addition to magnesium and calcium" (Singh et al. 2007).

Handling by fungi of minerals other than iron has not been as extensively studied and is comparatively uncertain. For example, Mendel and Bittner (2006) lamented that, "Molybdate transport is still poorly understood." Six years later, Mendel (2013) reported on molybdenum uptake and storage. He reported that, "Molybdenum is taken up in the form of its oxyanion molybdate. In the presence of competing anions, it requires specific uptake systems, which have been studied in detail in bacteria… In higher organisms such as in algae and plants, only recently have the first molybdate-transporting proteins been identified." Similarly, "Molybdate quantification in isolated vacuoles demonstrated that this organelle serves as an important molybdate storage compartment in *Arabidopsis thaliana* cells, where Mot2 was shown to be required for vacuolar molybdate export into the cytosol."

Another required micronutrient is copper, and Stoj and Kosman (2003) suggested that the Fet3 protein in *Saccharomyces cerevisiae* required for iron homeostasis "may play an essential role in copper homeostasis." Reporting on kinetic analyses, the authors reported that, "ferroxidase and cuprous oxidase activities are due to the same electron transfer site on the enzyme. These two ferroxidases are fully competent kinetically to play a major role in maintaining the cuprous-cupric redox balance in aerobic organisms."

Uptake and storage of molybdenum, copper, and zinc have been studied in fungi, and, like other minerals, "organisms have evolved with homeostatic mechanisms to maintain a relatively constant intracellular environment in the face of changing levels of extracellular zinc. One primary mechanism of zinc homeostasis is the control of zinc uptake across the plasma membrane. In addition, sequestration of zinc within intracellular organelles is an important strategy of zinc homeostasis. Organellar zinc sequestration is clearly needed for zinc homeostasis in fungi" (Simm et al. 2007). Specifically, the vacuole was identified as a major site of zinc sequestration in the cell (Devirgiliis et al. 2004).

Studies of magnesium regulation by *S. cerevisiae* determined that "cytosolic Mg^{2+} is maintained by the regulation of Mg^{2+} fluxes across both the vacuolar and plasma membranes" (Beeler et al. 1997).

In summary, the available evidence suggests that *A. oryzae* and other fungi handle all minerals in generally similar fashions. In mineral-depleted environments, they possess one or more active transporter mechanisms, but in mineral-enriched media, they rely on low uptake mechanisms to maintain homeostasis. In all cases, primary storage is in the vacuoles.

2.2.3. Method of Manufacture of Koji Mineral Products

All manufacturing takes place consistent with current Good Manufacturing Practice (cGMP). The facility has been certified by NSF International (Ann Arbor, MI) as being in compliance with 21 CFR Part 111. The manufacturing process is described in detail below. The first step is preparation of Koji spores as inoculum by propagating the spores under controlled conditions. In step 2, the spores are transferred into a fermenter where they are allowed to germinate. In step 3, the germinated Koji culture is transferred to a large fermenter where fermentation continues in a mineral-enriched medium, which is closely controlled to meet pre-set specifications, until maximum biomass is achieved. At that point, the temperature of the fermentation tank is raised to ≥65°C for approximately 20 minutes to inactivate the Koji cells ("kill step"). In step 4, the content of the fermenter is filtered to separate the fungal mycelium from the liquid residue. The biomass is mechanically broken up and fed into a flash drier to reduce the moisture content to <8%, after which the "cake" is ground to produce powder with the desired particle size. The manufacturing process is illustrated in Figure 2.

2.2.3.1. Initial Slant Preparation

Slants (cultures on agar in tilted test tubes) are used to propagate spores from the original spores ampoules. Each slant is evenly coated with potato dextrose agar and about 0.02 g spores and cultured at 28±2°C for 5-12 days. The culture is cooled to 1-8°C and can be maintained at that temperature for up to 90 days.

2.2.3.2. Preparation of Slanted Spore Suspension

10-20 mL of sterile water is added to the cultured mature slant spores and the surface spores are scraped into sterile water using an inoculation needle or inoculating spatula. The spore suspension is transferred into sterile flasks, which are stored at 1-8°C for up to 24 hours.

2.2.3.3. Preparation of Ampule Tubes and Sand Tubes

The spore liquids are transferred from sterile flasks to pre-prepared ampoule tubes and sand tubes after freeze drying and vacuum drying, respectively. The ampoule tubes can be stored for 5 years and the sand tubes can be stored for 2 years. Both sand tubes and ampoule tubes prepared from the strain can be used directly in the fermentation process or used to make to secondary slants for production.

2.2.3.4. Preparation and Inoculation of Solid Culture Medium

Solid culture is sterilized and cooled to a temperature of <30°C, and a sterilized scoop is used to place spore cultures from slants, tubs, or ampoules in the solid culture medium bottles.

2.2.3.5. Incubation of Solid Culture Medium

The solid culture bottles are incubated at <30°C for 5-12 days. The fungal spores can be preserved at 1-8°C for up to 30 days.

2.2.3.6. Transfer to Seed-Culture Fermenter and Germination

Sterile water is added to the mature fungal spore bottles and the incubated fungal culture spores and media are transferred to a seed tank. The spores are germinated in a medium consisting of sterile water and the following sterilized food-grade components: dextrose monohydrate, sugarcane molasses, yeast extract, and micronutrients. Temperature and pH are controlled and cell-growth is monitored. Samples are taken throughout the process for quality control.

2.2.3.7. Transfer to Large Fermenter and Addition of Minerals

The germinated seed culture is pumped aseptically into a large fermentation tank and sterilized food-grade fermentation ingredients (dextrose monohydrate, sugarcane molasses, yeast extract, and urea) and food-grade processing aids (alkaline and acid for pH control and polypheylene ether to control foam) are added. Finally, sterile food- or USP-grade inorganic minerals are added at required concentrations. Minerals include potassium dihydrogen phosphate, magnesium sulfate heptahydrate, calcium carbonate, calcium acetate monohydrate, ferrous sulfate heptahydrate, ferric pyrophosphate, manganese sulfate monohydrate, cupric sulfate pentahydrate, chromic chloride hexahydrate, sodium molybdate diehydrate, sodium selenite anhydrous, and zinc sulfate heptahydrate. Temperature and pH are controlled and cell-growth is monitored. Samples are taken throughout the process for quality control. At the end of fermentation, the temperature of the fermentation tank is raised to >65°C for approximately 20 minutes to inactivate the Koji cells ("kill step"). Samples are taken and checked microscopically to ensure completion of the kill step.

2.2.3.8. Filtration, Drying, and Grinding

The content of the fermenter is filtered to separate the fungal mycelium from the liquid residue. The biomass is mechanically broken up and fed into a flash drier to reduce the moisture content to <8%, after which the "cake" is ground to produce powder with the desired particle size.

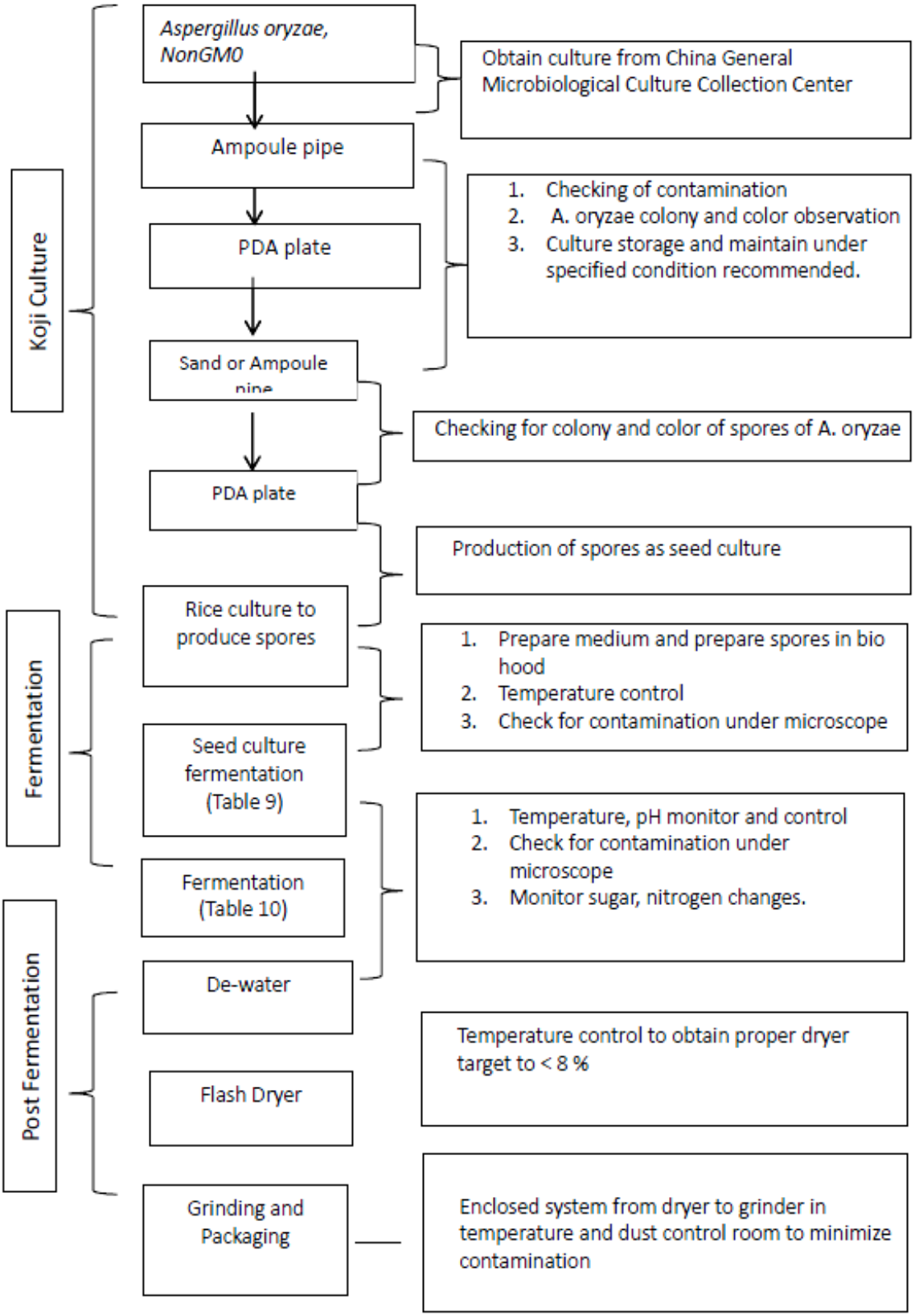


Figure 2. Koji Mineral Manufacture Process.

2.3. Specifications

Cura Global Health has established specifications for food-grade Koji Mineral products. Specifications for 8 examples of Koji Mineral products are shown in Table 2.

Data from physical and chemical analyses of 3 non-consecutive batches of iron-enriched Koji Mineral Fe-S and Koji Mineral Multi 7 are shown in Tables 3 and 4. Tables 5 and 6 display detailed analyses of contaminant levels in 3 non-consecutive batches of each product—heavy metals, polycyclic aromatic hydrocarbons, and mycotoxins. These data demonstrate that Koji Mineral is produced in compliance with established food-grade specifications and with levels of potential contaminants that are under levels of concern.

Table 2. Specifications for Examples of Koji Mineral Products.

Parameter	Fe-S	Fe-P	Zinc	Calcium	Selenium	Multi 6	Chromium	Multi 7
Identification	Mycellium	Mycellium	Mycellium	Mycellium	Mycellium	Mycellium	Mycellium	Mycellium
Apparence	Powder	Powder	Powder	Powder	Powder	Powder	Powder	Powder
Color	Light gray	Light gray	Light gray	Light gray	Light gray	Light gray	Light gray	Light gray
Loss on drying	≤10%	≤10%	≤10%	≤10%	≤10%	≤10%	≤10%	≤10%
Minerals								
Iron	7-10%	7-10%	N/A	N/A	N/A	N/A	N/A	3-6%
Zinc	Not Applicable (N/A)	N/A	7-10%			1.0-2.5%		1.0-2.5%
Selenium			N/A		4000-8000 ppm	45-200 ppm		45-200 ppm
Chromium					N/A	30-90 ppm	4000- 7000 ppm	30-90 ppm
Manganese						2300-4000 ppm	N/A	2300-4000 ppm
Copper						900-3000 ppm		900-3000 ppm
Molybdenum						36-100 ppm		36-100 ppm
Calcium			N/A	7-10%		N/A		N/A
Heavy Metals								
Lead	<3 ppm	<3 ppm	<3 ppm	<3 ppm	<3 ppm	<3 ppm	<3 ppm	<3 ppm
Cadmium	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm
Arsenic	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm	<1 ppm
Mercury	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Microbial Quality								
Total Plate Count	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g	<10,000 cfu/g
Yeast	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g
Mold	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g
Escherichia coli	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g
Salmonella spp.	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g
S. aureus	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g	neg/25g

Table 3. Physical and Chemical Characteristics of Iron-Enriched Koji Mineral Fe-S.

Physical and Chemical Parameter	Characteristics	Method	Tested Batch		
			[redacted]	[redacted]	[redacted]
Identification	DNA/Methyl blue staining-optical microscopy	External laboratory	Conforms	Conforms	Conforms
Appearance	Powder	Visual	Conforms	Conforms	Conforms
Color	Light gray/brown	Visual	Conforms	Conforms	Conforms
Bulk density	0.3 – 0.7 g/mL	Densimeter: CQ-MO-157	0.55	0.63	0.5
Iron content	8 – 10% (dry basis)	ICP: CQ-MO-247	8.7	8	8.1
Fibers content	>5% (indicative)	External lab.	33.2	31.2	31.5
Protein content	>30% (indicative)	External lab.	35.8	33.9	36.4
Particle size	>95% through 40 mesh (420 µm)	Sieve: CQ-MO-023	100	100	100
Loss on drying	<8%	IR balance: CQ-MO-018	4.95	5.8	5.3
Heavy metals					
Lead	<3 mg/kg	ICP: CQ-MO-247	<0.5	1.2	<0.5
Arsenic	<1 mg/kg	ICP: CQ-MO-247	<0.5	<0.5	<0.5
Cadmium	<1 mg/kg	ICP: CQ-MO-247	<0.1	<0.1	<0.1
Mercury	<0.1 mg/kg	ICP: CQ-MO-247	<0.005	<0.005	<0.005
Aflatoxin B1	<5 µg/kg	External lab.	<0.2	<0.2	<0.2
Aflatoxins B1+B2+G1+G2	<10 µg/kg	External lab.	<1	<1	<1
Pesticides	Complies w/USP	External lab.	Conforms	Conforms	Conforms
Irradiation	PPSL <700	PPSL: CQ-MO-572	Conforms	Conforms	Conforms
Microbiological Quality					
Total plate count	<10,000 cfu/g	Count: CQ-MO-231	<100	400	4500
Yeasts & molds	<100 cfu/g	Count: CQ_MO-244	<40	<40	<40
Salmonella spp.	Negative in 25 g	External lab.	Conforms	Conforms	Conforms
E. coli	Negative in 25 g	External lab.	Conforms	Conforms	Conforms

Table 4. Physical and Chemical Characteristics of Multi-7 Minerals Enriched Koji Mineral.

Physical and Chemical Parameter	Characteristics	Method	Tested Batch		
			[redacted]	[redacted]	[redacted]
Identification	DNA/Methyl blue staining-optical microscopy	External laboratory	Conforms	Conforms	Conforms
Appearance	Powder	Visual	Conforms	Conforms	Conforms
Color	Light gray/brown	Visual	Conforms	Conforms	Conforms
Bulk density	0.3 – 0.7 g/mL	Densimeter: CQ-MO-157	0.65	0.54	0.59
Iron content	3 – 6% (indicative)	ICP: CQ-MO-247	5	5.2	5
Zinc content	1.1% (indicative)	ICP: CQ-MO-247	1.9	1.5	1.6
Manganese content	>0.23% (indicative)	ICP: CQ-MO-247	0.23	0.24	0.29
Copper content	>0.09% (indicative)	ICP: CQ-MO-247	0.21	0.22	0.25
Selenium content	≥3.5 mg/kg (indicative)	ICP: CQ-MO-247	3.5	3.6	3.8
Molybdenum content	>45 mg.kg (indicative)	ICP: CQ-MO-247	50	63	58
Chromium content	>40 mg/kg (indicative)	ICP: CQ-MO-247	57	82	80
Particle size	>95% through 40 mesh (420 µm)	Sieve: CQ-MO-023	100	100	100
Loss on drying	<8%	IR balance: CQ-MO-018	3.95	4.8	3.7
Heavy metals					
Lead	<3 mg/kg	ICP: CQ-MO-247	<0.5	<0.5	<0.5
Arsenic	<1 mg/kg	ICP: CQ-MO-247	<0.5	<0.5	<0.5
Cadmium	<1 mg/kg	ICP: CQ-MO-247	<0.1	<0.1	<0.1
Mercury	<0.1 mg/kg	ICP: CQ-MO-247	<0.005	<0.005	<0.005
Aflatoxin B1	<5 µg/kg	External lab.	<0.2	<0.2	<0.2
Aflatoxins B1+B2+G1+G2	<10 µg/kg	External lab.	<1	<1	<1
Pesticides	Complies w/USP	External lab.	Conforms	Conforms	Conforms
Irradiation	PPSL <700	PPSL: CQ-MO-572	Conforms	Conforms	Conforms
Microbiological Quality					
Total plate count	<10,000 cfu/g	Count: CQ-MO-231	<100	<100	3500
Yeasts & molds	<100 cfu/g	Count: CQ_MO-244	50	<40	<40
Salmonella spp.	Negative in 25 g	External lab.	Conforms	Conforms	Conforms
E. coli	Negative in 25 g	External lab.	Conforms	Conforms	Conforms

Table 5. Heavy Metals, Polycyclic Aromatic Hydrocarbons, and Mycotoxins in Three Batches of Iron-Enriched Koji Mineral Fe-S.

Parameter	Method	Tested Batch		
Heavy metals				
Lead (mg/kg)	§64 LFGB L00.00-19/3	1.2	<0.5	<0.5
Arsenic (mg/kg)	§64 LFGB L00.00-19/3	<0.1	<0.1	<0.1
Cadmium (mg/kg)	§64 LFGB L00.00-19/3	<0.5	<0.5	<0.5
Mercury (mg/kg)	§64 LFGB L00.00-19/3	<0.005	<0.005	<0.005
Polycyclic aromatic hydrocarbons				
Benzo(a)pyrene (µg/kg)	GC/MS/MS	<0.5	<0.5	<0.5
Benzo(a)anthracene (µg/kg)	GC/MS/MS	<0.5	<0.5	0.8
Benzo(b)fluoranthene (µg/kg)	GC/MS/MS	<0.5	<0.5	0.9
Chrysene (µg/kg)		0.6	0.6	1.4
PAH sum (µg/kg)	GC/MS/MS	0.6	0.6	3.1
Mycotoxins				
Aflatoxin B1 (µg/kg)	IAC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin B2 (µg/kg)	IAC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin G1 (µg/kg)	IAC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin G2 (µg/kg)	IAC/LC/FLUO	<0.4	<0.4	<0.4
Aflatoxin sum (µg/kg)	IAC/LC/FLUO	<1	<1	<1
Ochratoxin A (µg/kg)	LC/MS/MS	<2	<2	<2

Table 6. Heavy Metals, Polycyclic Aromatic Hydrocarbons, and Mycotoxins in Three Batches of Multi-7 Minerals Enriched Koji Mineral.

Parameter	Method	Tested Batch		
Heavy metals				
Lead (mg/kg)	§64 LFGB L00.00-19/3	<0.5	<0.5	<0.5
Arsenic (mg/kg)	§64 LFGB L00.00-19/3	0.1	0.12	0.11
Cadmium (mg/kg)	§64 LFGB L00.00-19/3	<0.5	<0.5	<0.5
Mercury (mg/kg)	§64 LFGB L00.00-19/3	<0.005	<0.005	<0.005
Polycyclic aromatic hydrocarbons				
Benzo(a)pyrene (µg/kg)	GC/MS/MS	<0.5	<0.5	<0.5
Benzo(a)anthracene (µg/kg)	GC/MS/MS	<0.5	<0.5	<0.5
Benzo(b)fluoranthene (µg/kg)	GC/MS/MS	<0.5	<0.5	<0.5
Chrysene (µg/kg)		<0.5	<0.5	<0.5
PAH sum (µg/kg)	GC/MS/MS	<0.5	<0.5	<0.5
Mycotoxins				
Aflatoxin B1 (µg/kg)	AC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin B2 (µg/kg)	AC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin G1 (µg/kg)	AC/LC/FLUO	<0.2	<0.2	<0.2
Aflatoxin G2 (µg/kg)	AC/LC/FLUO	<0.4	<0.4	<0.4
Aflatoxin sum (µg/kg)	IAC/LC/FLUO	<1	<1	<1
Ochratoxin A (µg/kg)	LC/MS/MS	<2	<2	<2

2.4. Stability

In a 6-month accelerated stability study, 5 samples of Koji Mineral product (3 batches of Koji Multi-7 and 1 batch each of Koji Fe-S and Koji ZnSe) were taken from the packaging area of the production facility and placed in plastic bags which were in turn sealed in plastic-lined aluminum bags. All samples were stored in an incubator which maintained a temperature of 40±2°C and relative humidity of 75±5%. One sample bag of each product was tested for changes in appearance, moisture, total plate count, yeasts, and molds after 0, 1, 2, 3, and 6 months of storage; results were compared with the specifications shown in Tables 1 and 2.

The results of the testing (except the 6-month test of Koji ZnSe, which was cancelled due to failure of the storage bag) are shown in Table 7.

Table 7. Testing Results in 6-Month Accelerated Stability Study.

Product	Time (Months)	Parameter			
		Appearance	Moisture (%)	Plate Count (cfu/g)	Yeast & Mold (cfu/g)
Koji Multi-7a	0	Typical	6.2	400	25
	1	Typical	6.2	400	25
	2	Typical	6.3	800	20
	3	Typical	6.4	900	10
	6	Typical	7.1	850	10
Koji Multi-7b	0	Typical	5.1	100	10
	1	Typical	5.2	400	20
	2	Typical	5.3	900	30
	3	Typical	5.6	900	30
	6	Typical	5.6	300	20
Koji Multi-7c	0	Typical	4.6	400	30
	1	Typical	6.6	300	30
	2	Typical	6.6	800	20
	3	Typical	6.6	800	20
	6	Typical	6.7	650	20
Koji Fe-S	0	Typical	7.1	200	25
	1	Typical	7.0	200	25
	2	Typical	7.1	800	20
	3	Typical	7.2	700	30
	6	Typical	7.3	800	20
Koji ZnSe	0	Typical	6.2	350	10
	1	Typical	6.3	2000	20
	2	Typical	6.4	900	30
	3	Typical	6.5	3000	20
	6	NA[1]	NA	NA	NA

1. NA = not available due to problem with sample bag

Part 3: Intended Use and Dietary Exposure

It is intended to market a variety of Koji Mineral products based on the Koji strain of *A. oryzae* fermented in media enriched with different minerals. Some products, such as Koji Mineral Multi 7, result from fermentation in media with high concentrations of several minerals, while others, such as Koji Mineral Fe-S, are produced by providing the *A. oryzae* with growth media enriched in only a single mineral, in this case iron from iron sulfate. Because *A. oryzae* are living organisms, the mineral uptake is somewhat variable and so the mineral content of the biomass is expressed as a range.

In most cases, the objective is to add Koji Mineral to a food at a level that provides 20-25% of the Daily Value (DV) of the mineral or minerals per serving. (The exception is calcium, for which the DV of 1300 mg is too high to make 25% of it a feasible addition level in a single serving of food.) Examples of intended Koji mineral products are shown below.

Six examples of Koji Mineral products targeting single minerals are illustrated in Table 8. For each product, its mineral content is shown in the third column. The intended addition level and the resulting intake per serving of the target mineral are shown in the next two columns, while the last column shows 25% of the DV for that mineral. With the exception of calcium, intake per serving of each mineral exceeds 25% of the DV.

Table 8. Examples of Single Mineral Koji Mineral Products.

Product	Target Mineral	Mineral Content	Intended Koji Addition Level per Serving	Mineral Content per Serving	25% of DV of Target Mineral
Koji Mineral Fe-S	Iron (from iron sulfate)	7.0-10.0%	65 mg	4.55-6.50 mg	4.5 mg
Koji Mineral Fe-P	Iron (from iron pyrophosphate)	7.0-10.0%	65 mg	4.55-6.50 mg	4.5 mg
Koji Mineral Zn	Zinc	7.0-10.0%	40 mg	2.80-4.00 mg	2.75 mg
Koji Mineral Se	Selenium	4000-8000 ppm	3.5 mg	14.0-28.0 µg	13.75 µg
Koji Mineral Cr	Chromium	4000-7000 ppm	2.2 mg	8.8-15.5 µg	8.75 µg
Koji Mineral Ca	Calcium	7.0-10.0%	250 mg	17.5-25.0 mg	325 mg

An example of a multi-mineral product, Koji Mineral Multi 7, is shown in Table 9. Its intended addition level is 250 mg Koji Multi 7/serving of food.

Table 9. Koji Mineral Multi 7 Added at 250 mg/Serving.

Mineral	Mineral Content	Mineral Content per Serving	20-25% of DV of Target Mineral
Iron	3-6%	7.5-15 mg	3.6-4.5 mg
Zinc	1.0-2.5%	2.50-6.25 mg	2.2-2.75 mg
Manganese	2300-4000 ppm	575-1000 µg	460-575 µg
Copper	900-3000 ppm	225-750 µg	180-225 µg
Selenium	45-200 ppm	11.3-50 µg	11-13.75 µg
Molybdenum	36-100 ppm	9-25 µg	9-11.25 µg
Chromium	30-90 ppm	7.5-22.5 µg	7-8.75 µg

As can be seen in Table 9, at an addition level of 250 mg Koji Mineral Multi 7 per serving of food, the content in the food of each of the 7 minerals in Koji Mineral Multi 7 exceeds 25% of the DV for that mineral.

In the examples cited, the intended addition level of Koji Mineral ranges from 2.2 mg per serving (for Koji Mineral Cr) to 250 mg per serving (for Koji Mineral Ca and Multi 7), the highest intended addition level for Koji Mineral products.

In summary, Koji Mineral is intended to be added at a maximum concentration of 250 mg/ serving to conventional foods, excluding infant and baby foods and foods regulated by the U.S. Department of Agriculture. A "serving" of food is as given in 21 CFR §101.12, "Reference Amounts Customarily Consumed per Eating Occasion: General Food Supply." The technical effect of the addition of Koji Mineral is to serve as a nutrient supplement as defined in 21 CFR §170.3(o)(20), providing essential minerals.

The food categories to which Koji Mineral may be added include nonalcoholic beverages excluding carbonated beverages; breakfast cereals and bars; rice and pastas, powdered milk and yogurts; condiments; processed fruits and fruit juices; processed vegetables and vegetable juices; soups and soup mixes; and nutritional drinks. These food categories are consistent with the statement of purpose in FDA's Nutritional Quality Guidelines (21 CFR §104.20), where it is noted that FDA does not "consider it appropriate to fortify fresh produce; meat, poultry, or fish products; sugars; or snack foods such as candies and carbonated beverages."

Estimated daily intakes of Koji Mineral from its intended use in foods were derived based on food consumption records collected in the What We Eat in America (WWEIA) component of the National Health and Nutrition Examination Survey (NHANES) conducted in 2011-2012 and 2013-2014 (WWEIA/NHANES 2011-2014). The dietary assessment was conducted by the Exponent® Center for Chemical Regulation and Food Safety under contract with JHeimbach LLC.

Serving sizes for target food categories are shown in Table 10.

Table 10. Food Categories and Serving Sizes (RACCs).

Food Category	NHANES Food Selection	Serving Size
Beverages, nonalcoholic (excluding carbonated beverages)	Sport and energy drinks and enhanced or fortified waters	360 ml
	Fruit flavored drinks	240 ml
Breakfast cereals and bars	Hot cereals include grits, oatmeal, cream of rice, cream of wheat, whole wheat cereal, and oat bran cereal	240 g
	Ready-to-Eat (RTE) breakfast cereals, weighing less than 20 g per cup	15 g
	RTE breakfast cereals, weighing 20 g or more but less than 43 g per cup; high fiber cereals containing 28 g or more of fiber per 100 g	40 g
	RTE breakfast cereals, weighing 43 g or more per cup; biscuit types	60 g
	Cereal and nutrition bars including protein bars	40 g
Grain products, rice, pastas	Rice, dry/uncooked	45 g
	Pasta, dry/uncooked	55 g
Milk products—powdered milk, yogurts	Not reconstituted dried milk and not reconstituted milk beverage mixes	Amount to make 240 mL prepared
	Cocoa powder, not reconstituted	15 g
	Yogurt	170 g
Condiments	Fermented soybean product sauce	15 g
Processed fruits and fruit juices	Fruit smoothies and calcium fortified 100% juices or juice blends	240 ml

Processed vegetables and vegetable juices	Vegetable juice blends	240 ml
Soups and soup mixes	Soup, ready-to-serve	245 g
	Soup, condensed and dry mixes	Amount to make 245 g prepared
Nutritional drinks	Nutritional drinks such as Boost, Ensure, and Glucerna	240 ml

Based on a maximum addition level of 250 mg/serving for Koji Mineral products, Exponent® estimated 2-day average daily intakes on a *per user* basis, with intake expressed both per person and per kg body weight, as shown in Table 11.

Table 11. Estimated Daily Intake of Koji Mineral Added to Foods at 250 mg/Serving.

Population Group	% Users	Intake per User (mg/day)		Intake per Kg Bodyweight (mg/kg bw/day)	
		Mean	90th Percentile	Mean	90th Percentile
U.S. 2+ years	82	389	777	6.8	15.0
Children 2-8 years	94	338	638	16.6	32.2
Children 9-13 years	89	408	800	9.6	19.6
Adolescents 14-18 years	84	426	826	6.6	13.4
Adults 19+ years	80	391	786	5.1	10.4

The estimated daily intake of Koji Mineral is, not surprisingly, highest among adolescents aged 14-18 years, with an estimated mean intake of 0.43 g and estimated 90th percentile intake of 0.83 g. For the entire U.S. population over the age of 2 years, the estimated mean daily intake is 0.39 g and the estimated 90th percentile intake is 0.78 g.

Part 4: Self-limiting Levels of Use

There is no meaningful technological limitation to the concentration of Koji Mineral products in foods. The primary limitation is the maximum desirable mineral content, but at sufficiently high levels, depending on the type of food and its flavor properties, the fungal biomass would impart negative organoleptic characteristics to food. Cost-per-serving of the ingredient may also provide a self-limiting level of use.

Part 5: Experience Based on Common Use in Food

The conclusion that the intended use of Koji Mineral is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

The Koji strain of *Aspergillus oryzae* has been consumed in foods for many years world-wide. Products in which the strain is found outside of Asia, where it is ubiquitous in miso, sake, and other staples, include:

- U.S.: Café Galeos and Onai Fresh salad dressings, Tyson Glazed Chick Wings, Whole Foods Organic Miso Broth, Eden Organic Tamari Soy Sauce and Ponzu Sauce, Eden Five Flavor Arare Rice Puffs, Eden Organic Brown Rice Vinegar, La Choy Chicken Fried Rice Meal, AmaZake Cool Coconut Rice Shake with Almonds, Vanilla Soy Crème Rice Shake, Gimme Green Rice Drink, and Nature's Plus Acti-Zyme Digestion Aid
- Finland: Clearspring Organic Kagisa Shoyu Sauce and Urtekram Mugi Miso Stock
- France: Carrefour Bio Shoyu Soy Sauce
- Belgium: Oao Bio Organic Instant Miso Soup Mushroom, Ramen with Mushroom, and Soya & Citrus Sauce
- Canada: Edenblend Organic Rice & Soy Milk
- Czech Republic: Sunfood Ryorido Traditional Amazake Basic Dessert Oat, Basic Millet Dessert, and Muso Organic Amazake
- Netherlands: Clearspring Organic Brown Rice Amazake Sweet Grains Dessert and Ryorido Basic Dessert Rice
- Norway: Clearspring Organic Brown Rice Amazake Dessert
- Portugal: Clearspring Organic Brown Rice Amazake Dessert, Organic Millet Amazake Dessert, and Organic Amazake Oat Dessert
- U.K.: Clearspring Organic Amazake Oat Dessert

Part 6: Narrative

6.1. Studies of the Use of *Aspergillus oryzae* Derivatives as Animal Feed Supplements

A number of experiments were performed in which cultures of *A. oryzae* or *A. oryzae* products were used as animal feed supplements for cattle, swine, lambs, and poultry. While the *A. oryzae* interventions did not always report benefits, there were no reports of adverse effects.

A fermentation extract of *A. oryzae* was given as a top-dressing to 64 dairy cows at concentrations of 0, 1.5, 3, and 6 g/L feed (Denigan et al. 1992). Forty cows received the treatment for 70 days and 24 cows for 60 days. None of the levels of *A. oryzae* extract had a significant effect on milk yield or composition, bodyweight changes, or digestion coefficients. The authors concluded that, "Under the conditions of this study, none of the levels of [*A. oryzae* extract] affected the performance of lactating cows."

Varel and Kreikemeier (1994a) added *A. oryzae* extract to bromegrass and alfalfa cattle feed at a concentration of 3 g/L feed. Four beef cows fitted with ruminal and duodenal cannulas were fed the supplemented feed while 4 similar cows consumed unsupplemented feed for 28 days. The treatment had no effect on ruminal fiber degradation, but increased the count of ruminal anaerobic bacteria. No adverse effects on the cattle were reported.

In a follow-on study, Varel and Kreikemeier (1994b) again added *A. oryzae* extract at 3 g/L feed to bromegrass hay fed to 6 cannulated cows for 28 days. The authors concluded that, "The *A. oryzae* fermentation extract fed at nine times the recommended dosage did not produce any stimulatory effects, except for total VFA [volatile fatty acids], and was not inhibitory or toxic to ruminal metabolism and forage fiber degradation."

Four lactating Holstein cows with ruminal and duodenal cannulas received basal diet with or without 3 g/day of *A. oryzae* culture (Yoon and Stern 1996). Ruminal pH, ammonia nitrogen concentration, and total volatile fatty acid concentrations were similar among treatments and fiber digestion was similar, but fungal culture stimulated proteolytic and cellulolytic bacterial counts, regarded as beneficial effects.

In a study of the effects of steam-rolled vs. steam-flaked corn with or without added *A. oryzae* culture, Yu et al. (1997) reported a factorial design in which 32 Holstein cows were fed an assigned diet for 21 days. Fungal culture increased the percentage of protein and solids-not-fat in milk. *A. oryzae* culture had no effect on cows' bodyweight or milk production, and no adverse effects were reported.

McGilliard and Stallings (1998) reported a study in which 3417 dairy cows in 46 Virginia dairy herds received feed with or without a supplement consisting of the dried fermentation products of *A. oryzae, Bacillus subtilis, L. acidophilus*, and a yeast culture for 5 months. Milk production increased significantly with no reported adverse effects.

Feng et al. (2007a) reported on the effect of *A. oryzae*-fermented and unfermented soybean meal on growth and digestion of 60 5-week-oldcrossbred (Duroc x Landrace x Large White) piglets in a 2-week feeding trial. The *A. oryzae*-fermentation improved piglets' growth and feed utilization compared with control and was regarded as beneficial to growth performance, digestibility of dietary components, and activities of intestinal enzymes.

Three hundred twenty 1-day-old male Ross x Ross broiler chicks were randomly allocated to receive feed with or without soybean meal fermented by *A. oryzae* for 6 weeks (Feng et al. 2007b). Sixteen broilers of each treatment were sacrificed and pancreas, small intestine digesta, and duodenum, jejunum, and ileum segments were collected for evaluation of digestive enzymes and intestinal morphology. Protease activity was significantly increased and increased villus height and decreased crypt depth of jejunum mucosa was reported; no adverse effects were reported.

Zerby et al. (2011) reported on the effects of feeding 48 Dorset x Hampshire lambs with a diet supplemented with 1 g/day *A. oryzae* extract until they reached a predetermined weight; the treatment improved growth and feed utilization. In a second study, 168 crossbred steers were given 3 g/day *A. oryzae* extract and significant improvement was seen in feed utilization.

Fifty-two bull calves were randomly assigned to receive milk replacer with or without 2 g/day of a fermentation extract of *A. oryzae* from age 1 week to slaughter at 4 (n = 16) or 8 weeks (n = 36). Calves had *ad libitum* access to starter and water throughout the study. Feed intake and fecal and respiratory scores were recorded daily; body weight, withers height, and hip height were recorded weekly. Gross rumen measurements and rumen samples for gross and histological analyses were taken at 4 and 8 weeks. Growth, milk replacer consumption, starter feed consumption, total dry matter intake, gross and histological rumen measurements, rumen pH, and fecal and respiratory scores were not affected by treatment. A lower percentage of calves receiving *A. oryzae* fermentation extract required treatment for respiratory ailments. The authors concluded that, "Dietary inclusion (2 g/d) of an extract of *A. oryzae* did not affect calf growth, intake, and ruminal or health measurements."

To evaluate the effects of *A. oryzae* culture on milk performance and rumen fermentation of dairy cows, 64 Chinese Holstein cows were randomized to receive feed with or without 5 g/day of *A. oryzae* culture (Sun et al. 2017). No adverse effects were reported from the treatment, which increased cows bodyweight gain and stimulated rumen microbe populations.

6.2. Safety of *Aspergillus oryzae*

The genome of the strain of *A. oryzae* used in the production of Koji Mineral was published in 2005 (Machida et al. 2005). It comprises 37 million bases (Mb) distributed over 8 chromosomes and 12,074 genes. The *A. oryzae* genome is about a third larger than those of other *Aspergillus* species. Much of this expansion is in the amino acid-polyamine-organocation and the major facilitator superfamily transporter genes, which are concerned with transport of amino acids and transport of sugars, respectively. Machida et al. (2005) speculated that, because *A. oryzae* within the Koji culture grows on the surface of solid material such as steamed rice or ground soybean, where amino acids and sugars are deficient at the beginning, "The need for *A. oryzae* to get access to external nitrogen sources effectively and to degrade proteins and starches seems consistent with the observed expansion of the metabolism and transporter-related gene families."

Kobayashi et al. (2007) reported on the genome of *A. oryzae* and compared it with other species of the genus *Aspergillus*, reporting that it "has an approximately 2.5-times bigger genome size and approximately twice as many genes as *S. cerevisiae*." Further, it is "extremely enriched with genes involved in biomass degradation, primary and secondary metabolism, transcriptional regulation, and cell signaling." Kobayashi et al. (2007) discussed AflR, a regulator in the biosynthesis of aflatoxin, and noted that *A. oryzae*, the organism used in the fermentation industry, "does not produce aflatoxin since the *aflR* gene is not expressed and/or AflR is non functional in these strains." The authors' conclusion was, "It is a safe organism."

Shurtleff and Aoyagi (2012) reported that "Koji is a culture prepared by growing *A. oryzae* mold on cooked grains and/or soybeans in a warm, humid place," that was mentioned in the rites of the Zhou dynasty in China as long ago as 300 BCE. The same authors claim that koji entered the United States by 1906, when the California Miso Manufacturing Co. began to make miso, which requires koji, in San Francisco; by 1908 a different company, located in Los Angeles, advertised koji for sale (Shurtleff and Aoyagi 2012).

In a review of the safety of *A. oryzae,* Barbesgaard et al. (1992) reported that it is an aerobic, filamentous fungus that forms asexual spores and has an optimal growth temperature of

32-36°C. *A. oryzae* is principally found in China and Japan, and Barbesgaard et al. (1992) suggested that it is generally agreed that "cultivation through thousands of years has resulted in a 'domesticated variety' of *A. flavus* [a group to which *A. oryzae* belongs] with changed morphology and loss of certain metabolic activities, e.g., formation of aflatoxin." Barbesgaard et al. (1992) reported that *A. oryzae* has been used to produce koji for more than 2000 years, that the fungus and its enzymes are accepted as constituents of food, that it is generally regarded as a non-pathogenic fungus, and that "In Japan more than 30,000 persons are exposed to high concentrations of *A. oryzae* spores from the fermentation of soy products in small family workshops." The authors concluded that, "Invasive growth or systemic infections by *A. oryzae* in healthy humans have never been reported."

> Machida et al. (2008) reviewed the safety of A. oryzae and concluded that:
>
> "The long history of extensive use of *A. oryzae* in food fermentation industries prompted industrial applications of *A. oryzae* to be listed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) in the USA. The safety of this organism is also supported by World Health Organization (WHO). Although *A. oryzae* is genetically very close to *A. flavus*, which is known to produce the most potent natural carcinogen, aflatoxin, *A. oryzae* has no record of producing aflatoxin or any other carcinogenic metabolites. Fermented foods produced by *A. oryzae* ... have been shown to be aflatoxin free. The two species, *A. oryzae* and *A. flavus*, were traditionally distinguished based on morphological, physiological and culture-based characteristics. Recent DNA-based techniques have enhanced the potential for distinction… Homologs of aflatoxin biosynthesis gene cluster of *A. oryzae* are not expressed even under the conditions that are favorable to aflatoxin expression in *A. flavus* and *A. parasiticus*" (Machida et al. 2008).

The authors argued that, "These results suggest that the long history of industrial use of *A. oryzae* has removed genes unfavorable for human consumption. Alternatively, *A. oryzae*, by some means, might have been selected as a safe mutant from the beginning."

6.3. Safety of *Aspergillus oryzae* Products

In a table of enzymes used [safely] in food processing today, Pariza and Johnson (2001) included a number of enzymes derived from *A. oryzae*. They also provided a decision tree that can be applied as follows:

> 1. Is the production strain genetically modified? **NO**. *If no, go to 6.*
>
> 6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **YES**. If yes, the test article is ACCEPTED.

This decision-tree model indicates that enzymes and other products of *A. oryzae* are presumed to be safe.

Barbesgaard et al. (1992) reported that "No proven carcinogenic metabolites have been identified in *A. oryzae*," and "Aflatoxins are never formed." Three mycotoxins that may be produced by some strains are:

- β-nitropropionic acid, produced by about half of *A. oryzae* strains tested, which was not toxic in mice given acute intravenous doses of 15-60 mg/kg bw;
- Kojic acid, produced by less than half of *A. oryzae* strains tested, was reported to have an LD_{50} of 30 mg/mouse when administered intraperitoneally;
- Cyclopiazonic acid, produced by about half of *A. oryzae* strains tested, was reported to have an oral LD_{50} of 36 mg/kg bw in male rats.

The authors concluded that, "We therefore consider *A. oryzae* an excellent host for the safe production of harmless products by recombinant strains [based on them being as safe as the parent organism]."

Blumenthal (2004) reviewed potentially toxic secondary metabolites of *A. oryzae* (as well as *A. niger* and *Trichoderma reesei*). Those that were identified are:

- Aspergillomarasmine—not shown to be toxic by oral route
- Cyclopiazonic acid—ADI in humans estimated to be 700 µg/day (Burdock and Flamm 2000)
- Kojic acid—not toxic by oral route; evaluation by Burdock et al. (2001) concluded that ''consumption of kojic acid at levels normally found in food does not present concern for safety''
- Maltoryzine—not shown to be toxic by oral route
- 3-nitropropionic acid—ADI in humans estimated to be 25 µg/kg bw/day (Soni et al., unpublished)
- Violacetin—reported oral LD_{50} of 375 mg/kg bw in mice (Kobayashi 1966)

Despite the occasional isolation of these mycotoxins from *A. oryzae* cultures, the EPA (1997) concluded that, "under usual conditions of culture, well-established commercial strains of this species do not seem to produce significant levels of mycotoxins." Blumenthal (2004) suggested that potential production of these mycotoxins should be assessed when new production strains are under development, but "most industrial production strains of *A. niger, A. oryzae*, and *T. reesei* have successfully demonstrated a long history of safe use."

In 1987, The Joint WHO/FAO Expert Committee on Food Additives (JECFA) assessed the safety of enzymes produced by *A. oryzae*. The Committee summarized unpublished toxicological studies that had been submitted in support of the assessments. Two studies submitted in support of carbohydrases (Garvin et al. 1972) were summarized as follows:

> "Rats: Three groups, each containing 5 male and 5 female SPF Wistar rats, were maintained for 3 weeks on diets containing 0, 0.5, or 5% of the enzyme preparation. Only minor differences were observed among the groups in body-weight change and food intake. At termination of the study, haematologic measurements, organ-weights analyses, and gross post mortem examinations showed no compound-related effects" (WHO 1988).
>
> "In another study, two groups, each containing 10 male and 10 female ARS Sprague-Dawley rats, were fed diets containing 5 or 10% of the test enzyme (equivalent to 3.5 or 7 g enzyme/kg b.w./day) for 90-94 days. A control group of 20 male and 20 female rats was maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar among animals in the test and control groups. Differential blood counts were within the normal range at weeks 4 and 8 in all groups. At the end of the study, haematologic parameters, organ-weight analyses, and gross and microscopic pathology showed no compound-related effects" (WHO 1988).

Another toxicity study (Garvin et al. 1972) was submitted for a preparation containing 2 *A. oryzae* proteases:

> "Rats: Two groups of 10 male and 10 female ARS Sprague-Dawley rats, were fed diets containing 5 or 10% of the test enzyme (equivalent to 3.5 or 7 g enzyme/kg b.w./day) for 90 to 94 days. A control group of 20 male and 20 female rats was maintained on the diet alone. No signs of toxicity were observed during the test period. Body-weight gain and food consumption were similar in animals in the test and control groups. Differential blood counts were within the normal range at weeks 4 and 8 in all groups. At the end of

> the study serum clinical chemistry parameters, organ weight analyses, and gross and microscopic pathology showed no compound-related effects" (WHO 1988).

All assessed enzymes were reported safe and suitable for use in food.

Lane et al. (1997) evaluated the safety of tannase, an acylhydrolase enzyme preparation from *A. oryzae*, in a bacterial reverse mutation assay and a subchronic oral toxicity study. The enzyme was plated at concentrations of 0, 50, 150, 500, 1500, and 5000 µg tannase/plate and tested for mutagenic activity using *S. typhimurium* tester strains TA1535, TA1537, TA100, and TA98, with and without S-9 metabolic activation. The authors reported, "No increase in the number of revertant colonies occurred with any of the four test strains at any concentration, either in the presence or absence of metabolic activation," indicating an absence of mutagenic potential.

For the subchronic oral toxicity study (Lane et al. 1997), 5-week-old male and female F344/Du Crj rats (bodyweights not reported) were assigned at 20 rats/sex/group to receive diets containing 0, 0.01, 0.1, or 1% enzyme preparation for 90 days. Animals were single-caged and observed regularly; feed consumption and bodyweight were recorded weekly; ophthalmoscopic examinations were performed on high-dose animals at baseline and termination; hematology and clinical chemistries were obtained from 10 animals/sex/group at study termination. After sacrifice, rats were subjected to necropsy, selected organs were weighed, and organs from control and high-dose animals were examined histologically. There was no mortality and no clinical signs related to tannase enzyme, ophthalmologic lesions, differences in feed intake, differences in weight gain, or differences in hematology or clinical chemistries. There were no toxicologically significant differences in absolute or relative organ weights and no adverse histopathological effects. The no observed adverse effect level (NOAEL) of tannase enzyme for male and female F344 rats was 1% dietary concentration, corresponding to approximately 622 and 702 mg/kg bw/day for males and females, respectively.

A series of studies was conducted to assess the toxicological potential of 2 tripeptides (L-valyl-L-prolyl-L-proline and L-isoleucyl-Lprolyl-L-proline) found in *A. oryzae* protease casein hydrolysate (and in *L. helveticus*-fermented milk). Matsuura et al. (2005) reported on a mouse micronucleus test in which male Sprague-Dawley rats (5 per group) received doses of 0, 500, 1000, or 2000 mg casein hydrolysate/kg bw/day for 2 days. After 24 hours, bone marrow cells were fixed and examined for the presence of polychromatic erythrocytes (PCEs) and micronucleated PCEs (MNPCEs). The authors reported that, "Administration of [the test compound] to rats and mice produced neither changes in body weights nor signs of systemic toxicity. Similarly, neither [test article] caused statistically significant variations in the incidences of either PCEs or MNPCEs," indicating that "neither form of the tripeptides possesses the potential to induce micronuclei formation in these rodent species."

The 2 forms of the tripeptides were further tested in acute and subacute oral toxicity studies in male and female Sprague-Dawley rats (Maeno et al. 2005). Test articles were administered by gavage at a single limit dose of 2000 mg/kg bw and for 28 days at doses of 0, 500, 1000, or 2000 mg/kg bw/day. Evaluations of ophthalmology, clinical chemistry, hematology, urinalysis, necropsy, organ weights, and microscopic histopathology provided "neither in-life nor postmortem evidence that [casein hydrolysate] administration caused physiological or toxicological changes." The authors concluded that "The results of the repeated-dose study do not support identification of a target organ for [casein hydrolysate] toxicity. Similarly, there was no evidence to support establishment of either the LOEL [lowest observed effect level] or MTD [maximally tolerated dose]; both being greater than 2000 mg/kg/day for up to 28 consecutive days."

Mizuno et al. (2005) reported a subchronic study of *A. oryzae* protease casein hydrolysate in groups of 12 males and 12 female Charles River rats that were gavaged with doses of 0, 40, 200, or 1000 mg/kg bw/day for 91 days. Antemortem evaluative parameters included gross observations of

behavior and clinical signs; feed consumption and body weight gains; ophthalmologic examinations; clinical pathology (hematology, clinical chemistry); and urinalysis. Postmortem parameters included determination of absolute and relative organ weights and histopathological evaluation of approximately 50 organs and tissues from each animal.

All rats survived until the scheduled termination of the study and no treatment-related clinical signs were reported. Feed consumption was unaffected. There were no statistical differences between groups with respect to weight gain. There were no meaningful changes in hematological or coagulation parameters. Mid- and high-dose males had slightly increased serum chloride, but this was not regarded as significant. Mean relative kidney weight was decreased by 8% in low-dose males and mean relative uterus weight was elevated 46% in low-dose females. Absolute organ weights were not affected. Only naturally occurring microscopic changes were reported in all groups and none could be attributed to test-article administration. The authors concluded that, "under the conditions of these experiments, the maximally tolerated dose (MTD) and the no-observable-effect level (NOEL) for [casein hydrolysate] administered once daily for 13 weeks was greater than 1000 mg/kg BW/day."

A. oryzae produces the enzyme adenosine-5'-monophosphate deaminase, widely used in foods and beverages. Okado et al. (2015) reported on studies of the safety of this enzyme, including genotoxicity and subchronic oral toxicity. The genotoxicity studies included a bacterial reverse mutation test (Ames assay) compliant with OECD Guideline No. 471, using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and concentrations ranging from 0.5 to 4.0 µg/plate with and without metabolic activation, and an OECD Guideline No. 473 compliant *in vitro* mammalian chromosome aberration test with concentrations of 0.019 to 1.95 mg TOS [total organic solids]/mL. The authors reported that the enzyme "produced a negative response in both *in vitro* genotoxicity tests" and concluded that, "As a result of the bacterial reverse mutation test and mammalian chromosomal aberration test, [the enzyme] was considered non-mutagenic and non-genotoxic under the conditions of both *in vitro* genotoxicity tests." They concluded that the intervention was "without any noticeable adverse effects."

The subchronic oral toxicity study was conducted in accordance with OECD Guideline No. 408 (Okado et al. 2015). Eighty 4-week-old Sprague-Dawley Crl:CD specific-pathogen-free rats, 40 of each sex, were acclimatized for a week and randomly assigned (10 rats/sex/group) to receive 0, 19.8, 198.4, or 1984 mg TOS/kg bw/day by gavage for 90 days. At the start of dosing, male rats weighed 144-164 g and females weighed 122-140 g. Animals were individually caged and observed for clinical signs and mortality twice a day and weighed weekly. Feed intake was measured weekly. Ophthalmological examinations were conducted at baseline and on Day 87. At sacrifice on Day 90, blood samples were taken for hematology, coagulation, and clinical chemistry analysis; urinalysis was performed; organs were excised and weighed; and histopathological examinations were performed as specified in the OECD guideline.

There was no mortality and animals displayed good general condition throughout the in-life study; no differences were reported among the groups in feed consumption, body weight, body-weight gain, ophthalmology findings, clinical chemistries, or absolute or relative organ weights. The only significant differences in hematology were increases in mean corpuscular volume and mean corpuscular hemoglobin in high-dose males. The authors concluded that, "In summary, [the enzyme] was not associated with any adverse effects in Crl:CD(SD)[SPF] rats at doses up to 1984 mg TOS/kg bw/day in a 90-day repeated-dose oral toxicity study conducted in compliance with current guidelines."

The safety of another pair of enzymes produced by *A. oryzae*, a protease and a xylanase combined in a single preparation, was reported by Dillon et al. (2017) in acute and subchronic oral toxicity studies and 3 genotoxicity assays: bacterial reverse mutation test, *in vitro* mammalian

chromosome aberration test, and *in vivo* mammalian micronucleus test, all compliant with OECD guidelines. Acute oral toxicity was assessed in 6-week-old male and female CHS Swiss ICO:OFI (IOPS Caw) mice and specific-pathogen-free Sprague-Dawley Crl:CD rats. Ten mice or rats of each sex received single gavage administrations of 0 or 2000 mg/kg bw and were observed for 14 days. The authors reported that, "no mortality or clinical signs of morbidity occurred in Sprague-Dawley rats or Swiss mice" and "in both rat and mouse studies, body weight was unaffected by treatment and macroscopic examination of main organs showed no apparent abnormalities."

In the subchronic study (Dillon et al. 2017), 10 rats/sex/group were allocated to 5 dosing groups to receive daily gavage doses of 0, 3, 30, 300, or 1000 mg/kg bw/day and housed with 2 rats/cage. Animals were observed for clinical signs and mortality twice a day and weighed weekly. Feed intake was measured weekly. A functional observation battery (FOB) was performed between weeks 11 and 13. Ophthalmological examinations were conducted at baseline and at the end of the study. At sacrifice, blood samples were taken for hematology and clinical chemistry analysis; urinalysis was performed; organs were excised and weighed; and histopathological examinations were performed as specified in the OECD guideline.

No treatment-related mortality, clinical signs of morbidity, functional modifications, or ophthalmologic findings occurred in Sprague-Dawley rats administered the enzyme preparation for 13 weeks. Food consumption, blood biochemistry, hematology, urinalysis, organ weights, and microscopic findings were not affected by treatment. Most microscopic findings were within the range of expected pathology in Sprague-Dawley rats of this age and strain and occurred with similar incidence and severity in both control and treated rats. No discernible differences were reported in the cytological appearance of the outer renal medulla between the male control and 300-mg/kg bw/day groups or between the female control and 1000-mg/kg bw/day groups. Qualitative analysis of the stages of spermatogenesis did not reveal any treatment-related effects in the high-dose group, nor was there any discernible increase in haemopoiesis in the spleen. The authors concluded that "the no observed adverse effect level (NOAEL) for [the enzyme preparation] is 1000 mg/kg bw/day in female Sprague-Dawley rats, and 300 mg/kg bw/day in male rats" due to the treatment-related pathological changes seen in the kidneys of both sexes (but mainly males) and in the spleen of males at doses equivalent to 1000 mg/kg bw/day.

Dillon et al. (2017) reported that, "The battery of genotoxicity tests herein consistently showed [the enzyme preparation] to be nongenotoxic." These tests were as follows:

- Bacterial reverse mutation test (OECD Test 471) in *S. typhimurium* tester strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 at 0, 10, 100, 500, 1000, 2500, and 5000 µg/plate, with and without S9 metabolic activation;
- *In vitro* mammalian chromosome aberration test (OECD Test 473) in cultured human lymphocytes at 0, 10, 100, 500, 1000, 2500, and 5000 µg/ml, with and without S9 metabolic activation; and
- *In vivo* mammalian micronucleus test (OECD Test 474) in mouse bone marrow cells of 5 mice/sex/dose receiving 0, 500, 1000, or 2000 mg enzyme preparation/kg bw/ day for 2 days.

The safety of a water extract of Touchi, a traditional Chinese food obtained by *A. oryzae* (koji) fermentation of steamed soybeans, was assessed for mutagenic and genotoxic potential and subacute toxicity in OECD guideline-compliant research (Fujita and Yamagami 2007). In a bacterial reverse mutation assay with *S. typhimurium* tester strains TA98, TA1537, TA100, and TA1535 and *E. coli* WP2*uvrA*, Touchi extract was tested with and without metabolic activation at concentrations of 0, 313, 625, 1250, 2500, and 5000 µg/plate. The number of revertants did not increase to more than twice that of the negative control in any strain used, with or without S9 mix, and so did not exhibit mutagenicity under the conditions tested. Dose levels of 0, 500, 1000, and

2000 mg Touchi extract/kg bw/day were administered by gavage to 8-week-old male Sprague-Dawley Crl:CD specific-pathogen-free rats for 2 days in an *in vivo* bone marrow micronucleus test. No dose-dependent increase was reported in the incidence of polychromatic erythrocytes with micronuclei among Touchi-extract-treated groups, indicating that Touchi extract lacks clastogenic activity.

The subacute oral toxicity study (Fujita and Yamagami 2007) was performed with 5-week-old male and female Sprague-Dawley Crl:CD specific-pathogen-free rats assigned to 4 dose groups (10 rats/sex/dose) that received by gavage doses of 0, 250, 1000, or 2500 mg Touchi extract/kg bw/day for 28 days. Rats were individually caged and given free access to feed and water. Clinical observations were conducted twice a day and feed and water consumption and body weight were measured weekly. Urinalysis, clinical chemistry, hematology, necropsy, and histopathology analyses were all conducted as specified in OECD Guideline No. 407.

No clinical signs or changes in body weight or feed consumption related to the administration of Touchi extract were reported. No abnormal changes were reported in any urinalysis parameter. A statistically significant decrease was reported in mean corpuscular hemoglobin and mean corpuscular volume for males in the 1000 mg/kg bw group, whereas mean corpuscular hemoglobin concentration was statistically increased in these animals. However, these changes were considered to be unrelated to the test substance because no dose-dependent effects were noted and changes were within the range of background data. No other significant changes were reported in hematological parameters. Other than reductions in chloride levels in 2 groups of males and lower γ-glutamyltranspeptidase in low-dose females, both non-dose-dependent and judged non-toxico-logically significant, no significant effects on clinical chemistry parameters were reported. A statistically significant lower relative thymus weight was reported in mid-dose males, but was not dose-dependent and was within normal values. No other differences were reported in absolute or relative organ weights. No changes were reported upon pathological or histopathological examination other than unilateral pelvic dilation in the right kidney of one high-dose male, determined to be of spontaneous origin. The NOAEL for Touchi extract was determined to be 2500 mg/kg bw/day in male and female Sprague-Dawley rats.

In a prospective, randomized, double-blind, placebo-controlled study (Lim et al. 2015), 30 hyperlipidemic but otherwise apparently healthy adults (13 males, 17 females; mean age 42.0±7.7 years) were randomly assigned (n = 15 per group) to ingest 3 tablets a day for 12 weeks. Each test tablet provided 34.5 g/day of *A. oryzae*-fermented kochujang (Koji biomass, red powder, soybeans, starch, oligosaccharides, and salt that had been fermented for 15-30 days); the control tablets provided the same amount of placebo (rice flour, buckwheat flour, red pigment, caramel pigment, gardenia seed extract, salt, and spicy flavor). Fasting blood samples were drawn at baseline and study conclusion for measurement of total cholesterol, HDL cholesterol, LDL cholesterol, triacylglycerol, white blood cell count, red blood cell count, hemoglobin concentration, hematocrit, platelet count, total protein, albumin, creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase.

The volunteers ingesting the kochujang fermented by *A. oryzae* showed a significant reduction in total cholesterol and LDL cholesterol, with no significant changes in HDL cholesterol or triacylglycerol. The authors reported that, “At each of the clinic visit by the study subjects, the investigators interviewed subjects to discover any adverse events that had occurred since the previous visit. Except for one subject, no moderate or serious adverse events were reported during the 12-week study period. The evaluations were also expanded to include laboratory tests, electro-cardiogram, and measurements of vital signs (blood pressure and pulse) during the subjects' visits.”

6.4. Safety of Koji Mineral

6.4.1. Animal Studies

In a hemoglobin repletion study, Reddy and Armah (2018) fed 70 3-week-old male Sprague-Dawley rats weighing <100 g an iron-depleted diet for 24 days and then randomly assigned 10 rats/group to receive a control diet or diets providing 12, 24, or 36 mg iron/g feed from either Koji Mineral Fe-S or $FeSO_4$ for 14 days. Blood samples were taken from the control and the 2 high-dose groups and analyzed for hemoglobin, protein carbonyls, aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen. Rats were individually housed with free access to drinking water and weighed every other day; feed intake was also measured.

There was no difference between groups in pre- and post-repletion bodyweight or in pre-repletion hemoglobin concentration, but all groups receiving iron repletion except the Koji Mineral low-dose group had significantly higher post-repletion hemoglobin concentrations. There were no significant differences among the groups in weight gain or in aspartate aminotransferase, alanine aminotransferase, or blood urea nitrogen concentrations. Protein carbonyl levels (a measure of oxidative damage) were significantly higher than controls in the $FeSO_4$ groups; they were elevated, but not significantly, in rats receiving Koji Mineral. Based on increases in hemoglobin concentrations at different dose levels, it was determined that the relative iron bioavailability of Koji Mineral Fe-S was 60% of that of $FeSO_4$. The authors concluded that, "the above results showed no … toxic effects of Koji Mineral in liver, kidney, protein oxidation and growth within the study period."

6.4.2. Human Studies

Reddy et al. (2018) enrolled 16 apparently healthy women aged 20-28 years (mean age = 23.8±2.8 years) with serum ferritin levels <40 µg/L in a prospective, randomized, single-blind crossover study in which they received 10 mg elemental iron either as $^{57}FeSO_4$ or 2 mg ^{58}Fe and 8 mg natural abundance iron in Koji Mineral with a meal. After an overnight fast, they consumed a similar meal with the other form of iron supplement. Blood was drawn at baseline and 2 weeks after the second iron tracer administration for analysis of hemoglobin concentration, ferritin, C-reactive protein, and hepcidin. The second sample was also analyzed for erythrocyte incorporation of ^{57}Fe and ^{58}Fe.

The mean fractional iron absorption from Koji Mineral was 15.14±12.3%, while that from $FeSO_4$ was 17.18±14.2%; the difference was not significant. Iron absorption correlated negatively with serum ferritin and hepcidin concentrations, and women with high absorption of iron from one source tended also to have high absorption from the alternate source.

In a follow-up prospective, randomized, double-blind, placebo-controlled crossover study reported in the same article, Reddy et al. (2018) enrolled 17 apparently healthy women aged 18-31 years (mean age = 22.5±3.01 years). The 3 treatments, all provided as capsules taken with meals and administered in random order to different participants, were $FeSO_4$ providing 10 mg iron, Koji Mineral providing 10 mg iron, and Koji Mineral providing 20 mg iron. A blood sample taken before the meal was analyzed for serum iron concentration and C-reactive protein. After the meal, blood was drawn via an indwelling catheter every 30 minutes for 4 hours for measurement of iron concentration. This process was repeated 2 weeks apart with a different iron supplement consumed each time.

The area under the curve for serum iron concentration after ingestion of $FeSO_4$ providing 10 mg iron, 1674±376, was significantly higher than after intake of Koji Mineral providing 10 mg iron, 869±117, and Koji Mineral providing 20 mg iron, 900±193. C-reactive protein levels did not differ among the treatments. The authors suggested that the findings may indicate that iron from

Koji Mineral is released more slowly than that from $FeSO_4$ and concluded that this "may cause lower iron surges into the blood."

In a prospective, randomized, double-blind, placebo-controlled crossover study (Bries et al. 2018), 17 apparently healthy young women (ages not reported) with serum ferritin concentrations <40 µg/L consumed capsules providing 65 mg iron/day in the form of either $FeSO_4$ or Koji Mineral. Participants took one form of iron supplement for 3 weeks, followed by a 3-week placebo washout, and 3 weeks taking the other form of iron. Participants completed questionnaires regarding side effects (nausea, heartburn, abdominal pain, fatigue, headache, diarrhea, and constipation) during the study. Clinical chemistry measures included kidney function (blood urea nitrogen, creatinine, and estimated glomerular filtration rate), liver function (aspartate aminotransferase and alanine aminotransferase), oxidative stress (protein carbonyls and thiobarbituric acid reactive substances), and iron status (ferritin, hemoglobin, serum iron, iron saturation, and soluble transferrin receptor).

Compliance was assessed based on returned pills, and was higher with Koji Mineral (97.3%) than with $FeSO_4$ (93%), although this difference was not statistically significant. Reported nausea, abdominal discomfort, diarrhea, constipation, heartburn, and fatigue were non-significantly higher with $FeSO_4$ than with Koji Mineral. There was no difference between treatments in kidney and liver function indicators and oxidative stress. Transferrin saturation was significantly lower with Koji Mineral than with $FeSO_4$ and serum iron peaked an hour later with Koji Mineral than with $FeSO_4$ (at 4 hours vs. 3 hours), suggesting slower iron release. The authors concluded that, "[Koji Mineral] is a safe and effective iron supplement, without causing adverse side effects."

6.5. Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of Koji Mineral products is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of Koji Mineral products is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to Koji Mineral products under their intended conditions of use is not harmful. In the second step, the intended use of Koji Mineral products is determined to be GRAS by demonstrating that the safety of these products under their intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or organism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1. Data and information relied upon to establish the scientific element of safety must be generally available; and

2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the intended use of Koji Mineral products is safe and is GRAS.

6.5.1. Evidence of Safety

Genomic analysis of *Aspergillus oryzae* Koji strain established that the *aflR* gene is not expressed, showing that the strain cannot produce aflatoxin. No evidence of pathogenicity has been reported, and the species is generally regarded as non-pathogenic as well as non-toxigenic. The Koji strain of *A. oryzae* has been consumed in foods worldwide for many years; it is ubiquitous in miso, sake, amazake, and a large variety of other fermented foods. *A. oryzae* biomass, extracts, and products are widely used in animal feed supplements. Enzymes and other products of *A. oryzae* are presumed to be safe, based on a decision-tree analysis and extensive toxicological testing. The safety of Koji Mineral products was demonstrated in a single rat study and three human studies.

6.5.2. Conclusion of the Expert Panel

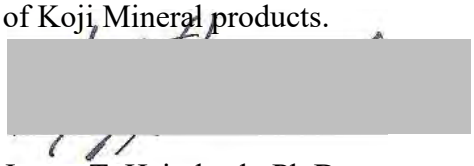
The intended use of Koji Mineral products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by genomic analysis of the Koji strain of *A. oryzae*, a long record of safe consumption of *A. oryzae* biomass and products, and studies of Koji Mineral products, concluding that the expected exposure to Koji Mineral products is without significant risk of harm. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the intended use of Koji Mineral products has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by James T. Heimbach, Ph.D., as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They independently critically reviewed and evaluated the publicly available information and the potential human exposure to Koji Mineral products anticipated to result from their intended use, and individually and collectively determined that no evidence exists in the available information on Koji Mineral products that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of Koji Mineral products.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion regarding the safety of Koji Mineral products under their intended conditions of use. Therefore, the intended use of Koji Mineral products is GRAS by scientific procedures.

6.6. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of GRAS status of the intended use of Koji Mineral products.



James T. Heimbach, Ph.D.

6.7. Conclusion of the Expert Panel

The intended use of Koji Mineral products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D. and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by James T. Heimbach, Ph.D., and other information deemed appropriate. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients.

The Expert Panel independently critically reviewed and evaluated the publicly available information summarized in this document, including the potential human intake resulting from the intended uses of Koji Mineral products, and have individually and collectively determined that Koji Mineral products produced in accordance with cGMP and complying with the specifications and use described in the GRAS monograph are GRAS (Generally Recognized As Safe) based on scientific procedures for addition to conventional foods at a maximum addition level of 250 mg/serving.

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia
Signature: [redacted] Date: 02 November 2018

Robert J. Nicolosi, Ph.D.
Professor Emeritus
University of Massachusetts—Lowell
Lowell, Massachusetts
Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin
Signature: _____ Date: _____

6.7. Conclusion of the Expert Panel

The intended use of Koji Mineral products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D. and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by James T. Heimbach, Ph.D., and other information deemed appropriate. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients.

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Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
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Signature: _____ Date: _____

Robert J. Nicolosi, Ph.D.
Professor Emeritus
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Lowell, Massachusetts
Signature: [redacted] Date: 04 Nov 2018

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin
Signature: _____ Date: _____

6.7. Conclusion of the Expert Panel

The intended use of Koji Mineral products has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D. and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by James T. Heimbach, Ph.D., and other information deemed appropriate. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients.

The Expert Panel independently critically reviewed and evaluated the publicly available information summarized in this document, including the potential human intake resulting from the intended uses of Koji Mineral products, and have individually and collectively determined that Koji Mineral products produced in accordance with cGMP and complying with the specifications and use described in the GRAS monograph are GRAS (Generally Recognized As Safe) based on scientific procedures for addition to conventional foods at a maximum addition level of 250 mg/serving.

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
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Signature: _____ Date: _____

Robert J. Nicolosi, Ph.D.
Professor Emeritus
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Part 7: List of Supporting Data and Information

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7.2. Unpublished

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From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: Supplement to GRN 000829 - Questions for Notifier
Date: Monday, April 6, 2020 2:44:04 PM
Attachments: image001.png
Hice Stephanie 20200406.pdf

As promised.

James T. Heimbach, Ph.D., F.A.C.N.
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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, April 6, 2020 12:47 PM
To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: Supplement to GRN 000829 - Questions for Notifier

Dear Dr. Heimbach,

During our review of the supplement to GRAS Notice No. 000829, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
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stephanie.hice@fda.hhs.gov

JHeimbach LLC

April 6, 2020

Stephanie Hice, Ph.D.
Staff Fellow (Biology)
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

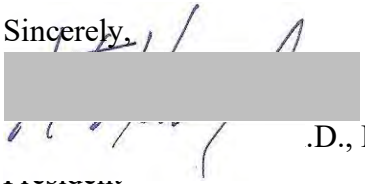
Dear Dr. Hice:

Earlier today you notified me that FDA reviewers of the supplement to GRN 000829 had requested an update of the relevant literature to cover the period since the original notice was submitted on November 28, 2018.

There is only one clearly relevant study (Bries et al., 2019), one of marginal relevance (Frisvad et al., 2018), one of interest but with no data regarding safety (Boczonadi et al., 2020), and several of essentially no relevance. This last category consists of research in which live *Aspergillus oryzae* were added to animal feed. I included one such study as an example, but do not believe that such studies contribute to determining the safety of the intended use of Koji mineral products.

The updated literature review appears on the next page.

Sincerely,



.D., F.A.C.N.

President

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Frisvad et al. (2018) reviewed the potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei* to produce mycotoxins and secondary metabolites. They explained that, "*Aspergillus oryzae* produce few recognized mycotoxins, and they are only produced by few strains. If they are produced, there are genetic means of inactivating the biosynthetic pathways, so isolates of the species can be exploited for production of enzymes and as a transformation host for industrially relevant secondary metabolites or enzymes." The authors' conclusion was that there are no safety concerns preventing the use of *A. oryzae* products.

In a prospective, randomized, double-blind cross-over study, Bries et al. (2019) gave 16 young females with low ferritin levels either $FeSO_4$ or iron-enriched *A. oryzae* biomass for 3 weeks. Participants completed questionnaires regarding side effects. Significantly fewer side effects such as nausea, constipation, or diarrhea were reported with iron-enriched *A. oryzae* biomass than with $FeSO_4$ and the authors concluded that "it is a safe oral iron supplement to treat IDA [iron deficiency anemia]."

Chuang et al. (2019) added *A. orzyae* and *Saccharomyces cerevisiae* to the diets of 240 broilers. Addition of these fungi had a number of beneficial effects and no adverse effects, leading the authors to conclude that they "can be suggested as a functional feed additive."

Boczonadi et al. (2020) did not administer *A. oryzae* biomass to humans or animals and so did not provide data regarding safety, but confirmed the efficiency of *A. oryzae* in accumulating minerals when grown in an enriched medium.

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From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: Supplement to GRN 000829 - Questions for Notifier
Date: Monday, April 13, 2020 2:34:49 PM
Attachments: image001.png

Dear Dr. Hice—

There is no change whatsoever in the identify and method of manufacture of *A. oryzae* or of the mineral enrichment. Absolutely the only change is the expansion of intended use to one more food category, meat analogs.

Regards,
Jim

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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, April 13, 2020 2:06 PM
To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: RE: Supplement to GRN 000829 - Questions for Notifier

Dear Dr. Heimbach,

For the administrative record, please state whether the identity and method of manufacture of powdered *Aspergillus oryzae* grown with one or more added minerals are the same as discussed in GRN 000829.

Thank you for your attention to our comments, and please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
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