Microorganisms

Handling/Processing

Identification of Petitioned Substance

Chemical Names:
There are many different microbial species used in processing and handling. Among the most common are: *Aspergillus oryzae.*, *Bacillus spp.*, *Bifidobacteria spp.*, *Pennicillium spp.* and *Rhizobus spp.*

Other Name:
N/A

Trade Names:

CAS Numbers:
Bacillus subtilis 68038-70-0
Bacillus coagulans 68038-65-3 *Lactobacillus bulgaricus* 68333-15-3
Lactococcus lactis 68814-39-1
Leuconostoc oenos 72869-38-6

Other Codes:
TSCA Flag XU [Exempt from reporting under the Inventory Update Rule]; TSCA UVCB

This technical report discusses the use of microorganisms in organic processing and handling. The focus of this report is the use of microorganisms in agricultural handling and processing of certified organic products such as probiotics, dairy and non-dairy fermented foods and beverages, bacteriophages, and as alternatives to sanitizers and cleaning agents for biological control. Yeasts are a type of microorganisms used in food production, but they are outside the scope of this technical report as they are listed separately on the National List. By-products and non-living components of microorganisms such as bacteriocins and enzymes are also outside the scope of this report.

Summary of Petitioned Use

Microorganisms are classified as nonagricultural (nonorganic) substances that are allowed as ingredients in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s)" (NOP Rule §205.605(a)). Any food grade bacteria, fungi, and other microorganisms are allowed for use without restrictions in processing & handling as stated at §205.605(a). Genetically modified/engineered microorganisms are prohibited, as stated at §205.105(c) & (e).

Characterization of Petitioned Substance

<u>Composition of the Substance:</u>
Microbial products are composed of identified organisms (OMRI 2013). Microbial products may be composed of a single strain, mixture of species, or contain a suite of microbes and their metabolites (Williams 2010); in some cases, the microbes provide a secondary function to the role of the metabolites (Stanton, et al. 2005). The European Food and Feed Cultures Association proposed the following definition, "Microbial food cultures are live bacteria, yeasts or molds used in food production" (Bourdichon, et al. 2012).

<u>Source or Origin of the Substance:</u>
Bacteria and fungi are ubiquitous in the natural environment, occurring in soils, water, air, and decomposing plant residue (Gest 2003). Microbial diversity is extremely high. An estimated 5 million microbial species exist; and between 20,000 and 40,000 species can be found in one gram of soil (Sylvia, et al. 2005). Abundance typically exceeds diversity; *Bacilli* populations range from 10^6 to 10^7 per gram of soil (EPA 1997a). The rhizobacterium *Bacillus subtilis* is the most studied gram-positive endospore-forming bacteria; several hundred wild-type strains have been collected (Stein 2005) and are most commonly found in soil (Sylvia, et al. 2005). Gram-positive microorganisms in soil were discovered as agents of fermentation

when buried vegetables such as cabbage fermented instead of spoiling, leading to "traditional peasant recipes for sauerkraut" (Dyer 2003). Microorganisms are also indigenous to the human body; "the colon is the main site of microbial colonization and, typically, the indigenous microbiota are considered to be made up of more than 500 different species of bacteria" (Tuohy, et al. 2003).

Microorganisms can be produced via natural and laboratory isolation methods or genetic engineering (Dyer 2003). Microorganisms have been isolated from vegetables, grains, and fruits; milk and yogurt; fermented products (Singh and Sinha 2012); and the human gut and breast milk (Williams 2010). Strains used for probiotics of human consumption have been isolated from the human gastrointestinal tract (Foulquié Moreno, et al. 2006). In the human colon, the main microbial species present are *Bifidobacterium adolescentis, B. bifidum, B. infantis, B. breve,* and *B. longum* (Champagne, Gardner and Roy 2005). Other *Bifidobacteria* species "have been isolated from fermented milk, the intestinal tracts of various animals and honeybees, and also found in sewage and anaerobic digesters" (Champagne, Gardner and Roy 2005). Lactic acid bacteria (LAB) can be isolated from a variety of natural habitats such as plant, dairy, and meat products; sewage and manure; and the intestinal tracts of humans and animals (Kahn, et al. 2011). Species of *Lactobacillus* can be isolated from the gastrointestinal tracts of animals and humans (Champagne, Gardner and Roy 2005; Kosin and Rakshit 2006). *Aspergilllus spp.* may be isolated from contaminated wheat, rice, and other grains; however, *A. oryzae* strains for industrial fermentations are typically from standard culture collections (EPA 1997b). *Penicillium spp.* can be isolated from soil, decaying organic matter, and plants (EPA 1997c). *Rhizopus spp.* are mass produced on rice in the fermentation industry (Esser & Bennett 2002).

<u>Properties of the Substance</u>:

Physical Properties

Because phenotypic information is subjective, microorganisms are grouped based on genetic variation (Woese, Kandler and Wheelis 1990). An American microbiologist, Carl Woese proposed the use of the small subunit ribosomal gene (ssu rRNA) for the classification of life in the 1970s (Ingraham 2010); ssu rRNA genes have a high degree of functional consistency, are easy to sequence, and all organisms possess these genes (Woese, Kandler and Wheelis 1990). Three groups or domains are used to classify all cellular organisms in the phylogenic tree of life: Archaea, Bacteria, and Eucarya (see Figure 1) (Ingraham 2010). Archaea and Bacteria are composed solely of microbes. Microbes that are ecologically and metabolically unique typify the group Archaea; for example, halophiles can live in environments with high salt concentrations and methanogens make methane gas (Ingraham 2010). Plants and animals belong to Eucarya. Of the three domains, only species within Bacteria are evaluated in this report.

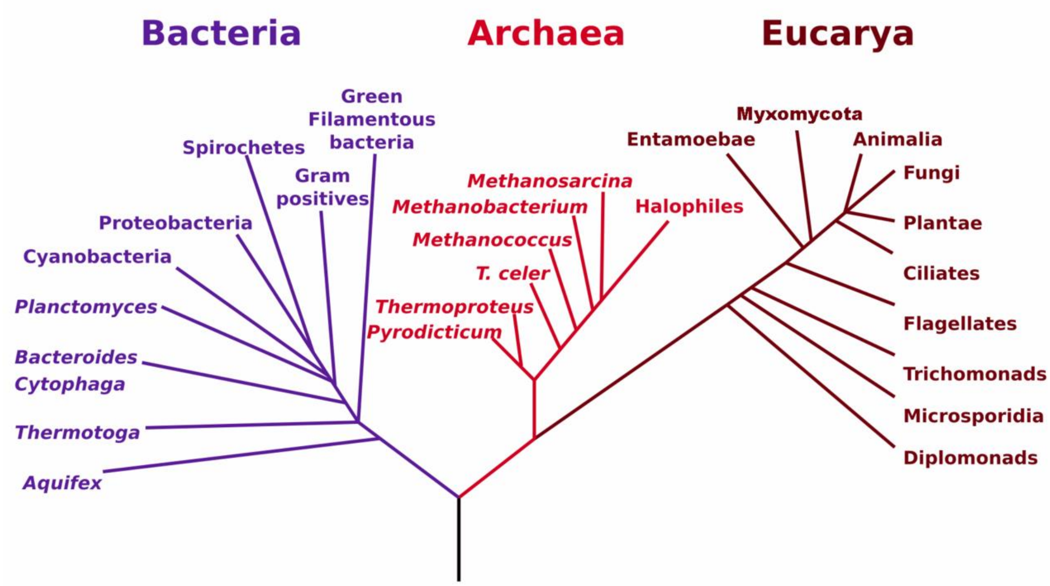

Figure 1. Phylogenetic Tree of Life

(New World Encyclopedia 2008)

Viruses and Bacteriophages

The scope of this technical report includes viruses and bacteriophages. Containing no cells, viruses are acellular and therefore are not classified in the phylogenetic tree of life (Ingraham 2010). Viruses are the most primitive form of life; they cannot reproduce independently of a host (Gest 2003). Viruses can multiply only inside susceptible living organisms. Once inside a cell, the virus replicates itself, kills the host cell, and thousands of virus particles spread to other cells. Bacteriophages are viruses that specifically infect bacteria (see Figure 2) (Sillankorva, Oliveira and Azeredo 2012).

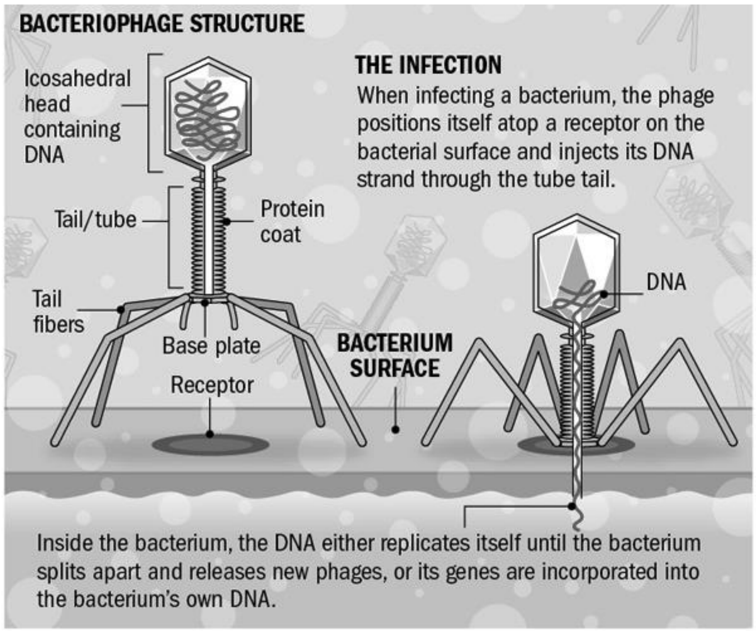

Figure 2. Physical attributes and biological action of bacteriophages
(Templeton 2013)

Bacteria

Bacteria are microscopic single-celled organisms with a cell wall, cell membrane, nucleoid, ribosomes, and flagella (Sylvia, et al. 2005). Flagella are used for motility. Microbial cell sizes range from 0.5-1μm wide and 1.0-1.2 μm long. The morphology of bacterial species is diverse, ranging from spherical (cocci), rod-shaped (bacilli), spiral-shaped (spirilla), or tightly coiled (spirochaetes) (Sylvia, et al. 2005). The bacteria within a genus typically have the same shape because microbial species were originally classified by phenotypic characteristics (see Figure 3). Bacteria reproduce by cell division and sporulation (Gest 2003). The production of endospores is an advantageous survival mechanism; soil populations of *Bacillus* can exist in the inactive spore state for many years (EPA 1997a).

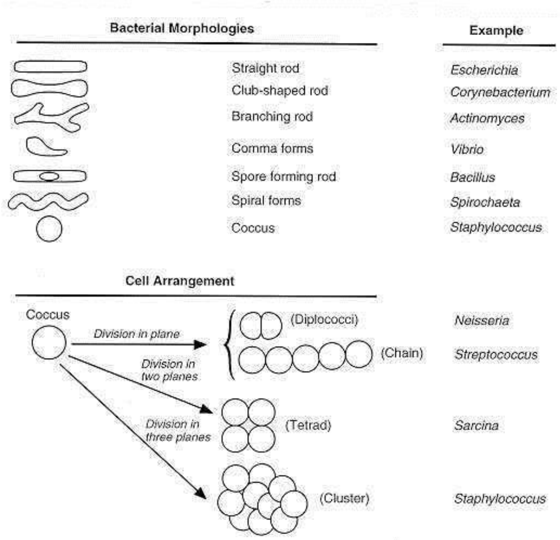

Figure 3. Typical shapes and arrangements of bacterial cells
(Rogers 1983)

In general, there are two major groups of bacteria, distinguished by cell wall type and named after the reaction of cells to the Gram stain identity test: gram-positive and gram-negative (see Figure 4) (Ingraham 2010). Gram-positive bacteria have a thick rigid cell wall that surrounds the cell membrane, which is not selectively permeable. This thick wall responds positively to a dye test and retains a purple color, distinguishing gram-positive cells from gram-negative cells (Ingraham 2010). A non-permeable outer membrane affords many ecological advantages such as greater osmotic shock, but it also increases antibiotic susceptibility (Sylvia, et al. 2005). Gram-positive bacteria typically have rod morphology, spore forming short rods, or cocci. *Bacillus*, *Clostridium*, and *Lactobacillus* are examples of gram-positive bacteria. Bacteria in the genus *Enterococcus* are gram-positive, non-sporeforming, facultative anaerobic cocci (Sylvia, et al. 2005). Examples of gram-positive bacteria genera used in industrial applications include: *Actinomyces, Actinoplanes, Arthrobacter, Bacillus, Brevibacterium, Clostridium, Corynebactrium, Lactobacillus, Lactococcus, Leuconostoc, Micrococcus, Mycobacterium, Nocardia, Propionibacterium, Streptococcus, Streptomyces* (Hansen 2011).

Gram-negative bacteria have a thin cell wall surrounded by an additional impermeable lipid membrane, the lipopolysaccharide layer (Gest 2003); this additional layer increases resilience to toxins in the environment and renders greater antibiotic resistance (Ingraham 2010). Examples of gram-negative bacteria genera used in industrial applications include: *Acetobacter, Acinetobacter, Agrobacterium, Alcaligenes, Azotobacter, Erwinia, Escherichia, Klebsiella, Methylococcus, Methylogphilus, Pseudomonas, Ralstonia, Salmonella, Sphinggomonas, Spirulina, Thermus, Thiobacillus, Xanthomonas, Zoogloea, Zymomonas* (Hansen 2011).

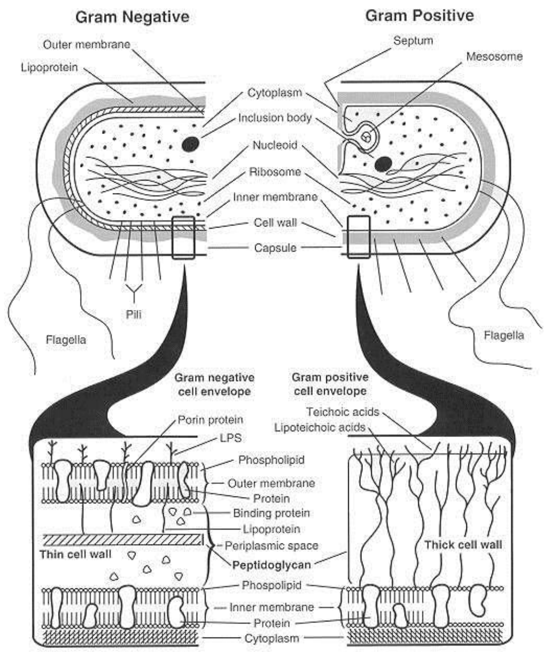

Figure 4. Physical attributes and differences between gram-positive and gram-negative bacteria (Rogers 1983)

Chemical Properties

Bacteria have broad metabolic capabilities; they can obtain carbon (C) from a variety of sources (Sylvia, et al. 2005). Bacteria can function as autotrophs (fix C from carbon dioxide (CO_2)) or heterotrophs (derive C from organic compounds) or chemotrophs (obtain energy from inorganic compounds, such as metals). Bacteria can use a variety of organic compounds as sources of energy (including highly complex and synthetic compounds), inorganic compounds, and electron acceptors (Sylvia, et al. 2005).

Heterotrophic bacteria that produce lactic acid as a byproduct of the consumption of carbohydrates for energy are commonly referred to as LAB (Lee, et al. 2011). *Lactobacillus* is the largest genus of LAB, followed by *Streptococcus* and *Enterococcus* (Foulquié Moreno, et al. 2006; Williams 2010; Franz, et al. 2011). Bacteriocins from LAB are divided into three major classes that include lantibiotics and bacteriocins with antilisterial effects (Teixeira de Carvalho, et al. 2006; Foulquié Moreno, et al. 2006). Bacteriocins are proteinaceous toxins produced by bacteria that prohibit the growth of similar or closely related bacterial strains (Foulquié Moreno, et al. 2006). The discussion of bacteriocin production by LAB is outside the scope of this report.

Fungi

The Fungi kingdom includes saprophytic and parasitic spore-producing eukaryotic (DNA is enclosed in nucleus) organisms that typically produce filamentous structures. They were formerly classified as plants that lack chlorophyll, but now are separated from plants. They include molds, yeasts, rusts, mildews, smuts, and mushrooms (Encyclopedia Britannica 2014). For the scope of this report, only those fungi considered to be “microorganisms” used in food production are included; thus, mushrooms are excluded from this report. Unlike plants, fungi do not make their own food energy via photosynthesis; instead, they derive energy from organic matter like rotting debris, and from the tissues of living plants and animals (American Society for Microbiology 2014). Fungi reproduce in several ways, but usually without sex. Yeasts reproduce asexually by budding, while fungi that produce hyphae may reproduce asexually when bits of the hyphae break off and grow as separate entitities. Less commonly, fungi may reproduce sexually by producing spores that mate and fuse together to form a spore stalk. Once the spores are free of the fruiting body, they may germinate wherever they find a suitable food source and conditions (American Society for Microbiology 2014).

Chemical Properties

The basis of all fungi is the hypha (mycelium). Hyphae are tubular structures surrounded by a rigid wall that separates the fungus from the environment. The walls are made up of a variety of components that support nutrient transport, metabolism, communication, and cell wall modifications. Typically, the walls contain small slender fibers or filaments that are bound together by sugars, proteins, lipids and polysaccharides. Approximately 80% of the wall consists of polysaccharides built on chitinous filaments. Proteins comprise the other 20%, mostly as glycoprotein (University of Sydney 2014). Some fungi produce metabolites that are used in food, such as colorants and citric acid.

Specific Uses of the Substance:

As stated, the focus of this report is the use of microorganisms in agricultural handling and processing of certified organic products such as probiotics, fermented foods and beverages, bacteriophages, and as biological control. Described as a probiotic effect (Gobbetti, Cagno and Angelis 2010), microorganisms are ingested directly in the form of supplements, prebiotics, probiotics, and symbiotics to increase microbial abundance and diversity in the gastrointestinal tract (Huffnagle 2007). Microorganisms can improve the taste, smell, and texture of food and provide health benefits to the consumer (Katz 2012). "The health benefits of fermented functional foods are expressed either directly through the interactions of ingested live microorganisms with the host (probiotic effect) or indirectly as the result of the ingestion of microbial metabolites synthesized during fermentation (biogenic effect)" (Gobbetti, Cagno and Angelis 2010). For a biogenic effect, food-grade bacteria are used for the bioconversion of food components into components with beneficial effects; the microorganisms are used as a mechanism to improve the palatability or nutritional value of food (Stanton, et al. 2005; Katz 2012). Microorganisms are added to a variety of food matrixes as starter cultures for the benefit of the metabolites produced during fermentation such as: fermented plant and animal products, Japanese sake, soy sauce, miso, tempeh, kimchi, cassava, vinegar, and cocoa (see Table 1) (Hansen 2011). Food-grade bacteria can also be used for improved vitamin production; raw food materials are often fortified with food grade bacteria that produce an excess of B vitamins *in situ* (Hugenholtz and Smid 2002). As biological control, bacteriophages are utilized as an antimicrobial to control bacteria during the production of foods on the farm, on perishable foods post-harvest, and during food processing (Greer 2005).

Microorganisms are utilized in a variety of applications not addressed in this report, including but not limited to: water treatment, energy production, odor control (OMRI 2012), biosynthesis of substances [chemicals, enzymes, vitamins, and sugars], bioremediation (Monachese, Burton and Reid 2012), food production and preservation, as biocontrol agents (OMRI 2012), and for improving agricultural fertility. Their capacity to supply nutrients to organic agricultural systems, such as the role of nitrogen-fixing bacteria in soils or their use as compost inoculants (OMRI 2012), is outside the scope of this report. With regard to food products, microorganisms are allowed for use in the production of dairy products, fermented soy products, tempeh, cheese, rice wines, xanthan gum, acetic acid, agar-agar, lactic acid, riboflavin, dextrans, sorbose, carbohydrase, breads, and flour (Esser & Bennett 2002; FDA 2013b). A flavor enhancer used in Asian cuisine, monosodium glutamate is produced from sugar by gram-positive *Corynebacterium* (Dyer 2003). Considered microorganisms, yeast are employed in the production of fermentation products such as beer and wine. Metabolites produced by microorganisms can be isolated and used in industrial scale vitamin production. The utilization of microorganisms for industrial scale vitamin production such as the production of Vitamin C by species of *Acetobacter* is also beyond the scope of this report. The use of microorganisms in dairy products, baked goods, and fermented beverages is common; however, it will not be addressed in this report because dairy cultures and yeast are listed separately at 205.605(a).

Table 1. Bacterial diversity of microorganisms with beneficial use and examples of primary uses in food production (Mantere-Alhonen, 1995; Hutkins 2006; Hansen 2011 and adapted from Bourdichon, et al. 2012)

Phylum	Family	Genus	Species	Primary Use
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	8	Probiotic supplements, yogurt, dairy, soy
	Brevibacteriaceae	Brevibacterium	3	Cheese
	Corynebacteriaceae	Corynebacterium	4	Cheese
	Dermabacteraceae	Brachybacterium	2	Cheese
	Microbacteriaceae	Microbacterium	1	Cheese
	Micrococcaceae	Arthrobacter	4	Cheese
		Kocuria	2	Cheese, dairy, meat, sausage
		Micrococcus	2	Cheese, sausage
	Propionibacteriaceae	Propionibacterium	5	Dairy, cheese; Production of vitamin B12, probiotic supplements
	Streptomycetaceae	Streptomyces	1	Meat
Firmicutes	Bacillaceae	Bacillus	3	Chocolate, yogurt, natto; Probiotics, fermented foods
	Carnobacteriaceae	Carnobacterium	3	Cheese, fish, meat, dairy
	Enterococcaceae	Enterococcus	3	Dairy, butter, cheese, cream, ham, miso, pickles, sausage, soy sauce, Manchego cheese
		Tetragenococcus	2	Miso, soy sauce, kimchi
	Lactobacillaceae	Lactobacillus	84	Fruit, vegetable products, sourdough bread, dairy, butter, yogurt, cheese, kefir, meat, fish, sausage, wine, rum, sake, cider, chocolate, chichi, kimchi, pickles, olive, cassava
		Pediococcus	3	Sausage, vegetable products
	Leuconostocaceae	Leuconostoc	12	Meat, fish, sauerkraut, coffee, kimchi, cheese, vegetable products, butter, pickles, buttermilk, wine, sour cream, olives
		Oenococcus	1	Wine
		Weissella	9	Cassava, kimchi, sausage, fish, chocolate
	Staphylococcaceae	Macrococcus	1	Cheese, sausage
		Staphylococcus	15	Cheese, sausage, soy, meat, fish, dairy
	Streptococacceae	Lactococcus	3	Buttermilk, chocolate, cheese, butter
		Streptococcus	3	Dairy, yogurt, meat
Proteobacteria	Acetobacteraceae	Acetobacter	9	Chocolate, vinegar, coffee, vegetable products

		Gluconacetobacter	9	Chocolate, coffee, vinegar
	Enterobacteriaceae	Hafnia	1	Cheese
		Halomonas	1	Meat
	Sphingomonadaceae	Zymomonas	1	Wine, pulque
Total Number of Species			**195**	

Probiotics

The bacterial species from the genera *Lactobacillus* and *Bifidobacterium* are most commonly used in probiotics (Williams 2010; Franz, et al. 2011), including: *Lactobacillus acidophilus*, *L. casei*, *L. reuteri*, *L. rhamnosus*, *L. johnsonii*, and *L. plantarum* and *Bifidobacterium longum*, *B. breve*, and *B. lactis* (Gupta and Abu-Ghannam 2011). Probiotics are administered orally via pharmaceutical preparations in the form of capsules, tablets, alginate gels, or dry powder (Franz, et al. 2011; Amalaradjou and Bhunia 2012) as dietary supplements (Williams 2010) or over the counter products.

The incorporation of probiotics into non-dairy foods is an increasing trend (Soomro, Masud and Anwaar 2002; Champagne, Gardner and Roy 2005). Probiotics are added to a variety of foods such as baked goods (Cutting 2011), meats (Kahn, et al. 2011), beverages (Gupta and Abu-Ghannam 2011), and snack foods such as muesli bars and chocolates (Franz, et al. 2011). Non-dairy probiotic foods are more challenging to produce and pose challenges to maintaining probiotic viability during heat treatment and unrefrigerated storage (Gupta and Abu-Ghannam 2011). Events that compromise the defense mechanisms afforded by naturally occurring healthy gut flora, like antibiotics, chemotherapy, or chronic disease "has led to the development of foods specifically designed to fortify gut microbiota" (Tuohy, et al. 2003).

Prebiotics are fiber food for bacteria (Gupta and Abu-Ghannam 2011). Prebiotics are not digested in the small or large intestine of humans; they provide food for probiotics in the colon and stimulate the growth of beneficial bacteria (Gupta and Abu-Ghannam 2011). Examples of prebiotic substrates are inulin, lactulose, various galacto, fructo, xylo-oligosaccharides and sugar alcohols (Soomro, Masud and Anwaar 2002; Kosin and Rakshit 2006). Symbiotics are a combination of prebiotics and probiotics (Franz, et al. 2011). "Many of the functional foods contain a combination of probiotic culture with a prebiotic substrate that favors its growth" (Soomro, Masud and Anwaar 2002). Paraprobiotics are "non-viable microbial cells" that are inactivated or dead microorganisms (Taverniti and Guglielmetti 2011).

Fermentation

Many bacteria are "involved in the manufacture and preservation of fermented feed and foods from raw agricultural materials (such as milk, meat, vegetables, and cereals) in which they are present as contaminants or deliberately added as starters in order to control the fermentation process" (Ammor, et al. 2007). Starters are added to preserve foods or to develop specific flavors or textures (Champagne, Gardner and Roy 2005). Bacteria are used to make yogurt, cheese, hot sauce, pickles, olives, fermented sausages and salamis, and dishes such as kimchi and sauerkraut (Caplice and Fitzgerald 1999). The use of LAB to preserve foods via fermentation is well documented (Caplice and Fitzgerald 1999; Dyer 2003; Leroy and De Vuyst 2003; Champagne, Gardner and Roy 2005; Ammor, et al. 2007; Kahn, et al. 2011; Katz 2012). The common fermenting bacterial species are members of the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Micrococcus*, and *Bacillus* (Gupta and Abu-Ghannam 2011). The LAB *Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Lactobacillus delbrueckii*, *Lactobacillus fermenti*, *Lactobacillus lactis*, and *Pediococcus cerevisiae* have been found to be responsible for many fermentation processes (Ammor, et al. 2007; Gupta and Abu-Ghannam 2011). Meat starter cultures are used for color and flavor, suppressing food pathogens (*Listeria*), lactic acid production, and acidification (Allied Kenco Sales 2013). *Bacillus spp.* are used in a variety of Korean traditional fermented soybean foods (Cho, et al. 2011). *Aspergillus oryzae* has been used for hundreds of years in the fermentation of soy sauce, miso, and sake (EPA 1997a). *Rhizopus spp.* are used to ferment and provide flavor and texture to a variety of Asian foods such as rice wine, sufu (toufuru) and tempeh (Esser & Bennett 2002). Bacteria in the genus *Enterococcus* are present in fermented foods such as cheese, olives (Foulquié Moreno, et al. 2006), and raw and cultured meat products such as sausage (Kahn, et al. 2011; Gazzola, et al. 2012). Present at high levels in a variety of traditionally fermented plant products, enterococci are tolerant of high pH conditions and of salt. The species *E. faecalis* and *E. faecium* are commonly found in Asian and African fermented plant products (Franz, et al. 2011). Poi, a traditional Hawaiian food made from taro root, is fermented by *Geotrichum spp.* and other yeasts and bacteria that occur naturally on the root and in the environment (Allen and Allen 1933; Brown and Valiere 2004). *Pencillium spp.* are added to ferment cheeses in order to produce pungent and unique flavors. Examples of cheeses fermented by *P. roqueforti* include Roquefort, Gorgonzola, Stilton Blue and Danish Blue. The crust on the outside of Brie and Camembert cheeses is the mycelium layer from *P. camemberti* (Seidl 2006). Various *Pediococcus spp*. work synergistically with other micrørganisms to control pH and enhance the flavor of the final product. Lambic beer for example, is acidic due to *Lactobacillus* and *Pedioccoccus spp* (Hui and Khachatourians 1995).

Bacteriophages

Bacteriophages are viruses that specifically infect bacteria. Bacteriophages are utilized as an antimicrobial to control bacteria during the production of foods on the farm, on perishable foods post-harvest, and during food processing (Greer 2005). Phages have been applied to control the growth of pathogens such as *Listeria monocytogenes*, *Salmonella*, and *Campylobacter jejuni* in refrigerated foods such as fruit, dairy products, poultry, and red meats (Greer 2005; Micreos 2013). Bacteriophage products are typically sprayed directly on food products prior to packaging (GRN 468; GRN 218).

<u>Approved Legal Uses of the Substance</u>:

FDA

As defined by the FDA, "*Microorganisms* means yeasts, molds, bacteria, and viruses and includes, but is not limited to, species having public health significance. The term 'undesirable microorganisms' includes those microorganisms that are of public health significance, that subject food to decomposition, that indicate that food is contaminated with filth, or that otherwise may cause food to be adulterated within the meaning of the act" (21 CFR Part 110.3(i)). Enzymes, microbes, and microbial products permitted for use by the FDA are listed at 21 CFR Part 173.11-173.170. Allowed food additives that are enzymes dominate this section of the regulation. One reference to permitted enzymes produced by microbes is present; the bacterial catalase derived from *Micrococcus lysodeikticus* is allowed for use in the manufacture of cheese (173.135). The FDA regulates the use of microorganisms in food products such as milk (21 CFR Part 131), cheese (21 CFR Part 133), and frozen desserts (21 CFR Part 135). A complete list of food ingredients composed of or derived from microorganisms that are GRAS is available online[1] and included in Evaluation Question #4 of this report. In the GRAS Notice Inventory, the FDA has no further questions about numerous microbial products used in food production (Table 6). For example, the active ingredient in the product Lactiguard™ manufactured by Guardian Food Technologies is GRAS (GRN 463). Marketed as functional foods that optimize digestive tract function, "Activate Muffins" containing a probiotic with a GRAS active ingredient, GanedenBC[30], were launched by Isabella's Health Bakery in 2008 (Cutting 2011; GRN 399).

FSIS

"Harmless lactic acid producing bacteria" are allowed for use "to prevent the growth of *Clostridium botulinum*" in bacon (FSIS, USDA §424.21). Similarly, "harmless bacteria starters of the acidophilus type, lactic acid starter or culture of *Pediococcus cerevisiae*" are allowed for use "to develop flavor in dry sausage, pork roll, thuringer, lebanon bologna, cervelat, and salami" (FSIS, USDA §424.21).

Bacteriophages

Bacteriophage products are typically sprayed directly on food products prior to packaging. Listeria-specific bacteriophages are permitted for use in a food additive that is applied directly to food (21 CFR Part 172.785). Liquid chemical sterilants such as this that are used solely in processed foods are regulated by the FDA, and are not considered pesticides by the EPA (40 CFR 152.6(a)). In 2011, the FDA listed a bacteriophage used to reduce *Escherichia coli* O157:H7 (EcoShield made by Intralytix) in the Inventory of Effective Food Contact Substances (FCN # 1018). Other bacteriophage products have received GRAS recognition. In 2013, the active ingredient used in SalmoFresh™ received GRAS affirmation for direct application onto "poultry products, fish, shellfish, and fresh and processed fruits and vegetables at 10^7 plaque-forming units per gram of food" to reduce strains of *Salmonella enterica* (GRN 435). Used to reduce *Listeria monocytogenes*, the active ingredient used in ListShield™ has self-determined GRAS recognition for use to treat smoked salmon prior to slicing. ListShield™ is also EPA Registered (EPA #74234-1).

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[1] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm

<u>Action of the Substance</u>:

Probiotics

Preparations of identified, viable (living) microorganisms in sufficient numbers to alter the microflora in an intestinal compartment of the host and bring beneficial health effects are referred to as probiotics (Gupta and Abu-Ghannam 2011). Species of *Lactobacillus* and *Bifidobacterium* are part of normal human intestinal microflora and are known to have a positive effect on human health by improving intestinal microbial balance (Soomro, Masud and Anwaar 2002). Probiotics such as *L. acidophilus* prevent the proliferation of pathogenic bacteria at the mucosal surface by out-competing them for nutrients or producing antibacterial compounds and by lowering pH through production of short chain fatty acids (Tuohy, et al. 2003; Saliminen, et al. 2010). "Probiotics act through suppression of viable count by production of antibacterial compounds, competition for nutrients and adhesion sites, alteration of microbial metabolites, and stimulations of immunity" (Soomro, Masud and Anwaar 2002). *Bacillus spp.* are used as probiotic dietary supplements due to their ability to stimulate the immune system and produce antimicrobial compounds that inhibit pathogenic microorganisms (Tuohy, et al. 2003).

Many factors determine probiotic food viability in food matrixes: pH, oxygen levels, storage temperature, and the presence of competing or inhibiting microorganisms (Soomro, Masud and Anwaar 2002). "The selection criteria for probiotic LAB include: human origin, safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonize the GIT, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity" (Soomro, Masud and Anwaar 2002). Species of microorganisms used as probiotics belong to the genera *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and *Saccharomyces* (see Table 2).

Table 2. Microorganisms used as probiotics organized by genus (Adapted from Soomro, Masud and Anwaar 2002; Champagne, Gardner and Roy 2005)

Genus	Species
Lactobacillus	*acidophilus* *plantarum* *casei* *casei* subsp. *rhamnosus* *delbreuckii* subsp. *bulgaricus* *fermentum* *reuteri*
Lactococcus	*lactis* subsp. *lactis* *lactis* subsp. *cremoris*
Bifidobacterium	*bifidum* *infantis* *adolescentis* *longum* *breve*
Streptococcus	*salivarius* subsp. *thermophilus*
Enterococcus	*faecalis* *faecium*
Saccharomyces	*boulardii*

Fermented Products

Fermentation is one way that microorganisms can change a food or beverage. Food processing methods rely on fermentation for a number of beneficial functions: preservation of food, food safety via inhibition of pathogens, improved nutritional value, and enhanced organoleptic properties (taste, sight, smell, touch) (Champagne, Gardner and Roy 2005; Bourdichon, et al. 2012). Bacterial populations are present on the surface of vegetables and increase after harvesting and with fermentation (Cho, et al. 2011). Present in raw meat, *Enterococcus faecalis* and *Enterococcus faecium* increase in number during fermentation (Gazzola, et al. 2012).

The primary preserving action of fermenting bacteria on foods is acidification (Champagne, Gardner and Roy 2005). Fermenting bacteria produce a suite of antimicrobial substances such as organic acids (lactic, acetic, or propionic), CO_2, diacetyl, and broad-spectrum antimicrobials (reuterin and bacteriocins) (Caplice and Fitzgerald 1999; Gupta and Abu-Ghannam 2011). Acetic acid inhibits yeasts, molds, and bacteria; propionic acid inhibits fungi and bacteria. High levels of CO_2 create anaerobic conditions that are toxic to aerobic microorganisms. Diacetyl is a product of citrate metabolism and is used primarily in the production of dairy foods. Produced by *Lactobacillus reuteri*, reuterin is an antimicrobial that inhibits ribonucleotide reductase, which is essential for DNA synthesis, in viruses, fungi, protozoa, and bacteria (Caplice and Fitzgerald 1999). Produced by fermentation with *Lactococcus lactis*, nisin is a lantibiotic bacteriocin effective against gram-positive bacteria, spore-forming bacteria, and food pathogens such as *Listeria monocytogenes* and *Clostridium botulinum* (Gupta and Abu-Ghannam 2011).

Fermentation occurs through the microbial oxidation of carbohydrates for energy in the absence of oxygen (Caplice and Fitzgerald 1999). "If vegetables are submerged, *Leuconostoc mesenteroides* initiates fermentation" (Katz 2012). Lactic acid bacteria are either homofermentative (producing exclusively lactic acid) or heterofermentative. In addition to lactic acid, heterofermentative bacteria produce significant quantities of secondary metabolites. For example, *Leuconostoc mesenteroides*, a heterofermentative bacteria, produces lactic acid, CO_2, alcohol, and acetic acid (Katz 2012). Various *Rhizopus spp.* produce different levels of lactic acid (0-65 g/l), fumaric acid (3-30 g/l), and ethanol (0-25 g/l) (Soccol, Stonoga and Raimbault 1994). A result of fermentation is that the food product is less hospitable to other microorganisms, including pathogens and spoilage-causing microorganisms, thereby extending the food's shelf life (Bourdichon, et al. 2012).

Aspergillus oryzae is the key microorganism in the culturing of various fermented foods such as miso, shoyu, and sake. It is used in solid state cultivation (cultivation on solid foods as opposed to liquids) and the resulting mold/food combination culture is also known as koji or koji mold. This solid state cultivation is thought to be the secret to high productivity of hydrolases[2] essential to the fermentation process (Mchida, Yamada and Gomi 2008). *Penicillium spp.* used in cheese-making lend flavor and texture by producing methyl ketones, secondary alcohols, lipoxygenase (an enzyme) and butyric acid. The blue-green mycelium veins contribute the classic characteristic of blue cheeses (Karahadian, Josephson and Lindsay 1985). *Geotrichum candidum* produces sulfur compounds which are also important for the maturation of cheese and formation of distinct flavors similar to cabbage or garlic (Damarigny, et al. 2000).

Bacteriophages

Bacteriophages are viruses that attack and utilize bacteria as hosts in order to reproduce (Gest 2003). Bacteriophages are obligatory parasites; they cannot reproduce without another organism. When a single phage particle attacks a single bacterium, the bacterial cell bursts after a short period of time, liberating 100 to 200 new phage particles (Gest 2003). The lifecycle of a bacteriophage can be employed as the active ingredient in antimicrobial products (OMRI 2012). Phages offer numerous advantages as biocontrol agents against food borne pathogens (Sillankorva, Oliveira and Azeredo 2012). Namely, bacteriophages have a high specificity to target their host and will continuously adapt to defense mechanisms developed by their host bacteria. Because viruses cannot reproduce without a host, phage replication is limited by the availability of a bacterial host, which enhances their overall antimicrobial impact. Phages consist of nucleic acids and proteins and therefore have a low inherent toxicity (Sillankorva, Oliveira and Azeredo 2012).

<u>**Combinations of the Substance:**</u>

[2] Hydrolases are any one of a class of 200 enzymes that catalyze the hydrolysis of several types of compounds (Encylcopedia Britannica 2014) .

Commercial starter cultures for use in formulated products such as probiotics or dairy and non-dairy food products are typically produced via fermentation; "the main raw materials used in the production [of lactic or probiotic strains] are microbial strains, milk powder, lactose, and yeast" (Kable 2013). There are different methods of probiotics stabilization: freezing, freeze-drying, spray drying, encapsulation, fluidized bed drying, and vacuum drying (Goderska 2012). Cultures are deep frozen using liquid nitrogen (cryo-freezing) (Kable 2013). The cultures are sold as single strains or mixtures of species that are frozen, freeze-dried, in liquid form, or as a "concentrated, deep frozen culture in pellet form for direct inoculation" (Chr. Hansen 2013; Danisco 2013; Kable 2013). Cyroprotectants used to freeze-dry microorganisms include liquid nitrogen, magnesium sulfate, and sodium aspartate (Kable 2013; OMRI Products Database 2013). Of the pure commercial starter culture specification sheets reviewed, "milk (including lactose)" was the only reported allergen in the frozen cultures (Chr. Hansen 2013; Danisco 2013).

Microencapsulation has recently been investigated as a technique to reduce viability loss and improve the stability of probiotic bacteria in probiotic food products (Mortazavian, et al. 2007; Soma, Williams and Lo 2009; Goderska 2012). As the name implies, microencapsulation involves coating microorganism cells "with hydrocolloids to segregate the cells from the surrounding environment" and conditions of low pH, bile salts, and temperature extremes caused by processing conditions of freeze drying and cryo-freezing (Goderska 2012). Components used in microencapsulation include: alginate, starch, xanthan gum, carrageenan mixtures, whey proteins, gelatin and chitosan (Mortazavian, et al. 2007). "A number of non-starch polysaccharides, namely carrageenan, xanthan gum, locust bean gum, guar gum, microcrystalline cellulose, and carboxymethyl cellulose can serve as texture modifiers that protect products…from a wide range of processing conditions and surviving in the gastrointestinal tract" (Soma, Williams and Lo 2009).

Following the fermentation and freezing of the active ingredient, inert ingredients are often added to create a formulated product. These inert or ancillary ingredients used in biological agents include sucrose, yeast extract, rice protein, and dextrose (OMRI 2012). Ancillary ingredients are those ingredients (e.g., carriers, stabilizers, and antioxidants) that are combined with the "active" ingredient or substance listed on the National List to provide a ***necessary*** technical effect on the National List substance. After fermentation of meat with starter cultures such as *Pedicoccus pentosaceus* and *Staphylococcus xylosus*, manufacturers recommend curing with salt, sodium nitrite, and sodium nitrate (Chr. Hansen 2013). Ancillary ingredients present in formulated products serve a specific technical function that enables the microorganisms to retain viability. *Aspergillus oryzae* is available for purchase as a freeze dried or liquid cell culture in a medium that may include salts, vitamins, amino acids, carbohydrates and water. Frozen cultures may also contain dimethyl sulfoxide as a cryoprotecant (American Type Culture Collection 2014). It can also be purchased cultured on organic and nonorganic milled rice. Organic koji is available from three certified operations (NOP, 2013). Some examples of ancillary ingredients as specified in technical data sheets or product descriptions for products currently on the market are presented in Table 3.

Table 3. Function and type of ancillary ingredients added to or remaining in microorganism preparations

by Food Additive Functional Class	
Anti-caking & anti-stick agents	magnesium stearate, calcium silicate
Carriers and fillers, agricultural or nonsynthetic	lactose, maltodextrins, sucrose, dextrose, potato starch, non-GMO soy oil, rice protein, grain (rice, wheat, barley) flour

Carriers and fillers, synthetic	micro-crystalline cellulose, propylene glycol, stearic acid
Preservatives	sodium benzoate, potassium sorbate
Stabilizers	maltodextrin
Cyroprotectants used to freeze-dry microorganisms	liquid nitrogen, maltodextrin, magnesium sulfate, dimethyl sulfoxide, sodium aspartate, mannitol, sorbitol
Substrate that may remain in final product	milk, lactose, grain (rice, barley, wheat) flour, brewed black tea and sugar, soy

The viability of microorganisms may be affected during food manufacturing, making the production of probiotic non-dairy food products more difficult than cultured dairy products (Gupta and Abu-Ghannam, 2011). "Some ingredients added to foods, such as salts, sweeteners, aroma compounds, and some preservatives (nisin, natamycin or lysozyme), may influence growth of probiotic bacteria" (Champagne, Gardner and Roy 2005). To protect anaerobic microorganisms from oxidative environments, processing aids such as sodium chloride, calcium chloride, sodium alginate, or epsilon-polylysine are added (GRN 440). Probiotic mixes and starter cultures appear to be the most common types of microorganisms that regularly contain ancillary substances. The most common ancillary substances in these probiotic mixes are cryoprotectants and standardizers or carriers (OMRI Products Database, 2013). Other microorganisms (*A. oryzae, Rhizopus spp*., dairy cultures) are mostly packaged and shipped on an organic or nonorganic carrier such as milk, soy, wheat, barley, rice, tea, etc. (Danisco 2014; Oregon Kombucha 2012). The carrier and microorganism combination is then added to the desired food to begin the fermentation process.

Some microorganisms appear to be available without any additional ancillary ingredients. For example, *P. roqueforti* appears to be available in dry form without any additional ingredients (Danisco, 2014; Fromagex, 2014), although some forms labeled for research purposes contain cryoprotectants (American Type Culture Collection 2014).

Status

<u>Historic Use</u>:

Probiotics

In the early 1900s, Eli Metchnikoff made the first observation of the positive role of certain bacteria, specifically intestinal microbes (Gupta and Abu-Ghannam 2011). The term probiotics was likely first used by Vergio in his 1954 manuscript comparing the detrimental effects of antibiotics to the beneficial effects of probiotics on gut flora (Franz, et al. 2011). In the past few decades, probiotics have been used as pharmaceutical preparations and as animal feed additives (Franz, et al. 2011). Probiotics used as feed supplements are termed direct fed organisms (AAFCO 2013). Specifically, enterococci probiotics have been used in slaughter animals to treat or prevent stress-induced diarrhea during transitions and improve immunity (Franz, et al. 2011). The World Health Organization Working Group and Joint Food and Agriculture Organization outlined parameters for the material. Specifically, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Franz, et al. 2011); and a "probiotic must be alive and deliver a measured physiological benefit, which is usually strain-specific" (Bourchodin, et al. 2012).

Fermentation

One of the oldest forms of biopreservation used by humans, fermentation has been used as a means of preserving perishable raw food materials since the Neolithic period (around 10,000 years BC), with records dating to 6000 BC in the Middle East (Soomro, Masud and Anwaar 2002). Throughout human history, the types of food produced via fermentation varied widely and were regionally dependent (Caplice and

Fitzgerald 1999). Food fermentation techniques and knowledge were passed down within local villages, religious groups, and governments (Caplice and Fitzgerald 1999). Captain James Cook utilized the preservation qualities of fermentation to prevent scurvy caused by vitamin C deficiency by bringing barrels of sauerkraut to sea (Katz 2012). The production of fermented alcoholic beverages is well known and goes back thousands of years (Caplice and Fitzgerald 1999), while soy fermented products were available in the early 1500s (Yamasa Corporation U.S.A. 2014). Roquefort is one of the oldest reported fermented cheeses, appearing in literature as far back as AD 79 (Masui and Yamada 1996). During the 1950's, the study of microbiology resulted in improved understanding of the mechanics behind fermentation and revealed that bacteria, yeast, and fungi were responsible for these chemical processes. Fermentation processes were tested and production rates increased in scale and efficiency (Caplice and Fitzgerald 1999). Currently, there are hundreds to thousands of identified strains of microorganisms used in food fermentation, including types of microorganisms not included in this report, such as yeast. Food fermentation is used for a variety of purposes, which includes increasing shelf life by acting as a preservative, and also for imparting flavor, texture, and health benefits (Katz 2012).

Bacteriophages

Bacteriophages were first described around 1915 by F.W. Twort and F. d'Herelle (Greer 2005). Likely the first published use of biological control, F. d'Herelle employed bacteriophages for treatment of dysentery caused by *Bacillus shigella*. Since their discovery as biocontrol agents, "phage prophylaxis" has been used to treat a variety of human and animal diseases (Greer 2005).

A number of bacteriophages have been approved for use in organic agriculture (OMRI 2012). A bacteriophage is a virus that infects and replicates within bacteria (Ingraham 2010). Examples of products with bacteriophages as the active ingredient allowed for use as processing aids are Listex P100, ListSheild, SalmoFresh™, and Bio-Save® 10NT Biological Fungicide (OMRI 2012). For example, Listex P100 contains bacteria-eating viruses that target and kill *Listeria monocytogenes* (ICT 2011) and has been shown to eradicate Listeria in ready-to-eat meat and cheese products (Marsden 2011). SalmoFresh™ can be used to kill *Salmonella* on meat and poultry, or other food items (Intralytix 2013). Resistant to several environmental conditions, *Listeria monocytogenes* is a LAB and foodborne pathogen that can cause illness and "infects primarily pregnant women, elderly, and newborns" (Teixeira de Carvalho, et al. 2006). Bio-Save® 10NT Biological Fungicide is a biological fungicide used in post-harvest handling (OMRI 2012). The active ingredients in the abovementioned products are GRAS and the final products are OMRI Listed. However, because the USDA classifies them as processing aids, they do not have to appear on the label when used as an ingredient within a formulated product (21 CFR 101.100(a)(3)).

Organic Foods Production Act, USDA Final Rule:

Microorganisms are not specifically addressed in the Organic Foods Production Act. However, all food grade bacteria, fungi, and other microorganisms are allowed on the National List at §205.605(a) and are considered nonsynthetic substances. Section 7 CFR §205.2 defines synthetic as "a substance that is formulated or manufactured by a chemical process or by a process that chemically changes a substance extracted from naturally occurring plant, animal, or mineral sources, except that such term shall not apply to substances created by naturally occurring biological processes." Therefore, microorganisms which are found in nature, but not chemically or genetically altered from their original form are considered nonsynthetic and are allowed for use in organic production and handling.

International

European Union

Article 9 of Council Regulation (EC) No. 834/2007 of 28 June 2007 states, "GMOs and products produced from or by GMOs shall not be used as food, feed, processing aids, plant protective products, fertilizers, soil conditioners, seeds, vegetative propagating material, micro-organisms and animals in organic production." Article 19 states, "The following conditions shall apply to the composition of organic processed food: …(b) only additives, processing aids, flavorings, water, salt, preparations of micro-organisms and enzymes...may be used, and only in so far as they have been authorized for use in organic production in accordance with Article 21."

"In addition, the products and substances referred to in Article 19(2)(b) are to be found in nature and may have undergone only mechanical, physical, biological, enzymatic or microbial processes, except where such products and substances from such sources are not available in sufficient quantities or qualities on the market."

Canada - Canadian General Standards Board Permitted Substances List
Microorganisms are permitted in organic processed foods as nonorganic ingredients that are not classified as food additives. This appears in 32.311 Table 6.4 as follows: "Microorganisms (processing derivatives) derived from genetic engineering or with the addition of chemosynthetic substance are prohibited."

CODEX Alimentarius Commission, Guidelines for the Production, Processing, Labeling and Marketing of Organically Produced Foods (GL 32-1999) Joint FAO/WHO Food Standards Programme
Microorganisms, probiotics, and enzymes are allowed for use as additives and processing aids. "Substances found in nature from biological/enzymatic processes and microbial processes (e.g., fermentation)" are allowed for use "as additives or processing aids in the preparation or preservation of food" (Section 5.1(c)). Any preparation of microorganisms can be used in food processing except those derived from genetic engineering (Table 3.4).

European Economic Community (EEC) Council Regulation, EC No. 834/2007 and 889/2008
Microorganisms and enzymes "normally found in food processing" are permitted for use (Article 2y(1)(b)).

Japan Agricultural Standard (JAS) for Organic Production
Microorganisms do not specifically appear in the JAS standard for Organic Processed Food (Article 3) nor are they considered food additives (Table 1). However, the JAS Standard includes the following language that indicates that microorganisms are allowed: "Only physical method or method using biological function (except for those produced by the recombinant DNA technology; hereafter the same) shall be used for the manufacturing or processing." (Article 4: Criteria of Production Methods – Management concerning manufacturing, processing, packaging, storage and other processes). The term "biological function" indicates the permitted use of microorganisms. In addition, microorganisms are referenced in the Questions and Answers section; "since culturing materials for microorganisms are not considered to be direct ingredients of organic processed foods, in cases where it is unavoidable, it is permissible to use microorganisms cultured with: materials other than organic plants, organic processed foods and organic livestock products" and "materials modified with recombinant DNA technology. However, should culturing materials for microorganisms be used in significant quantity (5% or more) in the manufacturing of processed foods, and remain there without being removed, said materials will be viewed as ingredients" (Japanese Agricultural Standards for Organic Plants and Organic Processed Foods Q21-15). The JAS Standard for Organic Processed Food also includes the following language that indicates that microorganisms are allowed: "Only physical method or method using biological function (except for those produced by the recombinant DNA technology; hereafter the same) shall be used for the manufacturing or processing." (Article 4: Criteria of Production Methods – Management concerning manufacturing, processing, packaging, storage and other processes). The term "biological function" indicates the permitted use of microorganisms.

International Federation of Organic Agriculture Movements (IFOAM)
The IFOAM standard states that, "in cases where an ingredient of organic origin is commercially unavailable in sufficient quality or quantity, operators may use non-organic raw materials, provided that: a) they are not genetically engineered or contain nanomaterials, and b) the current lack of availability in that region is officially recognized or prior permission from the control body is obtained." Section 7.2.5 states, "preparations of micro-organisms and enzymes commonly used in food processing may be used, with the exception of genetically engineered micro-organisms and their products. Cultures that are prepared or multiplied in-house shall comply with the requirements for the organic production of microorganisms." Section 7.2.6 states, "yeast shall be included in the percentage calculations of organic ingredients by 2013." Additionally, the section titled Preparations of Micro-organisms and Enzymes for use

in food processing states, "these may be used as ingredient or processing aids with approval from the control body: organic certified micro-organisms, preparations of micro-organisms…"

Evaluation Questions for Substances to be used in Organic Handling

<u>Evaluation Question #1</u>: Describe the most prevalent processes used to manufacture or formulate the petitioned substance. Further, describe any chemical change that may occur during manufacture or formulation of the petitioned substance when this substance is extracted from naturally occurring plant, animal, or mineral sources (7 U.S.C. § 6502 (21)).

Probiotics

Microbial products manufactured for use in organic cropping systems typically utilize fermentation or incubation to propagate microorganisms (OMRI 2012). The microbial species is either collected from a natural source or purchased from a company that specializes in maintaining strains of specific microbial species (Danisco 2013; Chr. Hansen 2013). Commercial starter cultures for use in formulated products such as probiotics or dairy and non-dairy food products are typically produced via fermentation; "the main raw materials used in the production [of lactic or probiotic strains] are microbial strains, milk powder, lactose, and yeast" (Kable 2013). The primary method to propagate microorganisms from the obtained seed inoculum involves growth on nutrient rich media substrate. Factors affecting the growth of microorganisms that are managed during fermentation or incubation include temperature, moisture content, pH, presence or absence of oxygen, and source of nutrients. Species of microorganisms can survive a range of conditions. For example, enterococci will grow at temperatures between 10-45°C, at pH up to 9.6, and can survive heating at 60°C for 30 minutes (Foulquié Moreno, et al. 2006). There is evidence that the culture of probiotics requires or frequently includes pH adjustment. For example, the manufacture of *Bacillus coaguluns* strain GBI includes a pH adjustment using ammonium or sodium hydroxide (GRN 399).

Both synthetic and nonsynthetic growth media components are used to provide carbohydrate and nitrogen sources. Examples of growth media include glucose, lactose, dextrose, peptones, yeast extract, corn and rice syrup, milk, soy, rice and other grains, and specific vitamins and minerals, etc. Media is removed via physical or mechanical methods such as centrifugation, filtration, or consumption; growth media removal can be complete or partial. Centrifugation is a physical process that involves spinning the microbial preparation at high speeds to separate components of various weights (such as separating microbes from media). This is the most common method of removing growth media. Microbial consumption of the growth media is also a very common removal step, but it is a less precise method. When growth media removal is impartial, it is usually for reasons related to the manufacturer's desired packaging, and to technical feasibility of the removal step. For microorganism products where the manufacturer intends for the growth media to be removed, it is still typical to have residues of <1-200 ppm leftover, or sometimes up to 2% of the formula (OMRI Products Database, 2013).

After production, microorganisms may be stabilized prior to packaging and/or freezing (Kable 2013). There are many different methods used to stabilize probiotics: freezing, freeze-drying, spray drying, spray freeze-drying, encapsulation, fluidized bed drying, and vacuum drying (Goderska 2012; OMRI 2012). Spray freeze-drying involves spraying "a solution containing dissolved/suspended material (e.g., protein) by an atomization nozzle into a cold vapor phase of a cryogenic liquid, such as liquid nitrogen, so the droplets may start freezing during their passage through the cold vapor phase, and completely freeze upon contact with the cryogenic liquid phase." The frozen droplets are then freeze-dried (Goderska 2012). As discussed earlier, ancillary ingredients may be used in microbial products.

Starter Cultures (Dairy, Soy, Rice, Kombucha)

Starter cultures are "microbial preparations of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process" (Hati, Mandal and Prajapati 2013). Starter cultures are typically cultivated on the same raw material that will later be fermented with the starter culture. For example, dairy cultures for making yogurt, buttermilk and kefir are cultivated on a milk or soy base. The same is true for fungi used in soy and tempeh production, where the microorganism is cultivated on a nonorganic or organic carrier such as rice flour, barley, wheat, or soy (Cultures for Health 2014; Yamasa Corporation U.S.A. 2014). In most cases, the starter growth medium is also the carrier in the final packaged commercial microorganism product. For example, koji mold may be cultivated and packaged on grains or soy, and this microorganism/grain or soy combination is then added to the food that will undergo further fermentation (GEM Cultures 2014).

Starter culture preparation varies depending on the desired microbial growth. For cultured milk products, a starter may be produced from frozen microorganisms. A typical propagation process is carried out in three phases: First, raw milk may be skimmed using a milk separator and then sterilized by heating to 100ºC for 10-20 minutes while stirring. The milk is then cooled to room temperature and 1-2 grains of freeze-dried culture per liter is added. The inoculated milk is left to incubate at room temperature for 16-24 hours. The second phase requires another inoculation of newly sterilized milk with the first phase cultured milk at a rate of 2-3% mixture. This new mixture is left to incubate for 16-24 hours. The third phase again inoculates sterilized milk with the second phase cultured milk. This third phase cultured milk is considered the "mother culture." The mother culture can be preserved by deep freezing, or fresh freeze dried culture can be purchased (FAO, unknown year).

Koji mold production is a similar process, where steamed grain is innoculated with *A. oryzae* spores (about 3 tablespoons of mold powder per 350 lbs of grain). A sample of this innoculated grain is spread over more steamed grain in a cooling box. The entire mixture is transferred to a "crib" where it is stirred to oxygenate the growing mold. The mixed grain is scooped in measured amounts into "koji trays" and stacked in a room for incubation at 90ºF with high humidity. After 48 hours of initial steaming and innoculation, the koji is harvested. Chunks of koji are broken up by passing them through a screen. The crumbled koji is mixed with sea salt, which preserves the enzyme and food value of the koji (South River Miso Company 2013). This koji mold may be combined with rice flour as a carrier to facilitate uniform measurements for final fermentation of sake, miso, or soy sauce. Producing a "scoby" or mother culture for kombucha is simpler; leave a jar of existing kombucha covered with cloth out at room temperature for approximately a week, and a thin layer of skin (the scoby) will form on the surface (Katz 2012).

Bacteriophages

The manufacturing of bacteriophages begins with the culture and fermentation of the host bacteria such as *Listeria innocua*, *Listeria monocytogenes*, or *Salmonella* (OMRI Products Database 2013). Host cultures can be fed a variety of substances for sources of energy: nutrient broths, sugar sources, or plant derivatives (OMRI Products Database 2013). Specific growth media components include: soy peptone, sodium chloride, yeast extracts (GRN 468), tripticase, and casamino acids (GRN 463). During the fermentation process the optimum oxygen level and temperature is maintained. Cell density is measured via photo spectrometry to determine when the targeted optical density (cfu/ml) has been achieved (OMRI Products Database 2013). Next, the host culture is infected with the phage stock and incubated for a period of hours to a day in order to propagate the bacteriophage (OMRI Products Database 2013). Phages are separated from the host cells and cell debris using physical removal methods such as filtration, centrifugation, or anion exchange chromatography (GRN 468). The concentration of the phages is modified as needed with the addition of water (GRN 468), or the mixture is suspended in saline (Intralytix 2012; GRN 435).

<u>**Evaluation Question #2**</u>**: Discuss whether the petitioned substance is formulated or manufactured by a chemical process, or created by naturally occurring biological processes (7 U.S.C. § 6502 (21)). Discuss whether the petitioned substance is derived from an agricultural source.**

Microorganisms are ubiquitous in the natural environment, occurring in soils, water, air, and decomposing plant residue (Gest 2003). Although microorganisms are classified as nonagricultural (nonorganic) substances, they can be derived from agricultural sources. Microorganisms have been isolated from vegetables, grains, and fruits; milk and yogurt; fermented products (Singh and Sinha 2012); and the human gut and breast milk (Williams 2010). Lactic acid bacteria can be isolated from a variety of natural habitats such as plant, dairy, and meat products; sewage and manure; and the intestinal tracts of humans and animals (Kahn, et al. 2011). "Lactic acid bacteria, most commonly *Leuconostoc mesenteroides*, are found on all plants, though in relatively low numbers, averaging below 1 percent of the plants' microbial populations;" microbial abundance and diversity increase after the plant is harvested (Katz 2012). Using high temperatures, *Lactobacillus spp.* can be isolated from chicken feces (Kosin and Rakshit 2006). Strains used for probiotics of human consumption have been isolated from the human gastrointestinal tract (Foulquié Moreno, et al. 2006). The intestinal isolates, *Lactobacilli* and *Bifidobacteria*, are probiotic strains that are commonly used in the production of sausages (Kahn, et al. 2011). Prebiotics are found in fruits, vegetables, and whole grains (Gupta and Abu-Ghannam 2011). Stable in many environments, bacteriophages can be sourced from soil and water; sewage and feces; farm and processing plant effluents; and foods (Greer 2005). "On fresh and processed meat and meat products, more than 10^8 viable phages per gram are often present….and [phages] are also especially abundant in the gastrointestinal tract" (GRN 468). Isolation of phages from food requires high concentrations of bacterial populations greater than 5 log cfu/g, meaning that isolation of phages specific to less pathogenic pathogens is more likely when using foods as the source (Greer 2005). Genetically modified source organisms are not allowed for use in organic processing and handling (NOP Rule §205.105(c) & (e)). Once isolated, microorganisms are propagated via naturally occurring biological processes such as fermentation (Singh and Sinha 2012).

<u>Evaluation Question #3</u>: If the substance is a synthetic substance, provide a list of nonsynthetic or natural source(s) of the petitioned substance (7 CFR § 205.600 (b) (1)).

Microorganisms are nonsynthetic. Since microorganisms are found in nature, not chemically or genetically altered from their original form, and are products of naturally occurring biological processes, they are considered nonsynthetic. However, microorganisms may come formulated with ancillary substances that are synthetic.

<u>Evaluation Question #4</u>: Specify whether the petitioned substance is categorized as generally recognized as safe (GRAS) when used according to FDA's good manufacturing practices (7 CFR § 205.600 (b)(5)).

Foods in use prior to the establishment of the Food, Drug and Cosmetic Act of 1958 were given default GRAS status; "Prior sanctions were granted for the use of harmless lactic acid producing bacteria, such as *Lactobacillus acidophilus*, as optional ingredients in specified standardized foods. These bacteria are permitted for use in cultured milk (which includes buttermilk) (§131.112), sour cream (§131.160), cottage cheese (§133.128), and yogurt (§131.200), provided that the mandatory cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* are also used in the yogurt" (FDA 2013a). Given the tradition of fermenting foods, the microorganisms used for fermentation prior to 1958 are also said to be GRAS (Ammor, et al. 2007; Bourdichon, et al. 2012). Since the establishment of the GRAS review, strains of microorganisms, bacteria, and yeast have been approved for specific uses. However, it is important to note that a microorganism is only GRAS for the stated food uses, since it is the use of the substance rather than the substance itself that is GRAS. This also applies to the GRAS determination for the concentration or dosage of microorganisms in a product (Bourdichon, et al. 2012). No regulations currently cover the use of active cultures in probiotics and the FDA has received few submissions for probiotics (Mattia and Merker 2008). FDA regulations on microorganisms used as food cover LAB, flavor-producing bacteria, and glucose-fermenting bacteria (Table 4; Mattia and Merker 2008).

Table 4. Types of microorganisms found at CFR Title 21 (Adapted from Mattia and Merker 2008)

Type of Microorganism	**Regulated uses in food (Regulation number)**
Harmless LAB	Sour cream and acidified sour cream (131.160; 131.162) Bread, rolls, and buns (136.110)
A "characterizing bacterial culture that contains the LAB *Lactobacillus bulgaricus* and *Streptococcus thermophilus*"	Yogurt: lowfat and nonfat (131.200; 131.203; 131.206)
Harmless flavor-producing bacteria	Cheeses (133) [separate regulations for hard grating, hard, soft ripened, semisoft, and semisoft part-skim cheeses]

Penicillium roqueforti	Appears as part of the requirements for specific standardized cheeses (Blue, Gorgonzola, Nuworld, Roquefort, and other blue-mold cheeses) (133.106; 133.141; 133.164; 133.184)
Glucose-fermenting bacteria	Dried egg whites (optional glucose-removal procedure (160.145)

Members of the genera *Lactococcus* and *Lactobacillus* are most commonly given GRAS status; members of the genera *Streptococcus* and *Enterococcus* and some other genera of LAB contain some opportunistic pathogens (Salminen, et al. 2010). GanedenBC^{30} is composed of the spore-forming probiotic, *Bacillus coagulans* (GBI-30, 6086), which received FDA GRAS status in 2012 (GRN 399). This was the first *Bacillus* strain to receive GRAS status (Cutting 2011). The approved probiotic ingredient is intended for use in a wide variety of foods, from baked goods to beverages, and can be ingested at high concentrations, up to 2 x 10^9 CFUs per serving (AIBMR Life Sciences 2011).

Microorganisms & Microbial Derived Ingredients Used in Food (Partial List)
Table 5 contains excerpts of GRAS affirmed substances that are microorganisms or derived from microorganisms (in 21 CFR part 184). "Conditions for their use are prescribed in the referent regulations and are predicated on the use of nonpathogenic and nontoxicogenic strains of the respective organisms and on the use of current good manufacturing practice (184.1(b)). Please be aware that not all GRAS substances have been recorded as such and so this does not represent a complete list of all microbial derived GRAS food ingredients" (FDA 2013b). Normally, microorganisms with GRAS status require no further testing if used under acceptable cultivation conditions (Waites, et. al. 2001). When reviewing a microorganism for GRAS allowance, "the FDA considers general aspects of safety (e.g., exposure and method of manufacturing), as well as taxonomy, pathogenicity, potential toxin production, antibiotic-resistance potential, safe history of use in food, reports of adverse events, metabolic considerations, environmental presence, and any other information deemed relevant to the safety assessment" (Mattia & Merker 2008).

Table 5. Generally recognized as safe (GRAS) affirmed substances listed in 21 CFR part 184 (FDA 2013a)

Section in 21 CFR	Ingredient or Substance
§184.1005	Acetic acid may be produced by fermentation
§184.1012	Alpha-amylase enzyme preparation from *Bacillus stearothermophilus* used to hydrolyze edible starch to produce maltodextrin and nutritive carbohydrate sweeteners.
§184.1027	Mixed carbohydrase and protease enzyme product derived from *Bacillus licheniformis* for use in hydrolyzing proteins and carbohydrates in the preparation of alcoholic beverages, candy, nutritive sweeteners and protein hydrolysates
§184.1061	Lactic acid may be produced by fermentation
§184.1081	Propionic acid from bacterial fermentation
§184.1115	Agar-agar, extracted from a number of related species of red algae class *Rhodophyceae*
§184.1318	Glucono delta-lactone, by oxidation of D-glucose by microorganisms that are nonpathogenic and nontoxicogenic to man or other animals. These include but are not restricted to *Aspergillus niger* and *Acetobactor suboxydans*
§184.1372	Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplane missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic
§184.1387	Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplane missouriensis*,

	Streptomyces olivaceus, *Streptomyces olivochromogenes*, and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic
§184.1388	Lactase enzyme preparation from *Candida pseudotropicalis* for use in hydrolyzing lactose to glucose and galactose
§184.1420	Lactase enzyme preparation from *Kluyveromyces lactis* (previously called *Saccharomyces lactis*)for use in hydrolyzing lactose in milk
	Lipase enzyme preparation from *Rhizopus niveus* used in the interesterfication of fats and oils.
§184.1538	Nisin preparation from *Lactococcus lactis* Lancefield Group N for use as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads.
§184.1685	Rennet (animal derived) and chymosin preparation from *Escherichia coli* K-12, *Kluyveromyces marxianus* var. *lactis* or *Aspergillus niger* var. *awamori* to coagulate milk in cheeses and other dairy products
§184.1695	Riboflavin biosynthesized by *Eremothecium ashbyii*
§184.1848	Butter starter distillate from milk cultures of *Streptococcus lactis*, *Streptococcus cremoris*. *Streptococcus lactis* subspecies *diacetylactis*, *Leuconostoc citovorum*, *Leuconostoc dextranicum*
§184.1924	Urease enzyme preparation from *Lactobacillus fermentum* for use in the production of wine
§184.1945	Vitamin B12 from *Streptomyces griseus*
§184.1985	Aminopeptidase enzyme preparation from *Lactococcus lactis* used as an optional ingredient for flavor development in the manufacture of cheddar cheese.

Agency has no Questions

Examples of substances considered GRAS that the FDA has no further questions about are listed in Table 6. For example, the FDA responded to a submission by Cargill regarding the use of the ingredient *B. animalis lactis* in foods [dairy foods; baked goods; fruits and fruit beverages; cereals; meat substitutes; fermented foods; preserves; candies; drinks] at a maximum level of 10^{11} cfu per serving. The agency had no further questions at that time (GRN 377). Similarly, *Bifidobacterium animalis* subsp. *lactis* strains HN019, Bi-07, B1-04, and B420 were determined GRAS for use in juice, dry beverages, bakery products, and confectionary (Jumppanen 2013; GRN 445).

Table 6. Examples of microbial and bacteriophage substances considered generally recognized as safe (GRAS) and their intended use in processed and ready-to-eat (RTE) food products (FDA 2013a)

GRN #	Substance	Intended Use	Status
468	Bacterial monophages	Antimicrobial to control *Salmonella* in meat and poultry	Pending
463	*Lactobacillus acidophilus* (NP28, NP51, NP7) *Pediococcus acidilactici* (NP3)	Antimicrobial to control pathogenic bacteria in raw and RTE meat products	Pending
445	*Bifidobacterium animalis* subsp. *lactis*	Ingredients in RTE breakfast cereals, bars, cheeses, milk drinks, bottled water, fruit juices, chewing gum, confections	No questions
440	*Lactobacillus reuteri* (NCIMB 30242)	Ingredient in beverages, breakfast cereals, cheeses, dairy products, fats and oils, grain products, processed fruits and juices, sugar substitutes	No questions
435	Bacterial monophages	Antimicrobial to control *Salmonella* in meat, poultry, and processed fruits and vegetables	No questions
415	Heat-killed *Propionibacterium freudenreichii* ET-3	Ingredient in beverages, breakfast cereals, cheeses, coffee and tea, fats and oils, gelatins, puddings, grain products, milk products, processed fruit, soft candy	No questions
399	*Bacillus coagulans* GBI-30, 6086	Ingredient in baked goods, beverages, breakfast cereals, chewing gum, coffee and tea, condiments, confections, fruit juices, gelatins, puddings, grain products, candy, spices, preserves, nut products, snack foods, syrups	No questions
378	Cultured sugars, wheat, malt fermented with strains of *Lactobacillus, Streptococcus,* and *Bacillus*	Antimicrobial agents in variety of foods including meat and poultry	No questions
357	*Lactobacillus acidophilus* NCFM	Ingredient in functional beverages, dairy products, nutritional powders, juices, bars, RTE breakfast cereals, chewing gum, confections	No questions
305	*Carnobacterium maltaromaticum* CB1	Inhibitor of *Listeria monocytogenes* on a variety of foods as viable or heat-killed microorganism	No questions
288	*Lactobacillus rhamnosus* HN001	Ingredient in various foods (beverages, cheeses, RTE cereals, energy bars, fruit juices, hard candies)	No questions
254	*Lactobacillus reuteri* DSM 17938	Ingredient in cheeses, yogurt, ice cream, fruit juices, processed vegetables, energy bars and drinks, chewing gum	No questions
240	Corn, cane, or beet sugar cultured with *Lactobacillus, Bacillus,* or *Propionibacterium*	Antimicrobial agent in meat and poultry products	No questions
218	Bacteriophage P100	Control of *Listeria monocytogenes* in foods in general and meat/poultry	No questions

Evaluation Question #5: Describe whether the primary technical function or purpose of the petitioned substance is a preservative. If so, provide a detailed description of its mechanism as a preservative (7 CFR § 205.600 (b)(4)).

Chemical food preservatives are defined under FDA regulations at 21 CFR 101.22(a)(5) as "any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties"(FDA 2013).The use of microorganisms in food production indirectly meets the definition of a preservative; the preservative qualities afforded by the process of fermentation, for example, are due to the byproducts of microbial metabolism or metabolites, not the strains of bacteria themselves (Champagne, Gardner and Roy 2005). Bacteria are inherently present on the surface of vegetables and in raw meat; the populations of bacteria increase during fermentation (Katz 2012). Traditionally, fermentation was used to preserve perishable foods in the absence of refrigeration, utilizing the preservation qualities of microbial metabolites such as lactic, citric, or acetic acid (Caplice and Fitzgerald 1999). The primary preserving action of fermenting bacteria on foods is acidification of the food material caused by the organic acid byproducts of fermentation (Champagne, Gardner and Roy 2005). Fermentative microorganisms produce a variety of antimicrobial substances such as organic acids (lactic, acetic, or propionic), CO_2, and bacteriocins and have been used as "protective cultures" in the food industry (Soomro, Masud and Anwaar 2002; Maragkoudakis, et al. 2009; Gupta and Abu-Ghannam 2011). Acetic acid inhibits yeasts, molds, and bacteria; propionic acid inhibits fungi and bacteria. High levels of CO_2 create anaerobic conditions that are toxic to aerobic microorganisms (Gupta and Abu-Ghannam 2011). "In modern societies, increasing consumer awareness and desire for natural products and processes emphasizes the concept of biopreservation as a natural alternative for food preservation" (Maragkoudakis, et al. 2009). A result of fermentation is that the food product is less hospitable to other microorganisms, including pathogens and spoilage-causing microorganisms, thereby extending the food's shelf life (Bourdichon, et al. 2012).

The preservation properties of bacteria are among a suite of beneficial functions afforded by microorganisms. As mentioned, the primary effects of bacteria include inhibition of pathogens, increasing nutritional value of foods, preventing disease, increasing host immunity, and improving the organoleptic properties. Preservation is not the primary technical function or purpose of microorganisms.

There is no literature to suggest preservatives used in microbial preparations as ancillary substances exert any technical or functional preservative effect in the final fermented product. Typically, Good Manufacturing Practices (GMP) dictate that preservatives are added at a maximum level of 0.1% by weight of the finished product to exert the desired effect (FDA 2013b). Using a hypothetical microbial starter that has 0.1% preservatives added, the preservative present in the final product can be calculated. Microbial starters and probiotics are usually added at a very small percentage to the raw food. For example, to inoculate soybeans with *Rhizobus spp.* a ratio of 1:768 parts microbial preparation to raw soybean is employed (Cultures for Health 2014). This indicates that the microbial product is 0.13% of the fermented product, and thus, the preservative from the microbial preparation is 0.00013% or 1.3 ppm in the final cultured product.

Evaluation Question #6: Describe whether the petitioned substance will be used primarily to recreate or improve: Flavors, Colors, Textures, or nutritive values lost in processing (except when required by law) and how the substance recreates or improves any of these food/feed characteristics (7 CFR § 205.600 (b)(4)).

A review of the literature did not indicate that microorganisms are primarily used to recreate or improve flavors, colors, or textures lost in processing. However, an effect of fermentation is the increased palatability of raw foods by the 'pre-digestion' of bacteria (Katz 2012). "Food may be preserved, but its composition is altered by the digestive processes of the organisms involved. Organic compounds are metabolized into more elemental forms. Minerals become more bioavailable, and certain difficult-to-digest compounds are broken down." Soy protein is broken down into smaller amino acids that are more readily assimilated during digestion. When milk is cultured with LAB, the bacteria convert lactose into lactic acid. The enzymatic digestion of fermenting bacteria tenderizes meat and fish. (Katz 2012). Compared to the raw, unprocessed food product, fermentation can improve the flavor and digestibility of certain foods such as grain, legumes and cassava (Gupta and Abu-Ghannam 2011). Microbial cultures can provide or improve the beneficial traits of food products by improving flavor, texture, or smell (Bourdichon, et al. 2012). Enterococci occur in olive fermentations and facilitate the breakdown of the bitter oleuropein compound (Franz, et al. 2011). "LAB contribute to the organoleptic, rheological, and nutritional properties of fermented feed and foods" (Ammor, et al. 2007). *P. roqueforti* and *P. camemberti* are both used in cheese production to directly create the desired texture and flavor of the product (Karahadian, Josephson and Lindsay 1985).

There is no literature to suggest that microbial preparations with ancillary substances improve or recreate flavors, colors, textures, or nutritive values in the final product. For example, dairy cultures on milk substrate are added to vats of milk to further ferment the raw product; the milk substrate is blended in and fermented along with the rest of the milk. The same is true with grain flour that will be distributed along with the microbes when inoculating soy and rice for soy sauce, miso, and sake.

Evaluation Question #7: Describe any effect or potential effect on the nutritional quality of the food or feed when the petitioned substance is used (7 CFR § 205.600 (b)(3)).

Probiotics

Live cultures in probiotics are used to improve nutrition by improvement of immunity and gut health of the human host (Rodgers 2008; Cutting 2011). Microbial food cultures can provide or improve the beneficial traits of food products by increasing the nutritive value (Bourdichon, et al. 2012). Microorganisms produce amino acids, fatty acids, and certain vitamins that are absorbed when the food product is consumed (van Boekel, et al. 2010). The microbial activity may also reduce the content of anti-nutrients, substances present in certain foods (e.g., pulses [legumes], cereals, vegetables), which interfere with the absorption of nutrients. "Reducing the content of such components enhances absorption of nutrients from the food and thereby increases its nutritional value. One example is sourdough, which contains lactic acid bacteria with the ability to eliminate phytate. Phytate is an anti-nutrient present in wholegrain flour, which, through its capacity to form complexes with minerals, may prevent absorption in the intestine of essential nutrients such as calcium, iron, zinc and magnesium. The bioavailability of minerals is thus higher in sourdough bread than in bread leavened by yeast only" (van Boekel, et al. 2010). Probiotics used in cultured dairy products have been found to improve digestibility of the food product (Katz 2012). Certain lactic acid bacteria produce mannitol and sorbitol during fermentation, which lend low calorie sugars in fermented milk (Hati, Mandal and Prajapati 2013).

The literature suggests that microbial preparations with ancillary substances have very little effect on the nutritional quality of the food. The only clear effect that ancillary substances in microbial preparations have on the nutritional quality of food is to help to maintain the viability of the microbe during processing, packaging, and shipping (Wowk 2007). For example, without the use of cryoprotectants, the viability of live probiotics would decrease significantly. The compromised microbial preparation may have deleterious effects on the concentration and variability of the probiotic content of the final processed food to which it was added. There is no literature to suggest that preservatives in microbial preparations have any effects on the nutritional quality of the final fermented food.

Fermentation

The oxidation process of fermentation is incomplete; therefore, fermented foods "retain sufficient energy potential to be of nutritional benefit to the consumer" by either preserving or increasing the initial nutrient content of the raw food product (Caplice and Fitzgerald 1999). As utilized by Captain James Cook who prevented scurvy by bringing barrels of sauerkraut to sea, the fermentation process preserves the vitamin C content of vegetables (Katz 2012).

The fermentation process increases the availability of nutrients in plant and animal products by improving their digestibility (van Boekel, et al. 2010). The use of lactic acid fermentation in carrot juice production was shown to improve iron solubility 30 fold (Gupta and Abu-Ghannam 2011). Lactic acid fermentation of beetroot juice with three different *Lactobacillus* species improved the nutritive properties of the product while acting as a preservative; the fermented beetroot juice had optimum levels of vitamins, minerals, and pigments (Gupta and Abu-Ghannam 2011). Fermentation of soybean paste by the CS90 strain of *Bacillus subtilis* has been shown to increase the total phenolic content of the food; in addition, antioxidant and free radical scavenging activity increased significantly during fermentation (Cho, et al. 2011). "Fermentation pre-digests foods, making nutrients more bioavailable, and in many cases fermentation generates additional nutrients or removes anti-nutrients or toxins" (Katz 2012).

Biogenic microbial metabolites derived from fermentation increase the nutritional value of fermented foods (Stanton, et al. 2005). "Many bacteria used in food fermentations possess the biosynthetic capability to produce folate" (Stanton, et al. 2005). The levels of B vitamins increase in fermentation products, including thiamin (B_1), riboflavin (B_2), and niacin (B_3), when compared to the raw vegetables prior to fermentation (Katz 2012). Nutritionally significant levels of vitamin B_{12} were found in tempeh (fermented soybean) and ontjom (fermented peanut) that were not present in the bacterium or raw food alone (Liem, Steinkraus and Cronk 1977). Fermentation increases the concentration of amino acids; for example, the fermentation of cereal grains increases the availability of lysine, an essential amino acid (Katz 2012). Some microbial strains over-produce methionine, which is often deficient in vegetarian diets (Gobbetti, Cagno and Angelis 2010). However, commercial biosynthesis of methionine has not been successful (Kumar and Gomes 2005). Unlike the fermentation waste products of lactic and citric acid, the synthesis of methionine requires energy, adenosine triphosphate (ATP). As a result, overproduction of methionine is "tremendously wasteful to the microorganisms and only the methionine needed for growth is produced" (Kumar and Gomes 2005).

Manufacturing descriptions also seem to indicate that carriers help standardize microbial count within a desired measurement, so that the correct amount of microbes are used for the desired nutritional effect (such as formation of metabolites to ferment food) (Cultures for Health 2014; Danisco 2014; GEM Cultures 2014).

<u>Evaluation Question #8</u>: List any reported residues of heavy metals or other contaminants in excess of FDA tolerances that are present or have been reported in the petitioned substance (7 CFR § 205.600 (b)(5)).

Under the Toxic Substances Control Act (TSCA), microorganisms such as *Leuconostoc oenos* and *Bacillus subtilis*, *Bacillus coagulans*, *Lactobacillus bulgaricus*, *Lactococcus lactis*, and *Leuconostoc oenos*, are listed as exempt (TSCA Flag XU) from reporting according to the Inventory Update Rule (40 CFR Part 170), which was most recently updated in 2003 (Berger 2003). The TSCA inventory does not cover chemical substances addressed by other U.S. statutes such as foods and food additives.

Microorganisms have been employed as alternative solutions for decontaminating environmental sites with high concentrations of heavy metals such as lead, cadmium, arsenic, chromium, and mercury (Monachese, Burton and Reid 2012). Chemolithotrophic bacteria use inorganic sources of energy, such as metals, for the production of ATP. Bacterial cells are capable of binding large quantities of different metals (Mullen, et al., 1989) present in soil, water, and even the human gut (Monachese, Burton and Reid 2012). The negative charge of the bacterial cell wall adsorbs the cationic (positive) charge of many metals. This is especially true of gram-positive bacteria, which have a high peptidoglycan and teichoic acid content in their cell walls, and results in a high adsorptive capacity. Gram-negative bacteria contain lower proportions of these cellular wall constituents and are poorer metal absorbers (Mullen, et al. 1989). Recent research highlights the possibility of *Lactobacillus* for use in heavy-metal biosorption in the human body (Monachese, Burton and Reid 2012).

<u>Evaluation Question #9</u>: Discuss and summarize findings on whether the manufacture and use of the petitioned substance may be harmful to the environment or biodiversity (7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i)).

Fermentation

The EPA final risk assessments of *B. subtilis* and *B. licheniformis* in 1997 concluded that the use of this bacterium in fermentation constitutes a low potential risk to human health or the environment. The bacterium is ubiquitous in the environment, and human infections have only occurred in immunosuppressed individuals or post trauma (EPA 1997a). Toxicology studies performed on the strain *Bacillus coagulans* GBI-30, 6086, used in GanedenBC®, that evaluated whether the bacteria has mutagenic properties were negative (GRN 399). Most self-affirmed GRAS substances cite the long history of safe use of the specific microorganism in food production (GRN 305, 257, 378, 440). The EPA final risk assessment for *P. roqueforti* (1997c) indicates that environmental hazards of potential release of the fungus to the environment are low. It is not known to be a pathogen of plants nor soil organisms. The only anecdotal evidence of potential harm occurred when moldy silage was fed to animals and caused abortion. However, it is not known whether *P. roqueforti* was the specific fungus to affect the animal. Similarly, the EPA Risk Assessment for *A. oryzae* (1997b) indicates low risk for environmental damage. The main concern was the potential for toxin production by *A. oryzae* strains, although commercial strains do not seem to produce significant levels. It is only when extended culture and fermentation is carried out that increased risk of toxin contamination is observed, and this contamination only has negative effects on animals, rather than on plants.

There is no literature to suggest that the manufacture or use of microbial preparations with ancillary substances is harmful to the environment or biodiversity. The Select Committee on GRAS Substances opinion on sodium benzoate (1973) indicates that there is no evidence to show that sodium benzoate as a food ingredient constitutes a hazard to the general public when used at levels prescribed in 21 CFR 184.1733. Potassium sorbate is also considered to have little to no effect on the environment. The European Chemicals Agency (ECHA) found that it is readily biodegradable and has low potential for bioaccumulation (European Chemical Agency 2011). Carriers and culture mediums are food-grade materials and thus pose little to no environmental damage.

Bacteriophages

Designed to target only the cells of their host species (GRN 468), bacteriophages "generally do not cross species or genus boundaries, and will therefore not affect (a) desired bacteria in foods (e.g., starter cultures), and (b) commensals in the gastrointestinal tract, or (c) accompanying bacterial flora in the environment" (GRN 218). Bacteriophages are widely distributed in the environment, estimated in numbers exceeding 10^{31} virus particles. Bacteriophages and their decomposition products of amino and nucleic acids occur naturally in the environment (GRN 218).

Evaluation Question #10: Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i)) and 7 U.S.C. § 6518 (m) (4)).

The beneficial effects of probiotics are widely supported in the literature; these benefits include antimutagenic effects, anticarcinogenic properties, improvement of lactose metabolism, antimicrobial activities, and immune system stimulation (Soomro, Masud and Anwaar 2002; Foulquié Moreno, et al. 2006; Paturi, et al. 2007; Gobbetti, Cagno and Angelis 2010; Gupta and Abu-Ghannam 2011). Oral administration of *Lactobacillus acidophilus* and *Lactobacillus paracasei* was found to increase levels of immunoglobulin A, interleukin-10, and interferon gamma producing cells in the gut immune system of mice while inhibiting the growth of *Listeria monocytogenes* (Paturi, et al. 2007). Preliminary studies indicate that probiotics can contribute to the reduction of allergy development in children (Gupta and Abu-Ghannam 2011) and are used in strategies for decreasing phenomena of food intolerance (e.g., gluten) (Gobbetti, Cagno and Angelis 2010). Incidences of diarrhea caused by antibiotic therapy and travel-related indigestion can be alleviated and reduced with probiotics (Tuohy, et al. 2003; Foulquié Moreno, et al. 2006). Other documented benefits of probiotic use include: reduced risk of colon cancer, reduced cholesterol, and amelioration of lactose malabsorption symptoms, irritable bowel syndrome, colorectal cancer, candida, and urinary tract infections (Tuohy, et al. 2003; Foulquié Moreno, et al. 2006).

Scientific evidence indicates that inactivated microbes can positively affect human health, causing a response from the host comparable to the host's immune response to live, viable microbes (Taverniti and Guglielmetti 2011). Since the FAO/WHO definition of probiotic can only be used to describe products that contain live microorganisms, the new term 'paraprobiotic' has been used for products that contain inactivated microbial cells or cell fractions that confer a health benefit to the consumer (Taverniti and Guglielmetti 2011).

The occurrence of adverse effects caused by microorganisms in fermented food has been rare and dependent on either the food matrix or the susceptibility of the host (Bourdichon, et al. 2012). "The widespread use of LAB and bifidobacteria in fermented foods and dairy products has a long history of safety, and it is generally assumed that the risk of infection from ingested bacteria is very low (Ammor, et al. 2007). In a healthy population, an estimated 0.5/1 million *Lactobacillus* infections occur per year (Bourdichon, et al. 2012). Infections have been reported in immune-compromised patients with significant underlying problems, "specifically central venous catheter in place, metabolic disorders, organ failure, or invasive procedures such as dental work" (Bourdichon, et al. 2012).

Safety studies on probiotic bacteria evaluate components such as infectivity and pathogenicity (Table 7; Zhou, et al. 2000); microbial translocation and infection (Taverniti and Guglielmetti 2011); and gene transfer of antibiotic resistance (Gupta and Abu-Ghannam 2011). A study of three LAB strains with immune-enhancing and anti-infection properties reported "no effect on the animals' general health, haematology, blood biochemistry, gut mucosal histology, or incidence of bacterial translocation" (Zhou, et al. 2000). A study of LAB species identified 17 *Lactobacillus* isolates that were resistant to one or more antibiotics (Klare, et al. 2007). Although LAB used for several centuries in the fermentation of food products are considered GRAS by default, it is recommended that newly isolated strains of LAB are evaluated for their safety prior to use in food production (Zhou, et al. 2000; Salminen, et al. 2010).

A major concern with the use of microorganisms in food production is their potential to transfer antibiotic resistance to pathogenic bacteria (Ammor, et al. 2007). "The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial populations may play a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria." Enteroccoci and non-enterococcal LAB and bifidobacteria have been evaluated for the presence of antibiotic resistant genes and the susceptibility of "transfer to human pathogenic bacteria during food manufacture or during passage through the gastrointestinal tract" (Ammor, et al. 2007). Because of the association of enterococci with human diseases and antibiotic resistance, industrial applications of the bacteria have been slow to develop (Franz, et al. 2011; Gazzola, et al. 2012). *E. faecium* SF68 and *E. faecalis* Symbioflor 1 have a long history of safe use as probiotics; similarly, "there are no reports to date on diseases caused by enterococci probiotics that are currently on the market" (Franz, et al. 2011). Other strains of enterococci act as opportunistic pathogens and can cause nosocomial infections in immune compromised individuals or those with underlying disease (Franz, et al. 2011). LAB and strains of enterococci that are common in foods of animal origin have exhibited both intrinsic and transferable resistance to antibiotics (Ammor, et al. 2007; Gazzola, et al. 2012). Studies on the occurrence of virulence factors in bacteria have demonstrated that these factors are strain-specific (Ammor, et al. 2007; Franz, et al. 2011). Therefore, an important food safety criterion is the absence of transferable antibiotic resistance in individual strains evaluated for use in food production (Franz, et al. 2011).

According to the EPA Final Risk Assessments for *P. roqueforti* and *A. oryzae* (1997c and 1997b), the potential for human health hazards is low. The main concern in both fungi is their potential for producing mycotoxins[3]. *P. roqueforti* produces many mycotoxins but only two are considered to be of concern: roquetine and PR toxin. Animal exposure data for these mycotoxins indicate decreased motor activity and respiration rate, and hind leg weakness in rats. There have been no reported cases of actual human harm beyond a possible allergic reaction by a worker in a blue-cheese production facility. *A. oryzae* on the other hand is very closely related to *A. flavus*, which produces dangerous "aflatoxins." However, *A. oryzae* has

[3] Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease of death in humans and animals (Bennett and Kilch 2003).

not been found to produce the same aflatoxins as *A. flavus*; therefore it is not considered to be an increased concern based on this relation. *A. oryzae* does produce other mycotoxins, especially when left to ferment for longer periods than are typical for koji molds. It can produce kojic acid, maltoryzine, cylcopiazonic acid, and beta-nitropropionic acid. These mycotoxins can be controlled by following standard fermentation practices that reduce the incubation period for koji molds. There is no literature detailing any adverse effects of *Rhizopus spp.* used for tempeh production.

Table 7. Classification of probiotic organisms according to virulence and pathogenic potential (Adapted from Salminen, et al. 2010)

Organism	Infection Potential
Lactobacillus	Mainly non-pathogens, some opportunistic infections (usually in immune-compromised patients)
Lactococcus	Mainly non-pathogens
Leuconostoc	Mainly non-pathogens, some isolated cases of infection
Streptococcus	Oral streptococci mainly non-pathogens (including *Streptococcus thermophilus*); some may cause opportunistic infections
Enterococcus	Some strains are opportunistic pathogens with haemolytic activity and antibiotic resistance
Bifidobacterium	Mainly non-pathogens, some isolated cases of human infection
Saccharomyces	Mainly non-pathogens, some isolated cases of human infection

There is no literature to suggest that microbial preparations with ancillary substances have negative effects on human health. However, there are studies indicating that preservatives added as ingredients in final processed products do have an adverse effect on human health, especially in reference to Attention Deficit Hyperactivity Disorder (ADHD). Bateman, et al. (2004) found that there was a general adverse effect of benzoate preservatives on the hyperactivity behavior in preschool children, while Beezhold, Johnston, and Nochta (2012) found increased reporting of ADHD symptoms in college students that drank sodium benzoate rich beverages. There have been a number of studies from the 1960's on dimethyl sulfoxide (DMSO, cryoprotectant) used as a drug. The toxicology of DMSO was called into question after lenticular (eye lenses) changes occurred in animals that received DMSO; however, subsequent studies showed that DMSO at acute exposure rates is a relatively non-toxic compound (Rubin 1975). The FDA currently permits DMSO in the manufacture of sucrose fatty acid esters and sucrose oligoesters (direct food additives), as long as the total dimethyl sulfoxide content is not more than 2 parts per million (FDA 2013).

<u>Evaluation Question #11</u>: Describe any alternative practices that would make the use of the petitioned substance unnecessary (7 U.S.C. § 6518 (m) (6)).

The consumption of probiotics, the processes of fermentation, and the use of bacteriophages are alternatives to conventional practices and would not be possible without the use of microorganisms. Fermentation is a natural process that inhibits the growth of certain virulent microorganisms through the process of microbial interference (Caplice and Fitzgerald 1999). Without microorganisms, fermented food products and probiotics would be impossible; therefore, these processes and products are essential to organic production.

For microbial preparations with ancillary substances, there are alternative practices to using nonorganic carriers and/or growth substrates for cultures. Specifically, nonorganic carriers can be replaced with organic carriers and growth substrates. Certification agencies differ in whether growth substrates and carriers are required to be organic. This practice may include creating cultures in the same facility on organic products, instead of purchasing nonorganic microbial preparations.

The use of preservatives in microbial preparations appears to be limited to probiotic mixtures, and research indicates that their use is rare, even among this subset of microbial products. Alternative practices include those that are currently employed in the marketplace such as freeze drying. However, freeze drying requires the use of cryoprotectants in most cases (Wowk 2007).

<u>Evaluation Question #12</u>: Describe all natural (non-synthetic) substances or products which may be used in place of a petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (ii)). Provide a list of allowed substances that may be used in place of the petitioned substance (7 U.S.C. § 6518 (m) (6)).

Microorganisms provide an alternative to food preservatives that are not allowed for use in organic food processing and handling. Microorganisms replace chemical preservatives like sorbate, benzoate, lactate, and organic acids, such as citric acid, used in cooked sausages (Danisco 2013). Effective as antimicrobials, selected strains of *Enterococcus* and *Lactobacillus*, when applied as protective cultures on raw meat reduce the growth of the common food pathogens, *Listeria monocytogenes* and *Salmonella enteritidis* (Maragkoudakis, et al. 2009). In the livestock industry, "the use of LAB and bifidobacteria species as probiotics may help to reduce antibiotic use for therapeutic, prophylactic, and growth promotion in animal husbandry" (Ammor, et al. 2007). In some cases, such as in production of blue-mold cheeses, the use of microorganisms is essential to making an authentic product.

Microorganisms provide an alternative to cleaning agents, sanitizers, and antimicrobial products that are not allowed for use in organic food processing and handling. Antimicrobial agents approved for use by FSIS in meat and poultry products include: potassium lactate, sodium diacetate, sodium lactate, and trisodium phosphate (FSIS, USDA §424.21). Bacteriophages provide an alternative to broad-spectrum antimicrobials (Greer 2005) and "are considered for use as natural, 'green' decontaminating agents effective in reducing or eliminating contamination of various inanimate surfaces…contaminated with pathogenic bacteria" (Anderson, et al. 2011). The specificity of bacteriophage activity for one species of bacterial host provides a more targeted approach to food preservation (Greer 2005).

<u>Evaluation Information #13</u>: Provide a list of organic agricultural products that could be alternatives for the petitioned substance (7 CFR § 205.600 (b) (1)).

No alternatives to the petitioned substance were found among current organic products used for food processing and handling. While microorganisms are not commercially available in organic form, microorganisms are considered a non-agricultural substance. Similar to yeast, microorganisms can potentially be produced organically, depending on substrate and nutrient inputs.

For those microorganisms formulated with ancillary substances, the most common alternative to nonorganic carriers and growth media such as milk, soy, and other agricultural substances is to substitute an organic carrier or growth medium. For example, dairy cultures may be started on organic milk, koji mold on organic rice, and kombucha scoby on organic tea and sugar. There is no literature discussing the pros and cons of using nonorganic carriers and growth media for starter cultures as opposed to organic, therefore it is not known whether this is a feasible alternative. However, certification agencies differ in whether organic carriers or growth substrates are required in microbial preparations. Common standardization agents for certain starters, such as maltodextrin, may also be certified organic as an alternative to nonorganic standardizers. There are currently 16 certified entities that produce organic maltodextrin (NOP 2013).

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