6.4.2.2. Studies in Children and Infants with *Bacillus subtilis* other than R0179

To our knowledge there are no other documented studies describing the use of *B. subtilis* in infants or children. *B. subtilis* fermented soy (natto) is commonly fed to children in some countries such as Japan without adverse reactions.

6.4.2.3. Conclusions from Studies in Children and Infants

Data obtained from the clinical studies of the Medilac-Vita (Mamaia), which was administered to infants at doses providing *B. subtilis* R0179 at a range of $1.5x10^7$ to $9.0x10^7$ cfu/day, did not show any serious adverse event reports or complaints regarding the consumption of this product in neonates or infants.

A total of 2,011 infants received this product without any adverse effects that were observed which could reasonably be believed to be attributable to the treatment, indicating that the strain *B. subtilis* R0179 is safe for use in infants.

6.4.3. Animal Studies

6.4.3.1. Studies of *Bacillus subtilis* R0179 in Animals

One safety study with this strain was done in rats (Tompkins et al. 2008). This study was performed by Evic-Tox (Blanquefort, France) in accordance with animal ethical rules in the European Directive 86/609/EEC (European Union 1986) and was submitted to the internal animal ethics committee located at the facility. Ten male and 10 nonpregnant female specific pathogen-free Sprague–Dawley albino rats (Charles River Laboratories, 69592 L'Arbresle, France) aged 6–7 weeks were divided equally into 2 groups. One group received a concentrated suspension of *B. subtilis* R0179 at $2x10^9$ cfu/kg bw/day for 28 consecutive days while the control group was gavaged with an equal volume of vehicle. Animals were monitored daily for potential signs of toxicity and groups compared for mortality, morbidity, behavior, body mass, food consumption, anatomopathology, intestinal colonization, and infection. These parameters included observation for potential changes in skin, fur, eyes, mucous membranes, secretions, excretions, autonomic activity (lacrimation, piloerection, pupil size, and unusual respiratory patterns), changes in gait, posture, and handling response, as well as for the presence of clonic or tonic movements or bizarre behavior. The sensory reactivity to auditory, visual, and proprioceptive stimuli, grip strength, and motor activity were also assessed. At the end of the treatment, animals were necropsied, and the liver, kidneys, spleen, heart, and lungs subjected to histopathological and microbiological examination. Terminal portions of the small and large intestine from 4 males and 4 females per group were removed and sent to the Institut Supérieur des Techniques Agroalimentaires de Bordeaux (Bordeaux, France), where microbial contents were evaluated on PCA Difco medium.

No outward indications of toxicity or oral intolerance were observed in animals receiving long-term delivery of microbes nor were variations in body mass, feed consumption, or mortality observed in any group. No visible lesions or changes to organ mass were observed in gross post-mortem examination, except for lower heart mass in female rats receiving *B. subtilis* R0179. The organ mass/total animal mass ratios were not affected by treatment. In one control animal, slight lesions

of the liver (focal extra- medullar hematopoiesis and biliary stasis) and spleen (megacaryocyte hyperplasia) were noted. In microbial examination of the treated animals, *B. subtilis* were not observed in the liver, kidneys, spleen, or heart, although they were found at high levels ($1.5x10^6$ – $1.2x10^7$ cfu/g) in the intestinal content of all animals treated with this microbe.

There have been five published studies done in rats with *B. subtilis* R0179 but in combination with *Enterococcus faecium* R0026 (Guo et al. 2006; Guo et al. 2007; Yu et al. 2006; Yuan et al. 2004, Yu et al. 2008). Guo et al. (2006 and 2007) examined the prevention and treatment of acute gastroenteritis induced by *Shigella flexneri*. In this model, as a preventative the combination maintained the ratio of Treg / CD4+T cells in the rats but the treatment worked best when combined with antibiotics.

Yu et al. (2006) studied a nonalcoholic steatohepatitis model. Yu et al. (2008) showed that the impact of the combination in this model was related to the decrease in TNF-alpha (i.e., pro-inflammatory cytokine) and increase in peroxisomal proliferator activated receptor (PPAR)-gamma expression. Yuan et al. (2004) demonstrated that the mortality rate of the cirrhotic rats was not improved with treatment.

Recently, Wu et al. (2019) explored the mechanical action in intestinal flora caused by *B. subtilis* R0179. Male ICR mice between 6 and 8 weeks of age were divided into four groups which received different treatments. Chronic colitis-associated colon cancer (CAC) was induced by azoxymethane (AOM)/dextran sodium sulfate sodium (DSS) administration. On the day of the first AOM injection, the AOM/DSS + *B. subtilis* and Control + *B. subtilis* groups of mice were orally administered 10^9 cfu *B. subtilis* R0179 per animal each day until the end of the experiment. The mice of AOM/DSS and Control groups were orally administered sterile water. No adverse events were reported associated with *B. subtilis* R0179.

6.4.3.2 Studies of other *Bacillus subtilis* Strains in Animals

Several studies have been completed in a variety of animals as the use of *B. subtilis* as a feed additive has been accepted practice in Europe and North America for some time (EC Directorate-General for Veterinary and International Affairs 2011). The safety of three other strains of *B. subtilis* intended for use in humans has been evaluated (Hong et al. 2008; Sorokulova et al. 2008). The acute and chronic toxicity of these strains was tested in mice, guinea pigs, rabbits, and piglets and an attempt was made to determine the oral LD_{50}. Hong et al. (2008) examined the safety of two *B. subtilis* strains, PY79 (a prototrophic strain derived from *B. subtilis* 168) and a strain isolated from Japanese natto. The toxicity studies were done only on the natto strain; a short-term continuous exposure study was done in New Zealand white rabbits and an acute single dose study was done in guinea pigs. In the rabbit study, a suspension of 10^9 spores in 1 mL saline was given daily by gavage for 30 days. At the end of this period, blood was taken for hematological analysis and samples of various visceral organs and tissues (liver, kidneys, spleens, small intestines, and mesenteric lymph nodes) were collected for histological analysis. There were no adverse effects on the general health status of the animals or their feed intake. There were no observable changes to the visceral organs and

tissues, nor were there significant differences in the hematological indexes in blood from control and treated rabbits. In the acute study in guinea pigs, the animals were given a single dose of 10^{12} spores in 1 mL of saline. The animals were observed daily for behavior, appearance, and activity, and feces were collected for 14 days. Body weight was measured throughout. Blood was taken on day 17 by cardiac puncture from anesthetized animals for hematological analysis. Samples of the visceral organs and tissues (liver, kidneys, spleens, small intestines, and mesenteric lymph nodes) were collected for histological analysis. Comparison of treated and control (saline only) animals revealed a significant increase in weight gain in the female group at day 14 for those receiving natto spores. Histological analysis of organs and tissues revealed no signs of inflammation or pathological changes and there were no differences in the hematological indexes between the control and treated guinea pigs. The authors concluded that there were no signs of toxicity and that it was difficult to establish the LD_{50} due to the difficulty of physical constraints of providing higher doses of the bacteria.

Sorokulova et al. (2008) evaluated the strain *B. subtilis* VKPM B2335 (aka BS3) in acute toxicity studies in mice and short-term repeated-dose toxicity studies in mice, rabbits, and piglets. The acute toxicity studies were done by administering intravenously and intraperitoneally doses of $5x10^7$, $5x10^8$, and $5x10^9$ cfu/mouse and orally $5x10^7$, $5x10^8$, and $2x10^{11}$ cfu/mouse. Animals were observed for seven days before being euthanized. Histological analysis was done on organs and tissues. There were no deaths in any of the groups at any dose; thus, the LD_{50} was deemed to be greater than $2x10^{11}$ cfu. There was no evidence of inflammation or other pathological changes in the organs and tissues analyzed. Repeated-dose toxicity was studied for a period of ten days in mice at a dose of $1x10^6$ cfu/day, and in rabbits and piglets at doses of $1x10^9$ cfu/day. On day 11 the animals were euthanized and internal organs and tissues were removed for histological evaluation. In addition, one group of rabbits was dosed at $1x10^9$ cfu/day for 30 days before being sacrificed. There were no adverse effects on the general health, nor changes in organs or tissues. Hematological indexes did not significantly differ from the control animals.

From the toxicity studies of four different *B. subtilis* strains, including R0179, in mice, guinea pigs, rabbits, and pigs, there were no adverse events, inflammation, or pathogenesis in either acute or repeated-dose toxicity experiments. Studies in mice, chickens, and pigs showed that the spores can germinate, and the resulting vegetative cells can stimulate an immune response in the host.

6.4.3.3. Conclusions from Studies in Animals

The notified strain *Bacillus subtilis* R0179 has been widely studied in a variety of animal models. In none of these studies has administration of the strain evidenced indications of toxicity or pathogenicity. In other toxicological studies on *Bacillus subtilis* strains including R0179, there were no adverse events, inflammation, or pathogenesis in either acute or repeated-dose toxicity experiments.

6.5. Safety Evaluations of *Bacillus subtilis* by Authoritative bodies

The *Bacillus subtilis* microbial strain and substances derived from this microorganism were subjects of evaluation by different authoritative bodies for their safe and beneficial use in food and have been regarded as not presenting safety concerns. Following are some examples of status of safety attributed to the *Bacillus subtilis* species, strains, or derived substances, in the United States, Canada, Japan, and the European Union:

United States

In opinion letters issued in the early 1960s, the Food and Drug Administration (FDA) recognized as GRAS some substances derived from microorganisms, including carbohydrase and protease enzymes from *B. subtilis*. The opinions are predicated on the use of non-pathogenic and non-toxicogenic strains of the respective organism and on the use of current good manufacturing practice (FDA list of microorganisms 2002; http://www.fda.gov/Food/FoodIngredientsPackaging/ucm078956.htm)

In 1999, FDA affirmed the carbohydrase and protease enzyme preparations from *B. subtilis* as GRAS for use as direct food ingredients (21CFR 184.1148 and 184.1150).

FDA stated that non-toxigenic and non-pathogenic strains of *B. subtilis* are widely available and have been safely used in a variety of food applications, including the documented consumption of *B. subtilis* in the Japanese fermented soybean, natto, which strain is identical to *B. subtilis* R0179 as shown earlier in this report. FDA has also given a no-questions letter for another strain of *B. subtilis* in GRN 831. FDA concluded that these enzymes derived from the *B. subtilis* strain were in common use in food prior to January 1, 1958.

Other enzyme preparations derived from genetically modified *B. subtilis* were approved as food additives (21 CFR 173.115) or notified as GRAS with no questions from FDA related to their relevant GRAS Notices: GRN 20; GRN 114; GRN 205; GRN 274, GRN 406, GRN 476, GRN 579, GRN 592, GRN 649, GRN 714, GRN 746, GRN 751.

The Association of American Feed Control Officials (AAFCO) has listed *B. subtilis* as approved for use as a feed ingredient under Section 36.14 Direct-Fed Microorganisms. This microorganism was reviewed by the FDA Center for Veterinary Medicine and found to present no safety concerns when used in direct-fed microbial products.

Canada

The Novel food section of the Food Directorate at Health Canada has recognized the status of *B. subtilis* R0179 as “non-novel” food. Their decision was based on evaluation of a description of the specific strain R0179, its origin and history of use in food, its expected consumption, data on general safety, and a description of the manufacturing process. The letter from Health Canada detailing their decision is included in Appendix 8.

The Canadian Food Inspection Agency (CFIA)-Animal health and production Feed Section has classified *Bacillus culture dehydrated* approved feed ingredients as silage additives under Schedule IV-Part 2-Class 8.6 and assigned the International Feed Ingredient number IFN 8-19-119.

Japan

The natto product and *B. subtilis* natto as its principal component are FOSHU approved in Japan. The Foods for Specified Health Use (FOSHU) are foods approved by the Ministry of Health, Labor, and Welfare as effective for preservation of health by adding certain active ingredients or removing undesirable ones. They are regarded as safe and effective for the maintenance and improvement of health by incorporating them into one's diet (http://www.matsutani.com/fibersol2marketinjp.html). The Japanese FOSHU products are products where their safety and efficacy have been verified scientifically (Gibson 2005)

European Union

Noting that a wide variety of microbial species are used in food, some with a long history of apparent safe use, and facing the need to set priorities for risk assessment, the European Food Safety Authority (EFSA) proposed a system referred to as ''Qualified Presumption of Safety'' (QPS). This system proposed basing the safety assessment of a defined taxonomic group (e.g., a genus or a species) on 4 pillars: established identity, body of knowledge, possible pathogenicity, and end use. If the taxonomic group did not raise safety concerns that cannot be defined and excluded, the grouping could be granted QPS status. Thereafter, ''any strain of microorganism the identity of which can be unambiguously established and assigned QPS group would be freed from the need for further safety assessment other than satisfying any qualification specified'' (EFSA 2007, p1).

EFSA's Scientific Committee was asked to recommend organisms regarded as suitable for QPS status. The list of such organisms proposed by the Committee included *Bacillus;* the Committee stated that ''for decades, strains belonging to several species of *Bacillus* have been deliberately introduced into the food chain either as plant protection products or animal feed supplements. Their safety can therefore be assessed by the QPS methods, according to EFSA (2005)'' (EFSA *Annex 4: Assessment of Bacillus Bacteria with respect to a Qualified Presumption of Safety)*.

In the Assessment of *Bacillus* bacteria with respect to a QPS (EFSA *Annex 4: Assessment of Bacillus Bacteria with respect to a Qualified Presumption of Safety*), the Committee evaluated the criteria of identity, body of knowledge, safety concerns, and whether the safety concern can be excluded. In conclusion, the Committee proposed to include a number of *Bacillus* species notified to EFSA on the list of QPS granted units due to the substantial body of knowledge available about these bacteria. *B. subtilis* was one of these species as several strains of *B. subtilis* have been used in animal feed supplements or in aquaculture (Hong et al. 2005; SCAN 2002), for treatment of seeds and roots to protect or promote the growth of plants (Cavaglieri et al. 2005; Krebs et al. 1998), and in the preparation of traditional fermented dishes in Africa and Asia (Sarkar et al. 2002), and no foodborne cases or food safety problems have been linked to these usages (EFSA *Annex 4: Assessment of Bacillus Bacteria with respect to a Qualified Presumption of Safety*). The Committee observed that, because

all bacteria within the *Bacillus* species potentially possess toxigenic traits, absence of toxigenic activity needed to be verified for qualification.

Accordingly, several species of *Bacillus,* including *B. subtilis,* were added to the QPS list, with a qualification concerning the absence of food poisoning toxins and enterotoxic activities.

The Committee stated that ''where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion'' (EFSA 2007, p8) and that the recommendations are ''based on a thorough review of the available scientific literature and the knowledge and experience of the scientists involved'' EFSA 2007, p8).

In 2009, EFSA asked the Panel on Biological Hazards (BIOHAZ) to deliver a scientific opinion on the maintenance of the list of QPS microorganisms intentionally added to food or feed (2009 update). The opinion reviewed the previous assessments of microorganisms in the context of a proposal for QPS. The previous list of QPS microorganisms was reviewed and confirmed. In conclusion, no modification of the QPS list for *Bacillus* species was needed and the list still includes *B. subtilis* (EFSA 2009; P33). *B. subtilis* continued to be listed as QPS in the most recent update (EFSA 2019).

In addition, the International Dairy Federation, in collaboration with the European Food and Feed Cultures Association, assembled a list of microorganisms with a documented history of safe use in food. *B. subtilis* is one of the strains listed in this inventory and its subsequent updates, for its safe use in fermented soy products (Bourdichon et al. 2012).

6.6. Decision-Tree Analysis of the Safety of the Notified Strain

The decision tree published by Pariza et al. (2015) indicates that the notified strain, *Bacillus subtilis R0179*, "is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption" (Pariza et al. 2015).

The responses to each of the questions asked in the decision tree are as follows:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? - Yes

2. Has the strain genome been sequenced? - Yes

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? - Yes

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? - Yes

5. Does the strain produce antimicrobial substances? - No

6. Has the strain been genetically modified using rDNA techniques? - No

7. Was the strain isolated from a food that has a history of safe consumption for which the species to which the strain belongs is a substantial and characterizing component (not simply an 'incidental isolate')? - Yes.

8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? - No.

6.7. Safety Assessment and GRAS determination

6.7.1. Introduction

This section presents an assessment that demonstrates that the intended use of the strain *Bacillus subtilis* R0179 is safe and is GRAS.

This safety assessment and GRAS determination involves two steps. In the first step, the safety of the intended use of the strain *B. subtilis* R0179 is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of humans to this strain under its intended conditions of use is not harmful. In the second step, the intended use of this strain is determined to be GRAS by demonstrating that its safety under its intended conditions of use is generally recognized among qualified scientific experts and is based on generally available and accepted information.

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

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

1. Data and information relied upon to establish the scientific element of safety must be generally available; and
2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the addition of *Bacillus subtilis* R0179 at a maximum level of $1x10^9$ cfu/serving to the following food categories[1] is safe and is GRAS:

- whole grain yeast breads and rolls and specialty breads

[1] The first six categories listed were covered in the 2012 GRAS determination, while the remaining eleven categories are newly added in this GRAS determination.

- muffins and sweet quick breads
- Kombucha
- 100% fruit juices and nectars
- 100% vegetable juices
- diet salad dressings
- baked goods and baking mixes
- beverage and beverage bases
- breakfast cereals
- chewing gum
- confections and frostings
- dairy product analogs
- fruit and water ices
- nuts and nut products
- plant protein products
- processed fruits and fruit juices
- snack foods

6.7.2. Safety Evaluation

The identity of *B. subtilis* R0179 has been evaluated through phenotypic, genotypic, and genomic analysis. The results of these analyses have clearly shown the identity of *B. subtilis* strain R0179 as part of the *Bacillus subtilis* species strongly related to natto isolates. The natto fermented beans are largely consumed in Asian countries and are recognized for their contribution to a healthy gut flora and vitamin K_2 intake; during this long history of widespread use, natto has not been implicated in any adverse events potentially attributable to the presence of *B. subtilis*.

The genomic analysis and *in vitro* testing have shown the safety of strain R0179 by demonstrating its sensitivity to all antibiotics recommended by EFSA and CLSI. The genome of the strain is also free of toxin production based on PCR amplification of susceptible genes and by genomic analysis. No plasmid has been isolated by test kit and any associated DNA sequences were identified in the genome analysis. The bacterium is not able to hydrolyze bile salts, even though it can survive and grow in a bile solution and in very acid conditions. Both aspects represent major advantages for survival through the stomach transit down to the gut. The adherence of *B. subtilis* R0179 to intestinal cells (HT-29) was shown to be 10-100 times lower than that of the pathogenic *B. cereus*. This observation was confirmed by the genomic analysis, which confirmed the lack of functional adhesion genes. This characteristic limits the strain's pathogenicity and infectivity potential. *Bacillus subtilis* R0179 shows incomplete hemolysis (alpha-hemolysis) of 5% sheep blood agar compared to the type strain *B. subtilis* 168, which produces a beta-hemolysis. Finally, the safety of strain R0179 and other *Bacillus subtilis* strains is supported by published toxicity studies.

As described in section 6.4. *B. subtilis* R0179 has been investigated in extensive published clinical research in Asia. In addition, a published safety trial was conducted in North America with this strain. The studies uniformly show that products containing *B. subtilis* R1079 are safe, with no reports of serious adverse events in adults, children, and infants. Nearly all of this research has included patients with health conditions that greatly increase their potential vulnerability to infection or other adverse events, and the failure of *B. subtilis* R0179 to produce such events is powerful evidence of the safety of the intended use of the strain.

Moreover, evaluation of historical use of bacterial products on the market has not shown any adverse events putatively attributable to the use of *Bacillus subtilis*. Finally, traditional foods containing *Bacillus subtilis,* such as natto, are regularly consumed without issue and other jurisdictions such as Canada, Japan, and the European Union have recognized the use of *Bacillus subtilis* in food as safe.
In conclusion, the use of *Bacillus subtilis* R0179 in humans does not pose significant risk.

6.7.3. General Recognition of Safety

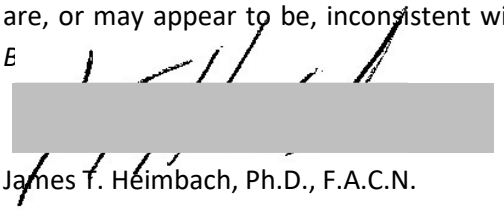
The intended use of *Bacillus subtilis* R0179, to be added to the food categories listed above in Section 6.7.1, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by establishing the identity and characteristics of the strain, demonstrating its freedom from pathogenic, toxicogenic, or other risk factors, and concluding that the expected exposure to *B. subtilis* R0179 by humans is without significant risk of harm. Finally, because this safety assessment is based on generally available information, and so satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the addition of *Bacillus subtilis* R0179 to the listed food categories has been made through the deliberations of a GRAS Panel consisting of Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., who reviewed this monograph, prepared by Lallemand Health Solutions and edited by JHeimbach LLC, as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients, including bacteria, intended for addition to different food categories. They critically reviewed and evaluated the publicly available information and the potential exposure to *B. subtilis* R0179 anticipated to result from its intended use, and individually and collectively concluded that no evidence exists in the available information on *B. subtilis* R0179 that demonstrates, or suggests reasonable grounds to suspect, a hazard to humans under the intended conditions of use *Bacillus subtilis* R0179.

It is the GRAS Panel's opinion that other qualified scientists reviewing the same publicly available data would reach a similar conclusion. Therefore, the intended use of *Bacillus subtilis* R0179 as described in this monograph, and produced under cGMP, is GRAS by scientific procedures.

6.8. Statement Regarding Information Inconsistent with GRAS

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



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

6.9. Conclusion of the GRAS Panel

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of the addition of ingredients, including bacteria, to conventional foods. We have individually and collectively critically evaluated the materials summarized above. We recognize that *B. subtilis* strains have a long history of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. We conclude that *B. subtilis* strain R0179 has been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of this bacterium, produced under cGMP, at levels up to $10x10^9$ cfu/day. Therefore, we conclude that addition of *Bacillus subtilis* strain R0179 to conventional foods as described is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach a similar conclusion. Therefore, the intended use of *Bacillus subtilis* strain R0179 is safe, and is GRAS, via scientific procedures.

Robert J. Nicolosi, Ph.D. _____ Date: March 16, 2021
Professor Emeritus
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Michael W. Pariza, Ph.D. _____ Date: _____
Professor Emeritus
University of Wisconsin—Madison
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