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Safety evaluation of HOWARU® Restore (*Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* Lpc-37, *Bifidobacterium animalis* subsp. *lactis* Bl-04 and *B. lactis* Bi-07) for antibiotic resistance, genomic risk factors, and acute toxicity

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ABSTRACT

Although probiotic lactobacilli and bifidobacteria are generally considered safe by various regulatory agencies, safety properties, such as absence of transferable antibiotic resistance, must still be determined for each strain prior to market introduction as a probiotic. Safety requirements for probiotics vary regionally and evaluation methods are not standardized, therefore methodologies are often adopted from food ingredients or chemicals to assess microbial safety. Four individual probiotic strains, *Lactobacillus acidophilus* NCFM®, *Lactobacillus paracasei* Lpc-37®, *Bifidobacterium animalis* subsp. *lactis* strains Bl-04®, and Bi-07®, and their combination (HOWARU® Restore) were examined for antibiotic resistance by broth microdilution culture, toxin genes by PCR and genome mining, and acute oral toxicity in rats. Only *B. lactis* Bl-04 exhibited antibiotic resistance above a regulated threshold due to a *tetW* gene previously demonstrated to be non-transferable. Genomic mining did not reveal any bacterial toxin genes known to harm mammalian hosts in any of the strains. The rodent studies did not indicate any evidence of acute toxicity following a dose of 1.7–4.1 × 10^{12} CFU/kg body weight. Considering a 100-fold safety margin, this corresponds to 1.2–2.8 × 10^{12} CFU for a 70 kg human. Our findings demonstrate a comprehensive approach of *in vitro, in silico,* and *in vivo* safety testing for probiotics.

1. Introduction

Lactobacillus acidophilus, Lactobacillus paracasei and *Bifidobacterium animalis* subsp. *lactis* are Gram-positive, non-spore forming, lactic acid producing bacteria. Both genera are often referred to as Lactic Acid Bacteria (LAB) even though bifidobacteria produce lactic acid through a different metabolic pathway than lactobacilli. Representatives of the three species are commonly included in probiotic foods and dietary supplements. Bifidobacteria tend to have higher abundance in the colon (Lyra et al., 2012) while lactobacilli are thought to be more prevalent in the small intestine (Li et al., 2015). *L. acidophilus* has also been found to be a minor but stable member of the fecal microbiota while *L. paracasei* appears to be a less stable member of the fecal microbiota (Rossi et al., 2016). Although *B. lactis* is commonly detected in fecal samples, it is not always clear whether it is an endogenous member of the fecal microbiota or originates inadvertently from consumption of fermented (dairy) foods or other probiotic products. However, *B. lactis* has also been detected in the feces of infants (Grzeskowiak et al., 2015) and may therefore be an endogenous species during different phases of life.

Because of their long history of safe use (Bourdichon et al., 2012) and presence in the fecal microbiota, strains of the species *L. acidophilus, L. paracasei* and *B. lactis* are usually considered safe for consumption. In the U.S., the Food and Drug Administration (FDA) has responded favorably with “no questions” to Generally Recognized As Safe (GRAS) notifications for a number of strains of these species (US FDA, 2002b, 2008a, 2011a, 2011b, 2012b, 2013b, 2014), and a number of other lactobacilli and bifidobacteria species (US FDA, 2008b, 2009a. 2009b, 2012a, 2013a, 2013c, 2013d, 2013e, 2015b). Similarly, in the European Union these three species have been given Qualified Presumption of Safety (QPS) status (Ricci et al., 2017). The safety status is further strengthened by the fact that systemic infections with these species are extremely rare or non-existent (Martinez et al., 2014;

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Abbreviations

ARDB	Antibiotic Resistance Genes Database
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
Contigs	Contiguous sequences
DBETH	Database of Bacterial ExoToxins for Human
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
LAB	Lactic Acid Bacteria
MvirDB	Microbial Virulence Database
MIC	Minimum Inhibitory Concentration
NCBI	National Center for Biotechnology Information
OECD	Organisation for Economic Co-operation and Development
PPTP	Phages, Prophages, Transposable elements, Plasmids
QPS	Qualified Presumption of Safety
RAST	Rapid Annotation using Subsystem Technology
VDD	Virulence, Disease, and Defense

Salminen et al., 2006).

Advances in next generation sequencing offer highly resolute analyses of probiotic identification (Morovic et al., 2016; Patro et al., 2016), and much of what is currently considered essential information for characterizing identity and safety by a number of regulatory agencies includes genomic analyses. For example, one European Food Safety Authority (EFSA) requirement is to determine the lack of transferable antibiotic resistance genetic elements, as this characteristic may contribute to the spread of the resistance in pathogens (Kazimierczak et al., 2006). The current EFSA-approved guidance document recommends quantitative *in vitro* testing to be coupled with subsequent analyses, such as genomic mining, if a strain is above the established threshold for a required antibiotic (EFSA, 2012). Genome mining also enables rapid screening for bacterial toxins using curated sequence databases (Zhang et al., 2012), although few regulatory agencies currently require this information. Furthermore, there is little guidance on how to screen the variety of bacterial toxins, like those that damage other bacterial cells (Dobson et al., 2012), single-celled eukaryotes (Matsubara et al., 2016), higher eukaryotic organisms (Middlebrook and Dorland, 1984), or the source cell itself (Prozorov and Danilenko, 2010). For probiotics, the absence of known endotoxins and exotoxins that directly damage higher eukaryotic cells and functions is critical for consumer safety.

Rodent-based toxicity studies of probiotic lactobacilli and bifidobacteria, ranging in duration from single-dose acute (~2 weeks) to chronic (> 3 months), and at high dose levels relative to anticipated human consumption, have been conducted historically to provide *in vivo* safety data despite the lack of probiotic-specific validated test methods, (Abe et al., 2009; Jia et al., 2011; Lara-Villoslada et al., 2007; Mukerji et al., 2016; Szabo et al., 2011; Yakabe et al., 2009; Zhou et al., 2000a). While this information is considered useful by some regulatory agencies to provide evidence of safety, it is noteworthy that no adverse effects have been identified in healthy animal studies conducted to date with probiotic lactobacilli and bifidobacteria species when preceding evaluations demonstrate a history of safe use, lack of novel functional or transferrable antibiotic resistance, and the absence of virulence factors, toxins associated with pathogenicity, and antimicrobial substances (US FDA, 2002b, 2008a, 2008b, 2009a. 2009b, 2011a, 2011b, 2012a, 2012b, 2013a, 2013b, 2013c, 2013d, 2013e, 2014, 2015b). These observations are consistent with the decision-tree approach described by Pariza and colleagues for evaluating the safety of microbial cultures for consumption, whereby these strains would be considered safe for human consumption in the absence of *in vivo* safety evaluation studies (Pariza et al., 2015). For additives with a low level of concern, which is not well defined, the US FDA (2006) recommends short term toxicity tests. Since LAB have a low level of safety concern, here an acute toxicity model was appropriate.

While *in vivo* safety studies can vary in design (i.e. purpose, test system, sample number, exposure duration, dosage, etc.), an acute oral toxicity study using a limit dose of 5000 mg/kg body weight (mg/kg), as specified in Organisation for Economic Co-operation and Development (OECD) and U.S. FDA testing guidelines (US FDA, 2003; OECD, 2008), provides rapid evidence of a lack of overt toxicity, and supplements the genomic screening and antibiotic resistance evaluations. Specific toxicity studies with the three aforementioned bacterial species are often not performed according to regulatory guidelines (Jia et al., 2011; Zhou et al., 2000a, 2000b, 2001), and the safety evaluation of strain combinations as a blended product and comparison with its individual, constituting strains has been evaluated even more infrequently (Mukerji et al., 2016; Shokryazdan et al., 2016).

Because of the long history of safe use in foods, and the QPS status of these four strains, the goal of this report was to assess the potential presence of transferable antibiotic resistance, genomic risk factors harmful to human hosts, and the acute toxicity of the probiotic strains *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* Lpc-37, *Bifidobacterium animalis* subsp. *lactis* Bl-04, and *B. animalis* subsp. *lactis* Bi-07, in addition to the combination product, HOWARU® Restore, which is a blend of these same probiotic strains. Antibiotic resistances were determined as recommended by the European Food Safety Agency (EFSA, 2012), genomic analysis was performed using public databases with commercial bioinformatics software, and toxicity tests were performed as described by the OECD (2008) and US FDA (2003).

2. Materials and methods

2.1. HOWARU® Restore concentrate production

HOWARU® Restore is a mixture of four probiotic strains, *Lactobacillus acidophilus* NCFM (ATCC SD5221), *Lactobacillus paracasei* Lpc-37 (ATCC SD5275), *Bifidobacterium animalis* subsp. *lactis* Bl-04 (ATCC SD5219) and *B. animalis* subsp. *lactis* Bi-07 (ATCC SD5220), in a 1:1:1:1 ratio. HOWARU® Restore is manufactured under 21 CFR 111, Current Good Manufacturing Practice in Manufacturing, Packaging, Labeling, Or Holding Operations for Dietary Supplements (US FDA, 2015a). The proprietary manufacturing process is a batch-type fermentation using sterilized media comprising proteins, carbohydrates, vitamins, and minerals in water prior to inoculation with the selected bacteria further described in the GRAS statements (US FDA, 2011a and 2013b). Each batch of each strain is fermented and freeze-dried individually and required to pass quality checks for enumeration, identity, and contamination before blending together to produce HOWARU® Restore. The product is always formulated to contain viable cells at or above the label claim until the labeled expiration date at recommended storage conditions.

2.2. In vitro testing

2.2.1. Biogenic amines

In lactic acid bacteria, production of histamine results from the catabolism of histidine by a histidine decarboxylase (*hdc*; EC 4.1.1.22) and production of tyramine results from the catabolism of tyrosine by a tyrosine decarboxylase (*tdc*; EC 4.1.1.25). A specific PCR-based detection method for *hdc* and *tdc* genes has been developed internally by DuPont based on scientific literature (Coton and Coton, 2005; de Las Rivas et al., 2005, 2006). Primers, thermocycler settings, and reaction criteria are outlined in Supplementary Table 1. Additionally, amino acid sequences of known *hdc* and *tdc* genes were collected from UniProt

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