Article

Evaluation of commercial probiotics for antimicrobial resistance genes

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Abstract — The objective of this study was to determine if transferable antimicrobial resistance (AMR) genes are present in commercial animal probiotics. DNA was extracted from 50 probiotics, tested for the presence of bacterial DNA, and analyzed by polymerase chain reaction (PCR) for the presence of 8 transferrable AMR genes, including tetracycline, erythromycin, aminoglycoside, sulfonamide, and trimethoprim. Samples that were positive by PCR were confirmed by genome sequencing. Forty-seven (94%) products contained bacterial DNA. Of these, 97% contained at least 1 AMR gene, and 82% contained 2 or more. These results indicate that further evaluation of the risk for transmission of these AMR genes may be warranted.

Résumé — Évaluation de probiotiques commerciaux pour des gènes de résistance aux antimicrobiens. L'objectif de la présente étude était de déterminer si des gènes transférables de résistance aux antimicrobiens (RAM) sont présents dans des probiotiques pour animaux du commerce. L'ADN a été extrait de 50 probiotiques, testé pour la présence d'ADN bactérien et analysé par réaction d'amplification en chaîne par la polymérase (PCR) pour la présence de huit gènes RAM transférables, incluant la tétracycline, l'érythromycine, les aminoglycosides, le sulfonamide et le triméthoprime. Les échantillons positifs par PCR ont été confirmés par séquençage du génome. Quarante-sept (94 %) produits contenaient de l'ADN bactérien. Parmi ceux-ci, 97 % contenaient au moins un gène RAM et 82 % en contenaient deux ou plus. Ces résultats indiquent qu'une évaluation plus approfondie du risque de transmission de ces gènes RAM peut être justifiée.

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Introduction

The increasing emergence of antimicrobial resistant bacteria is considered a global health concern for humans and animals (1). Potential sources contributing to the development and spread of antimicrobial resistant pathogens include selective pressure due to both the widespread use and misuse of antimicrobials in both human and veterinary medicine, use for prophylaxis, and the use of antimicrobials for the promotion of growth (2).

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit to the host (3). The threat of introducing antimicrobial resistance (AMR) genes to the gastrointestinal microbiota *via* probiotics has been recognized in Europe. The European Food Safety Authority has stated that the absence of acquired or potentially transferable resistance factors must be established for probiotics to be declared safe for human and animal consumption (4). Similar regulatory testing is neither performed nor required for probiotics in the United States. Genes for AMR have been identified in commercially available probiotics marketed for humans (5). Recent work by these authors identified a transferrable vancomyocin resistance gene *(vanA)* in probiotics marketed for use in animals (6). However, to the authors' knowledge, products marketed for veterinary species have not been evaluated for the presence of other transferable AMR genes. Thus, the objective of this study was to evaluate commercially available probiotics marketed for various animals for known AMR genes. We hypothesized that probiotics marketed for animals contain AMR genes detectable by polymerase chain reaction (PCR).

Materials and methods

Selection of probiotics

A list was compiled of commercially available probiotics marketed for animals and available for purchase by owners

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Table 1. Summary of transferrable AMR genes detected in commercial probiotics.

Species	Product	*TetM*	*TetK*	*ErmB*	*ErmT*	*Aph(2)-1D*	*Sul 1*	*Sul 2*	*dfrG*	Total
Cattle	1[d]	+	+	+	−	−	−	+	−	4
	2[c]	+	+	+	−	−	+	+	+*	6
	3[d]	+	+	+	+	−	−	−	−	4
	4[b]	+	−	+	−	−	+	+	+*	5
	5[a]	−	−	−	−	−	−	−	−	0
	6[b]	−	−	−	+	−	+	+	+*	4
Camelids	7[b]	+*	+*	−	−	−	−	−	−	2
	8[b]	−	+*	−	−	−	−	−	+*	2
	9[d]	+*	+*	−	−	−	−	−	−	2
	10[d]	+*	+*	+*	−	−	−	+*	−	4
	11[d]	+*	+*	−	−	−	−	+*	−	3
Dogs	12[b]	+*	−	−	−	−	−	−	−	1
	13[b]	+*	+*	+*	−	−	−	+*	+*	5
	14[b]	+	+*	+	−	−	+*	+*	+*	6
	15[a]	+*	+*	−	−	−	+*	+*	−	4
	16[d]	−	+*	−	−	−	−	−	−	1
	17[c]	+*	−	−	−	−	−	+*	−	2
	18[b]	+*	+*	−	−	−	−	+*	−	3
	19[b]	+*	+*	−	−	−	−	+*	−	3
	20[b]	+*	−	+*		−	−	+*	−	3
Dogs/Cats	21[b]	−	−	+*	−	−	−	−	−	1
Goats	22[d]	−	+*	−	−	−	+*	+*	−	3
Horses	23[d]	−	+*	−	−	−	−	+*	−	2
	24[d]	−	−	+*	+*	−	−	−	+*	3
	25[d]	+*	−	−	−	−	+*	+*	−	3
	26[c]	−	−	−	−	−	−	−	−	0
	27[d]	+*	+*	−	−	−	−	+*	+*	4
	28[d]	−	+*	−	+*	−	−	−	+*	3
	29[d]	−	+*	−	−	−	−	+*	−	2
	30[d]	−	+*	−	−	−	−	+*	−	2
	31[d]	+*	+*	−	−	−	−	−	−	2
	32[d]	−	+*	−	−	−	−	+*	−	2
	33[c]	+*	+*	−	−	−	−	+*	−	3
	34[e]	+*	+*	−	+*	−	−	+*	−	4
	35[d]	−	−	−	−	−	−	−	−	0
	36[e]	+*	+*	−	−	−	−	+*	−	3
	37[c]	−	+*	−	−	−	−	+*	−	2
	38[e]	−	+*	−	−	−	−	−	−	1
	39[b]	+*	+*	+*	−	−	+*	+*	−	5
Multiple*	40[‡]	−	+*	−	−	−	−	+*	−	2
Cats	41[d]	+*	+*	−	−	−	−	−	+*	3
	42[a]	+*	−	−	−	−	−	−	−	1
	43[d]	−	−	+*	−	−	+*	+*	−	3
	44[a]	−	+*	−	−	−	+*	−	−	2
	45[a]	+	+*	+	+*	−	−	−	−	4
	46[b]	+*	−	−	−	−	−	+*	−	2
	47[a]	−	−	+	−	−	−	−	+	2
	Totals	23	29	10	4	0	7	24	8	

Gene's marketed as + were identified in that product by AMR gene specific PCR and the PCR product was confirmed to match the gene of interest by sequencing. The total number of AMR genes identified in each product is listed in the column to the far right (total) and the total number of products with each AMR gene is listed in the bottom row (total). The letters indicate which extraction kit was utilized, as follows: [a] DNeasy PowerFood Microbial Kit (QIAGEN, Valencia, California, USA); [b] DNeasy PowerSoil Pro Kit (QIAGEN); [c] DNeasy Blood & Tissue Kit (QIAGEN); [d] QIAamp PowerFecal (QIAGEN).

* Indicates which PCRs had a visible product after repeating the reaction, as described in the Materials and methods. Products are:

1 — Achieve Pro Calf Paste (Huvepharma, Peachtree City, Georgia, USA); 2 — Bovine IGG Immu-start 50 (Imu-Tek Animal Health, Fort Collins, Colorado, USA); 3 — Fastrack Ruminant Gel (Conkin Company, Kansas City, Missouri, USA); 4 — Manna Pro Calf Care (Mann Pro Products LLC, Chesterfield, Missouri, USA); 5 — Probios Ruminant Bolus (Vet Plus, Menomonie, Wisconsin, USA); 6 — TDN Rockets Mini with Inulin (Vet Plus); 7 — Llama-zyme (Alpacas All Naturale, San Marcos, California, USA); 8 — Soothe my Tummy (Alpacas All Naturale); 9 — Golden Blend (Custom Milling, Davisboro, Georgia, USA); 10 — ProGuard (The Holistic Horse, Irving, California, USA); 11 — Truval Feed Supply (Laboratori Truval Italia, Udine, Italy); 12 — Dr. Lyon's Probiotic Daily (Dr. Lyon's LLC, Dania Beach, Florida, USA); 13 — Nutri-vet Pre and Probiotics Dog Soft Chews (Nutri-Vet Wellness, LLC, Boise, Idaho, USA); 14 — Probios intelliflora Dog (Vet Plus); 15 — Smart Canine Digestion (SmartPak Equine LLC, Plymouth, Massachusetts, USA); 16 — Tomlyn Pre and Pro for Dogs (Vétoquinol USA, Fort Worth, Texas, USA); 17 — Tropiclean Life Dog Supplement (TropiClean, Wentzville, Missouri, USA); 18 — Ultracruz Canine Probiotic Supplement (Santa Cruz Animal Health, Dallas, Texas, USA); 19 — Vetone Advita Probiotic Dog (MWI Animal Health, Boise, Idaho, USA); 20 — Purina Proplan Veterinary Diets Fortiflora (Purina, Neenah, Wisconsin, USA); 21 — Pet Dophilus (Jarrow Formulas, Los Angeles, California, USA); 22 — Goats Prefer Probiotic Plus Paste (Vet Plus); 23 — Bug Check (The Natural Vet LLC, Unicol, Tennessee, USA); 24 — Equ-Bac Probiotic Oral Gel for Horses and Foals (Kaeco Group, Savannah, Missouri, USA); 25 — Foal Colostrum Oral Gel (Kaeco Group); 26 — Gut Digestive and GI Support for Horses (Uckle Health & Nutrition, Blissfield, Mississippi, USA); 27 — Senior Weight Accelerator Manna Pro (Manna Pro Products LLC, Chesterfield, Missouri, USA); 28 — SmartDigest Ultra Paste (SmartPak Equine LLC); 29 — The Missing Link Well Blend Senior (W.F. Young, East Longmeadow, Massachusetts, USA); 30 — Total Pre and Pro for Horses (Ramard, New Richmond, Ohio, USA); 31 — SmartGut Ultra (SmartPak Equine LLC); 32 — Exceed 6-Way (Med-Vet Pharmaceuticals, Eden Prairie, Minnesota, USA); 33 — Equiotic Paste (SmartPak Equine LLC); 34 — SmartDigest Ultras (SmartPak Equine LLC); 35 — Probios (Vet Plus); 36 — BioEZ (GGBC, Huntington Beach, California, USA); 37 — Command Probiotic Supreme (Brookside Equine LLC, Marysville, Kansas, USA); 38 — Absorbine Maximize (W.F. Young); 39 — Pro Weight MVP Equine (Med-Vet Pharmaceuticals); 40 — Fastrack (Conklin Company, Kansas City, Missouri, USA); 41 — Catsure Meal Replacement (Pet-Ag, Hampshire, Illinois, USA); 42 — Drs Foster and Smith Soft Chews for Cats (Doctors Foster and Smith, Rhinelander, Wisconsin, USA); 43 — Nature's Farmacy Catzymes Probiotic (Nature's Farmacy, Jasper, Georgia, USA); 44 — Nexabiotic Probiotics for Cats (DrFormulas, Huntington Beach, California, USA); 45 — Probios Intellifora Cat (Purina, Nennah, Wisconsin, USA); 46 — Vetone Advita Probiotic (feline) (MWI Animal Health); 47 — Probonix for Cats (Humarian, Carmel, Indiana, USA).

Table 2. Summary of PCRs used for AMR gene detection.

Antimicrobial	Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)	NCBI reference sequence
Tetracycline	*tetM* (8) *tetK* (9)	GGT GAA CAT CAT AGA CAC GC GTA GCG ACA ATA GGT AAT AG	CTT GTT CGA GTT CCA ATG C GCA ACT TCT TCT TCA GAA AG	401 278	X90939 NG_055987
Erythromycin	*ermB* (10) *ermT* (9)	CAT TTA ACG ACG AAA CTG GC TAT TAT TGA GAT TGG TTC AGG G	GGA ACA TCT GTG GTA TGG CG GGA TGA AAG TAT TCT CTA GGG ATT T	405 395	NG_047795 NG_047840
Aminoglycosides	*aph(2″)-Id* (11)	GTG GTT TTT ACA GGA ATG CCA TC	CCC TCT TCA TAC CAA TCC ATA TAA CC	641	*
Sulfonamide	*sul1* (12) *sul2* (12)	GCG AGG GTT TCC GAG AAG GTG CGG CAT CGT CAA CAT AAC C	TGG TGA CGG TGT TCG GCA TTC GTG TGC GGA TGA AGT CAG	790 722	AY444815 KX900483
Trimethoprim	*dfrG* (13)	TTT CTT TGA TTG CTG CGA TG	CCC TTT TTG GGC AAA TAC CT	405	NG_047756.1

* Probiotic DNA did not have evidence of *Aph(2″)-Id* based on PCR analyses and thus sequence comparison was not performed.

using common online sources. A random number generator (www.random.org) was used to select 50 products for further evaluation.

DNA extraction from commercial probiotics

The DNA from probiotics was extracted using one of several commercially available DNA extraction kits (QIAGEN, Valencia California, USA) (Table 1). Due to the heterogenous nature of probiotic substrates, multiple kits were used and the extraction determined to have the best DNA, based on quantity and purity indicators (i.e., A260:A280 ratio), was used for further analyses. The DNA concentration was quantified and quality was assessed using spectrophotometry (Nanodrop; Thermofisher, Waltham, Massachusetts, USA).

Extraction of DNA from positive control bacteria for AMR genes

Bacteria containing known AMR genes of interest were generously provided by the Center for Disease Control and Food and Drug Administration Antibiotic Resistance Isolate Bank (Centers for Disease Control, Atlanta, Georgia, USA) and propagated according to manufacturer recommendations. Subsequently, DNA was extracted using a commercial microbial DNA extraction kit (QIAGEN) according to the manufacturer's protocol. DNA concentration was quantified, and quality assessed using spectrophotometry (Nanodrop; Thermofisher).

Confirmation of the presence of bacterial DNA

The presence of bacterial DNA in each DNA extraction from the probiotics was assessed using a bacterial 16S rRNA PCR, as described (7), with the following modifications. Amplification was performed by an initial denaturalization at 95°C for 3 min, then 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The final extension was performed at 72°C for 10 min, then the sample was held at 4°C until it was analyzed by gel electrophoresis. The PCR products were evaluated by electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized to evaluate for a corresponding 994 base pairs (bp) PCR product. Polymerase chain reactions that did not result in an amplicon were considered negative and excluded from further analyses.

Selection of AMR genes, PCR validation, and PCR of genes in probiotics

As listed in Table 2, previously published PCRs for 8 AMR genes that have demonstrated transferability were evaluated in this study: 2 tetracycline resistance genes *(tetM, tetK),* 2 erythromycin resistance genes *(ermB, ermT),* 2 sulfonamide resistance genes *(sul1, sul2),* 1 aminoglycoside resistance gene [*Aph(2″)-Id*], and 1 trimethoprim resistance gene *(dfrG).* Selection of AMR genes was made based on previous reports of identifying these genes in human probiotics. All PCRs were performed with no-template negative control containing only Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer and according to the protocols provided in the citations, with the exception for *dfrG* (8–11). This PCR was modified to use the following conditions: amplification was performed by an initial denaturation at 94°C for 5 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The final extension was performed at 72°C for 7 min, then the sample was held at 4°C until it was analyzed by gel electrophoresis. The PCRs were validated using DNA extracted from the bacterial cultures with known AMR genes of interest, as described. The reactions were confirmed to have DNA product of the expected size using gel electrophoresis and PCR products were confirmed to correspond with the AMR gene of interest by comparison of known AMR gene sequences to PCR product sequences, as described below. Once PCRs had been validated with positive control DNA for the AMR genes of interest, the extracted DNA from probiotics was evaluated. Each PCR included a negative control as described above and a positive control, using the DNA extracted from the known isolates.

The PCR products were evaluated by electrophoresis in 2% agarose gel, stained with ethidium bromide and visualized for an appropriately sized PCR product. For products without a corresponding band of interest, the PCR was repeated with the same parameters using 10 μL of the initial PCR as template, including 10 μL of the initial negative control and positive

control. The second PCR was again evaluated by electrophoresis in 2% agarose gel, stained with ethidium bromide and visualized for an appropriately sized PCR product.

Sequencing of amplified AMR genes

Positive control and randomly selected probiotic PCRs with appropriately sized PCR products were enzymatically treated (ExoSap-it; Affymetrix Life Science Reagents, Waltham, Massachusetts, USA). Sanger sequencing samples were analyzed on a 3730XL DNA Analyzer (Applied Biosystems, Dublin, Ireland) at the DNA sequencing core facility, Laboratory for Biotechnology and Bioanalysis at Washington State University. Sanger sequencing of the treated PCR products was performed using the corresponding forward primer listed in Table 2 and a commercial mixture (Big Dye 3.1 reagent mix; Applied Biosystems). Sequence data were analyzed (Sequencher 5.2 Software; GeneCodes, Ann Arbor, Michigan, USA) and confirmed to represent the gene of interest by comparing the PCR product sequence to the known genetic sequence of the AMR gene of interest using the BLAST sequence analysis tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The NCBI references for the reference AMR gene sequence used for comparison are provided in Table 2. Samples were considered positive for the AMR gene if they had both PCR product size consistent with the gene of interest and the sequence of the PCR product was confirmed to correspond with the AMR gene of interest.

Results

All PCRs for the AMR genes of interest were appropriately validated and confirmed to amplify DNA corresponding to the gene of interest based on sequencing analysis.

Of the 50 probiotic samples evaluated, 47 were confirmed to have bacterial DNA and were used for further analyses. The AMR genes identified in the 47 products are summarized in Table 1. Of the 47 products with successful extraction of bacterial DNA, 94% (44/47) had at least 1 AMR gene. All positive PCR products were confirmed to have the correct sequence. None of the products contained the aminoglycoside resistance gene evaluated in this study; however, at least 1 product contained each of the other genes that were evaluated. Of these products, 89% (42/47) contained more than 1 AMR gene, with 2 products containing 6 of the 8 genes evaluated in this study. The AMR genes for tetracycline *(tetK)* and sulfonamide *(sul2)* resistance were found most frequently, being present in 32/47 (68%) and 28/47 (60%) products, respectively.

Discussion

Results from this study supported our hypothesis and confirmed that most (94%) probiotics marketed for animals that were evaluated in this study contained at least 1 transferable AMR gene. The presence of multiple transferable AMR genes in most of the probiotics evaluated in this study is potentially concerning. First, there is no barrier preventing the transfer of AMR genes to the animal's gastrointestinal microbiota which includes commensal, potentially pathogenic, and pathogenic bacteria. Furthermore, the transfer of AMR genes from probiotic bacteria to other species has been well-documented *in vitro* (12). Second, if AMR genes are present in commercial probiotics, the dose and frequency at which probiotics are routinely administered may result in frequent (perhaps daily) administration of AMR genetic material to residential gastrointestinal microbiota.

Interestingly, we only amplified bacterial DNA by PCR from 47 of the 50 chosen products, despite using numerous DNA kits and modifications. It is possible that those samples contained PCR inhibitors and did contain bacterial DNA. However, differences between label claims and actual contents in probiotics marketed for use in animals have been demonstrated repeatedly (13,14) including lack of culturable bacteria (13).

There are several weaknesses of this study that are worth consideration when interpreting the results. First, this study was qualitative, not quantitative; therefore, although we identified the presence of known transferable AMR genes in veterinary probiotics, the quantity of genetic material per dose of probiotic was not measured and was beyond the scope of this work. Quantitative information may be valuable when determining the risk of AMR gene transmission. Lacking culture to validate both bacterial viability and bacterial AMR gene content is another weakness of the study. Given that the presence of AMR genes in live bacteria is not a requirement for the genetic material to be transferred, we believe that these results are relevant and valuable without culture information. In addition, the identification of genotypic antimicrobial resistance does not always correspond to phenotypic resistance when the bacteria are cultured and *in vitro* minimum inhibitory concentrations of antimicrobials are evaluated (15). Future studies evaluating this as well as which micro-organisms contain the identified AMR genes are warranted. Next, AMR genes used in this study were chosen because they had been identified in other probiotics; however, this does not exclude the possibility of identifying additional AMR genes; this is an area in need of additional investigation.

In conclusion, 93% of the probiotics evaluated in this study contained at least 1 transferable AMR gene and 7 of the 8 AMR genes evaluated in this study were identified in multiple products. The identification of AMR genes in these widely used products warrants further evaluation and consideration.

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