

SHORT REPORT

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Detection of antibiotic resistance in probiotics of dietary supplements

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Abstract

Background: Probiotics are live microorganisms that confer nutrition- and health-promoting benefits if consumed in adequate amounts. Concomitant with the demand for natural approaches to maintaining health is an increase in inclusion of probiotics in food and health products. Since probiotic bacteria act as reservoir for antibiotic resistant determinants, the transfer of these genes to pathogens sharing the same intestinal habitat is thus conceivable considering the fact that dietary supplements contain high amounts of often heterogeneous populations of probiotics. Such events can confer pathogens protection against commonly-used drugs. Despite numerous reports of antibiotic resistant probiotics in food and biological sources, the antibiogram of probiotics from dietary supplements remained elusive.

Findings: Here, we screened five commercially available dietary supplements for resistance towards antibiotics of different classes. Probiotics of all batches of products were resistant towards vancomycin while batch-dependent resistance towards streptomycin, aztreonam, gentamycin and/or ciprofloxacin antibiotics was detected for probiotics of brands Bi and Bn, Bg, and L. Isolates of brand Cn was also resistant towards gentamycin, streptomycin and ciprofloxacin antibiotics. Additionally, we also report a discrepancy between the enumerated viable bacteria amounts and the claims of the manufacturers.

Conclusions: This short report has highlighted the present of antibiotic resistance in probiotic bacteria from dietary supplements and therefore serves as a platform for further screenings and for in-depth characterization of the resistant determinants and the molecular machinery that confers the resistance.

Findings

Introduction

Probiotics are live microorganisms usually bacteria or yeasts, that confer health-promoting benefits to the host if consumed in adequate amounts. These benefits range from improvement of intestinal health [1] and immune response [2], to prevention of acute and antibiotic-associated diarrhea [1], and cancer [3]. The health-promoting attributes, mechanisms and strain-specific benefits have been extensively reviewed elsewhere [4]. Probiotic foods have gained widespread acceptance and popularity [5, 6] as reflected by an estimated growth of more than 10 % in economic value from 2009 to 2014 [7]. Due to increasing consumer demand for natural non-drug approaches to improve health,

probiotic bacteria have been included in overwhelming number of food products especially in dairy foods such as the probiotic-containing yoghurt, cheese, milk and ice-cream [8].

Widespread use of probiotic bacteria in conjunction and in close association with antibiotic use or rather misuse, can over time establish a reservoir of antibiotic resistant genes in probiotic bacteria [9]. While intrinsic antibiotic resistance can be a desirable trait as probiotics help restore host gut microflora during a course of antibiotics, however the transfer of resistant genes to pathogenic bacteria offers serious clinical threats [10]. There is already a wide collection of literature reporting resistance of lactic acid bacteria towards antibiotics of the beta-lactams, macrolide, aminoglycoside, chloramphenicol and tetracycline classes [6, 11] and in some, their corresponding resistant genes and mechanisms have been characterized [9, 12]. Importantly, resistant gene transfer that occurs at high frequency and persist

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across generations have also been demonstrated *in vitro* and/or *in vivo* among *Lactobacilli* and from *Lactobacilli* to pathogens [13] and vice versa [14]. These reports have established probiotic bacteria as reservoir of antibiotic resistant genes that can be transferred to pathogenic strains.

While the detection of antibiotic resistance in probiotic strains from food and biological sources have intensified, such reports from dietary supplements have remained somewhat elusive. This is surprising considering the fact that probiotic dietary supplements contain high amount and often a heterogeneous population of probiotic bacteria both of which, are conditions that encourage the trafficking of resistant genes. Therefore, we hypothesize that antibiotic resistant probiotics may be present in dietary supplements.

Methods

Probiotic dietary supplements designated here as Bi, Bn, Bg, Cn and L, were purchased from local pharmacy or retail outlets (Table 1) and the ampicillin, aztreonam, erythromycin, gentamycin, streptomycin, clindamycin, vancomycin, ciprofloxacin, cephalixin and tetracycline antibiotic discs were purchased from HiMedia, India

Table 1 The information of probiotic bacteria in dietary supplements

Product	Country manufactured	Probiotic content	Probiotic amount ($\times 10^6$ CFU/capsule)
Bi	Malaysia	<i>B. longum</i> , <i>B. bifidum</i> , <i>B. infantis</i> , <i>L. bulgaricus</i> , <i>L. rhamnosus</i> , <i>L. casei</i> , <i>L. acidophilus</i>	5,000*
Bn	Austria	<i>B. longum</i> , <i>B. bifidum</i> , <i>L. gasseri</i>	10
Bg	Malaysia	<i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. longum</i>	2,000*
Cn	U.S.A	<i>L. acidophilus</i> , <i>L. salivarius</i> , <i>B. bifidum</i> , <i>S. thermophiles</i> ,	8
L	Malaysia	<i>L. acidophilus</i> , <i>L. bulgaricus</i> , <i>L. casei</i> , <i>B. longum</i> , <i>S. thermophilus</i>	10,000*

*One capsule contains equal amounts of each probiotic strain. In products where such information is not stated, equal contributions from each probiotic content is assumed

(Table 2). In order to recover and enumerate probiotic bacteria [15], one capsule of dietary supplement was dissolved in sterile double distilled water [16] and immediately plated on the de Man, Rogosa and Sharpe (MRS) media (Difco, USA) which is selective for *Lactobacilli*. We note that *Lactobacilli* constitute the majority of the probiotic bacteria population in the dietary supplements (Table 1) and have therefore excluded contributions of other probiotic strains from the bacteria count and from the subsequent antibiotic resistance screening (Additional file 1). One capsule contains equal amounts of each probiotic strain. In products where such information is not stated, equal contributions from each probiotic content is assumed. The dissolved samples (10^6 CFU) were cultured overnight in MRS broth for enrichment of probiotic bacteria after which the overnight culture was adjusted to uniform concentrations of 7×10^6 CFU/mL of bacteria by spectroscopy (OD_{690nm}) prior to antibiotic susceptibility tests using commercial antibiotic discs and according to the manufacturer's instructions (Additional file 1).

Results and discussion

Viable bacteria were recovered on MRS media and their amounts were enumerated prior to antibiotic susceptibility tests. Probiotic bacteria, particularly the *Lactobacilli* strains, from all brands of dietary supplements were successfully recovered on MRS agar (Fig. 1a). The recovered bacteria have different colony morphologies of which two are particularly distinctive. One, a larger sized opaque colony is present in all brands at similar amounts to the other colony type that is smaller in size and translucent, but appears to be far fewer in brands Bn and L respectively (Fig. 1a). This suggests that there is more than one type of probiotic strain in the respective products and this is consistent with the claims of the manufacturers (Table 1). The bacteria enumeration revealed that with the exception of brand Bn, samples from all brands have bacteria amounts that are fewer than that claimed by their manufacturers (Fig. 1b). Most significant of these are samples from brands Bi, Bg and L which have viable bacteria of only 6 %, 22 % and 3 % of the amounts stated on their respective data-sheets. The bacteria concentration of brand Cn is 57 % of that claimed by the manufacturer while brand Bn has a surprising bacteria count that is more than double the stated amount. The enumerated bacteria amounts were however above the recommended minimum threshold of 10^6 CFU/capsule [17]. The over-estimation of probiotic amounts in four of the five products tested suggest that 1) a significant amounts of bacteria have been compromised during the processing stages which may include improper handling and storage conditions or 2) the manufacturers have

Table 2 Classification and mode of action of antibiotics

Antibiotic	Class	Spectrum
Ampicillin (10 mcg)	Semi-synthetic beta lactams	Gram positive and Gram negative
Aztreonam (30 mcg)	Mono-bactams	Gram positive and Gram negative
Erythromycin (15 mcg)	Macrolides	Gram positive and Gram negative
Gentamicin (5 mcg)	Aminoglycosides	Gram positive and Gram negative, especially <i>Pseudomonas</i>
Streptomycin (10 mcg)	Aminoglycosides	Gram positive and Gram negative
Clindamycin (2 mcg)	Lincomycins	Gram positive and Gram negative, especially anaerobic <i>Bacteroides</i>
Vancomycin (30 mcg)	Glycopeptides	Gram positive and Gram negative, especially <i>S. aureus</i>
Cephalexin (30 mcg)	Cephalosporin	Gram positive
Tetracycline (30 mcg)	Tetracycline	Gram positive and Gram negative
Ciprofloxacin (10 mcg)	Fluoroquinolones	Gram positive and Gram negative, especially <i>Bacillus anthracis</i>

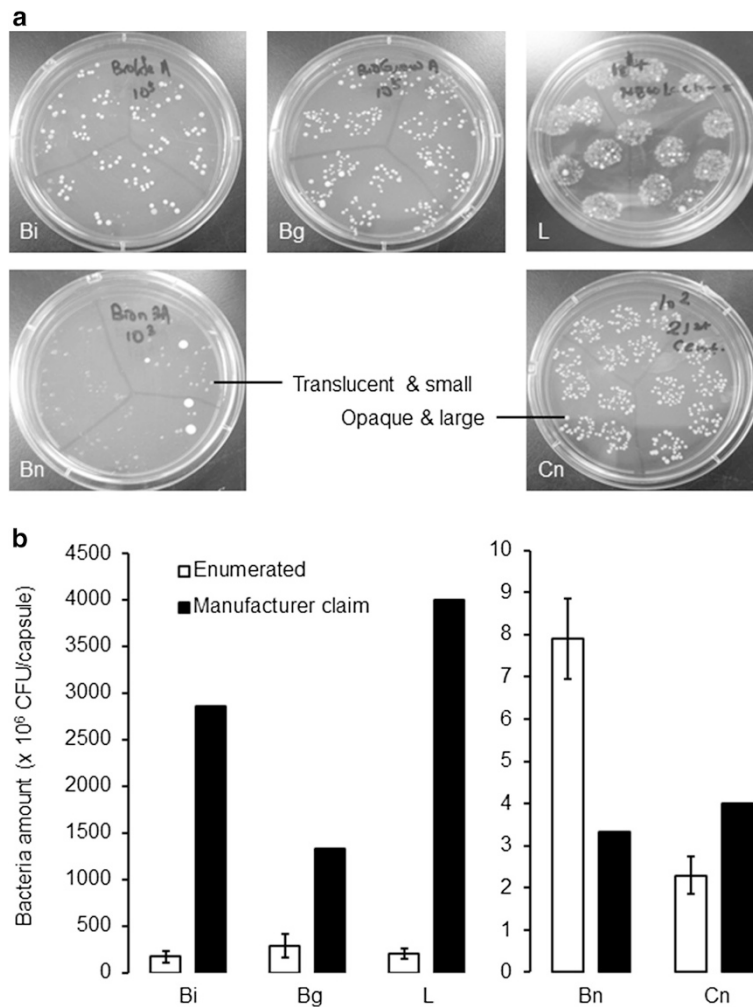
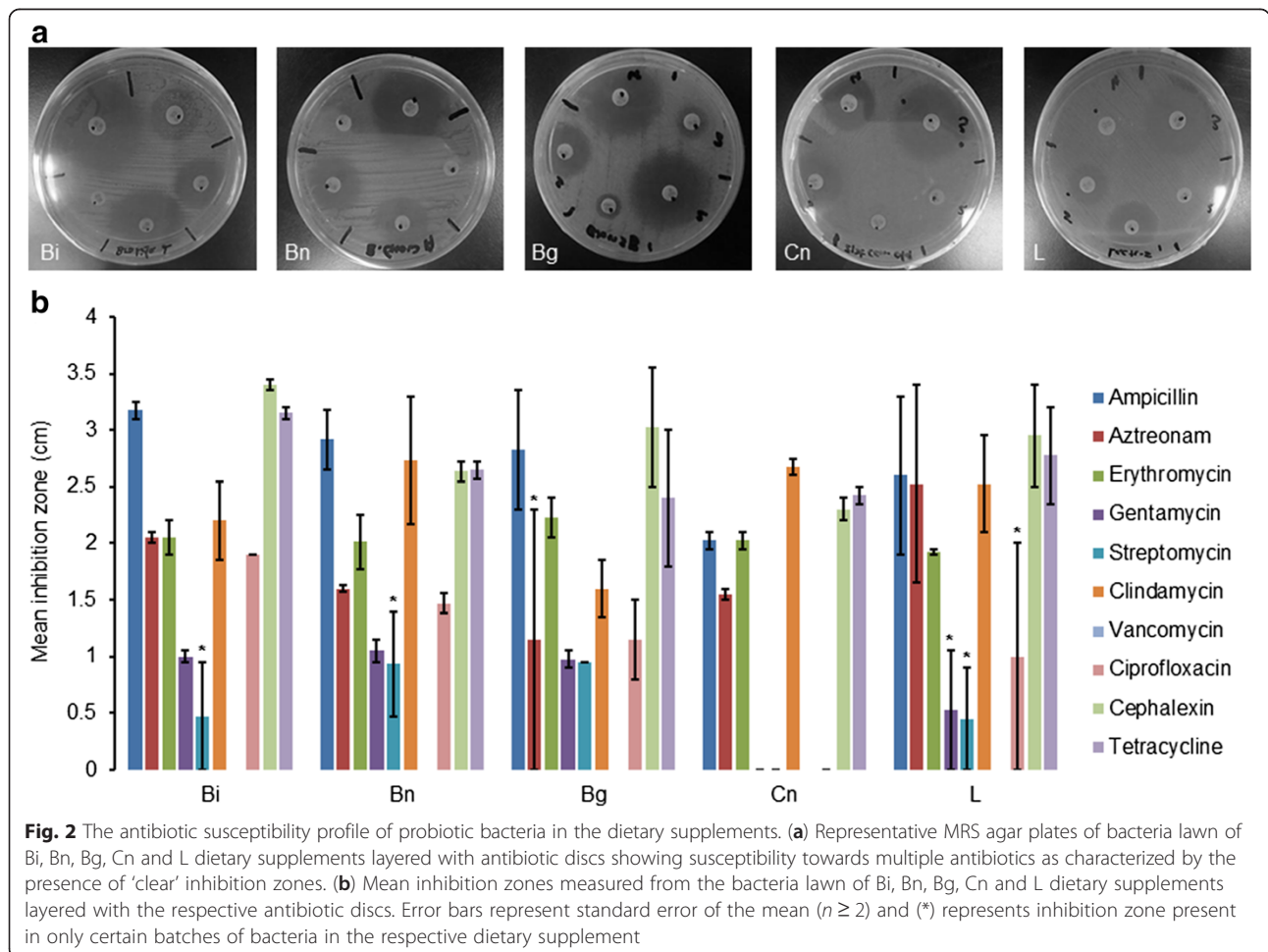


Fig. 1 The recovery and enumeration of probiotic bacteria from dietary supplements. **(a)** Representative MRS agar plates of probiotics bacteria recovered from Bi, Bg, L, Bn and Cn dietary supplements showing different colony morphologies and densities. **(b)** Comparison of the enumerated probiotics bacteria to that claimed by the manufacturers of the respective products (Table 1). Error bars represent standard error of the mean (n = 2)

deliberately overestimated the probiotic content of the products in an attempt to compete for consumers [18]. This finding seems to agree with other reports concerning mislabeling, overestimation and misidentification of probiotics in various food and health products [19]. It is therefore doubtful that the health claims of the respective products can be achieved by the inferior amounts of viable probiotic bacteria although some reports have claimed that viability is not essential and that probiotic DNA or cell wall materials may be sufficient to confer certain health effects [20]. This enumeration provides useful preliminary data shedding light on the accuracy and reliability of the probiotic bacteria information detailed on the datasheet of dietary supplements as regulations and legislations concerning product labeling are currently lacking [21].

In the antibiotic resistant screening, clear inhibition zones of > 0.5 cm were measured from the bacteria lawn of isolates from all batches of brands Bi, Bn, Bg, Cn and L towards ampicillin, erythromycin, clindamycin, cephalixin and tetracycline antibiotics (Fig. 2b), suggesting no

resistance towards these antibiotics. Meanwhile, inhibition zones detected in only certain batches of the probiotic products were that of brands Bi and Bn, Bg and L in the presence of the respective streptomycin, aztreonam and gentamycin, streptomycin and ciprofloxacin antibiotics (Fig. 2b). This batch-to-batch variation implies that these resistances were not conferred by intrinsic genes but more likely a result of acquired mobile genetic elements by a transfer event and this is a concern because mobile elements such as plasmids and transposons can be transferred from one cell to another by conjugation [10]. Indeed, this mechanism has been demonstrated *in vitro* where antibiotic resistant gene transferred from one *Lactobacillus* to another and more worryingly, also from *Lactobacilli* to other species including pathogenic strains such as *Staphylococcus* [13] and vice versa [22]. Streptomycin and gentamycin belong to the aminoglycosides and resistance towards this group of antibiotic may be conferred by intrinsic *Lactobacilli* aminoglycoside resistant genes *aac(6)-aph(2'')*, *ant(6)*, and *aph(3)-IIIa* respectively [11]. Consistent



with this speculation, bacteria of brand Cn also showed no inhibition zone against the gentamycin and streptomycin antibiotics besides also being resistant towards ciprofloxacin (Fig. 2b). While resistance towards aztreonam, a derivative of the beta-lactam antibiotics, may be conferred by the *blaZ* genes which have also been previously found in *Lactobacilli* [2], detection of ciprofloxacin resistance could not be attributed to known fluoroquinolone resistance genes as they have not been identified in *Lactobacilli*. Although probiotic bacteria (predominantly *Lactobacilli*) of all products were able to grow in the presence of vancomycin (Fig. 2b), this is attributed to intrinsic vancomycin resistant genes in *Lactobacilli* strains rather than a transfer event. This intrinsic resistant gene prevents vancomycin binding at the cytoplasmic end of their cell walls due to replacement of the terminal amino acid residue [23]. Notably, tetracycline and erythromycin resistance were not detected in isolates of all brands although genes conferring resistance towards these classes of antibiotics (i.e. the tetracycline and macrolides) are well-characterized in *Lactobacilli* [24]. Although reports on antibiotic resistance in probiotic isolates of dietary supplements are elusive, such data from foods [12] and biological sources [25] are widely reported [6, 9, 16]. Further, resistant determinants have also been identified with the resistance genes of tetracycline (>10), aminoglycoside (at least 4) and beta-lactam [12] constituting an expanding list of genetic elements in *Lactobacilli*.

In summary, this is a pilot study that reports the present of antibiotic resistance in probiotic bacteria in dietary supplements thus expanding the screening of antibiotic resistance to include this largely unexplored but increasingly popular group of functional foods. This study also serves as a platform for further screenings and in-depth characterization of the resistant determinants and the molecular machinery that confers the resistance. In particular, resistant probiotics can be isolated and identified by a combination of biochemical tests and 16S rRNA gene sequencing analysis and the antibiotic resistant genes characterized using degenerate primers and/or a motif-based identification approach [26]. Manufacturing companies of dietary supplements should incorporate antibiotic susceptibility screening using diagnostic tools such as microarray chips and PCR approaches in the production chain. Additionally, curative strategies such as the removal of genetic elements that harbor antibiotic resistance could also be applied to the relevant probiotic strains. The latter has been applied to the probiotic *L. reuteri* DSM 17938 whereby two resistant plasmids were successfully removed from the parent *L. reuteri* (ATCC 55730) [27] while not affecting the probiotic properties of the strain.

Additional file

Additional file 1: Extended Materials and Methods.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

RL conceived the project and supervised the work; DN and DLA performed the experiments; AW and AO performed the data analysis; and AW and RL wrote the manuscript. All authors read and approved the manuscript.

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