ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 5, Issue, 02, pp. 3246-3249, February, 2015

International Journal of
DEVELOPMENT RESEARCH

Full Length Research Article

PRESENCE OF ANTIBIOTIC RESISTANT PROBIOTICS IN HEALTH SUPPLEMENTS

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ARTICLE INFO

Article History:
Received 30th November, 2014
Received in revised form
31st December, 2014
Accepted 08th January, 2015
Published online 27th February, 2015

Key words:
Probiotics,
Antibiotic resistance,
Health supplements.

ABSTRACT

Probiotics are known for their health-promoting benefits and are widely used in food and health products. However, they serve as reservoirs for antibiotic resistant genes and risks clinical complications if transferred to pathogenic strains. Antibiotic resistant probiotic strains have been reported from various food and biological sources but the antibiogram of the corresponding probiotics from health supplements have remained largely unknown. Here, we report resistance towards tetracycline, erythromycin and kanamycin antibiotics from probiotic isolates of health supplements and discuss the implications of this finding.

INTRODUCTION

Foods fortified with probiotic strains have positive effects on health including the stimulation and regulation of the immune system (Herich and Levkut, 2002), enhancing mineral absorption (Scholz-Ahrens *et al.,* 2007), reducing lactose intolerance, preventing antibiotic-induced diarrhea (Marcel, 2000) and improving intestinal microbial balance by competitive exclusion of pathogens (Kaur *et al.,* 2002). It is therefore unsurprising that probiotic products have not only gained widespread acceptance but also increasing popularity (Stanton *et al.,* 2001) as reflected by a rapid increase in the global probiotics market which has a projected economic value of 31.2 billion US dollars in 2014 – a growth of 11.7% from 2009 (Markets and markets, 2009). Despite the overwhelming benefits, probiotics serve as reservoirs for antibiotic resistant genes which can be transferred to pathogens that share the same intestinal habitat or via food chain (Egervarn, 2009). In addition, probiotics may themselves acquire these resistant genes from human commensals and become pathogenic, thus giving rise to infections in immune-compromised patients (Courvalin, 2006). In addition to probiotic-fortified foods, consumption of high amount of probiotics especially in the form of health supplements encourage the spread of antibiotic resistant genes and this becomes a clinical problem because it limits the options of antibiotics for effective prophylactic applications. The transfer of mobile genetic materials such as plasmids coding for various antibiotic resistance mechanisms may over time, accumulate in the gut and leads to multiple-drug resistance (MDR). Cross-resistance to other antibiotics within the bacteria population can also happen when one resistance mechanism confers resistance to another antibiotic usually derived from the same parent compound. Given that the pipeline of new antibiotics is gradually exhausted, the emergence of MDR and 'pan-resistant' strains threatens to end the antibiotic era (Cirz *et al.,* 2005).

While efforts to detect antibiotic resistance in probiotic strains of various food sources have intensified, reports on antibiotic resistant probiotics from health supplements have remained somewhat elusive. Since probiotic health supplements contain both a high amount of probiotic bacteria and a heterogeneous population of bacteria, it is therefore conceivable that some of these probiotic strains harbor antibiotic resistance since these are conditions that encourage the transfer of genetic materials

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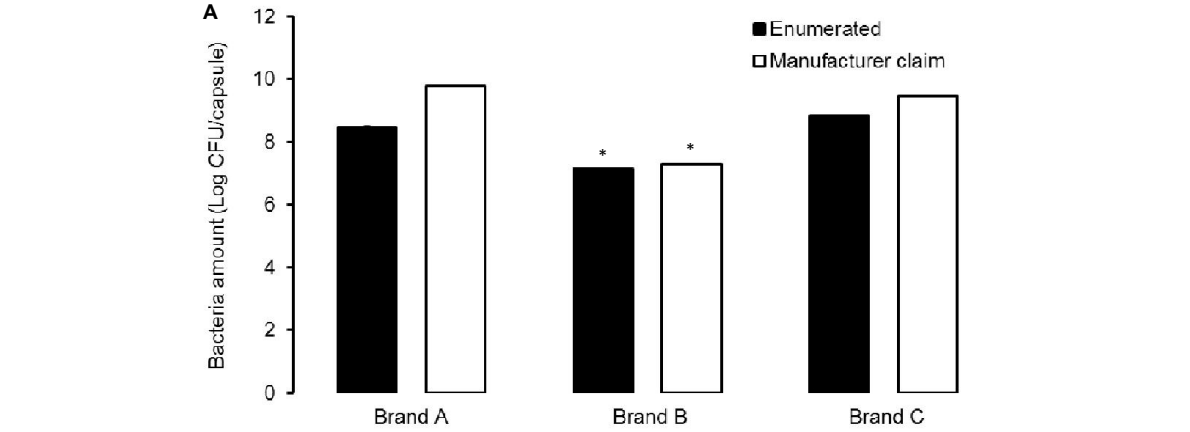
including those that confer resistance to antibiotics. We hypothesize that antibiotic resistant probiotics occur in health supplements and demonstrate that they serve as an excellent platform not only for the detection of antibiotic resistance, but also for molecular characterization of resistant genes and resistant conferring mechanisms. In this short communication, we screen for antibiotic resistant probiotics from three health supplements subsequently referred to as Brand A, B and C and of which two, Brand A and B contain heterogeneous population of probiotic strains and are commonly available over-the-counter in pharmacies and health outlets throughout Malaysia. Brand B probiotic health supplement consists of only *Lactobacillus reuteri* strain and was obtained as a prescription from a local hospital.

The antibiotics used in this study, tetracycline, kanamycin and erythromycin have contrasting mode of actions and resistant mechanisms. The identities of the tested products were blinded to protect the interests of both the manufacturing companies and the affiliating institution where this work was performed.

RESULTS AND DISCUSSION

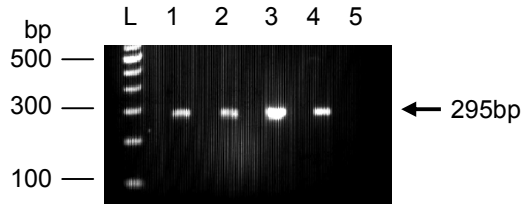
In order to first determine if the probiotic bacteria in the test supplements were viable, we performed an initial enumeration using the drop plate method (Munsch-Alatossava *et al.,* 2007). Bacteria growth was observed in all brands of supplements with Brand A and C demonstrating mixed colony morphologies, suggesting the presence of a heterogeneous



B

Staphylococcus aureus M769 *ErmC*gene, partial cds
Length=382

Score = 466 bits (252), Expect = 2e-128
Identities = 253/254 (99%), Gaps = 0/254 (0%)
Strand=Plus/Minus

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290PCRProductCTATCAAAAACAATTTTGCGTATTATATCCGTACTTATGTTATAAGGTATATTACCAAAT  68
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S. aureusCTATCAAAAACAATTTTGCGTATTATATCCGTACTTATGTTATAAGGTATATTACCAAAT  208

290PCRProductATTTTATAGGATTGGTTTTTAGGAAATTTAAACTGCAATATATCCTTGTTTAAAACTTGG  128
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S. aureusATTTTATAGGATTGGTTTTTAGGAAATTTAAACTGCAATATATCCTTGTTTAAAACTTGG  148

290PCRProduct   AAATTATCGTGATCAACAAGTTTATTTTCTGTAGTTTTGCATAATTTATGGTCTATTTCA  188
  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S. aureusAAATTATCGTGATCAACAAGTTTATTTTCTGTAGTTTTGCATAATTTATGGTCTATTTCA  88

290PCRProductATGGCAGTTACGAAATTACACCTCTGTACTAATTCAAGGGTAAAATGCCCTTTTCCTGAG  248
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S. aureusATGGCAGTTACGAAATTACACCTCTGTACTAATTCAAGGGTAAAATGCCCTTTTCCTGAG  28

290PCRProductCCGANTTCAAAGAT  262
|||| |||||||||
S. aureusCCGATTTCAAAGAT  14
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Figure 1. Bacteria enumeration and detection of erythromycin resistant gene

(**A**) Bacteria enumeration was performed using the drop plate method (Munsch-Alatossava *et al.,* 2007). Samples, powdered (Brand A and C) and liquid (Brand B), were prepared in phosphate buffer saline (PBS) and 10 µL from the appropriately diluted samples were dropped onto De Man Rogosa and Sharpe (MRS) (Difco, USA) agar plates and incubated at 37 ºC for 24 hrs. Bacteria amounts were compared to that claimed by the manufacturers. A total of two, one and three independent batches of Brand A, B and C respectively was platted each in triplicates, and the bacterial amounts were averaged. Only *Lactobacillus* probiotic strains were cultured and enumerated. Error bars represent standard deviations and (*) represents bacteria amount in log CFU/mL. (**B**) A representative DNA sequence of the 295 bp PCR product (Brand B erythromycin resistant isolate) with high sequence identity (98%) to the *erm(C)* gene from *Staphylococcus aureus* M769. Detection of erythromycin resistant gene was performed by PCR using the *erm(C)* gene-specific primer (Jensen *et al.,* 1999). Genomic DNA was extracted from identified erythromycin-resistant isolates, and then subjected to the PCR detection. PCR products were analyzed on (inset) a 1.5 % agarose gel and bands corresponding to the expected *erm(C)* size (295 bp) were excised, purified and sent for sequencing (First Base Laboratories, Malaysia). Lanes 1 – 4 corresponds to the PCR products of Brand A, B and C respectively, lane M corresponds to the 100 bp DNA ladder (New England Biolabs, USA) and lane 5 represents products of PCR reaction of genomic DNA isolated from non-erythromycin resistant *L. casei*. The DNA sequence was aligned against the GenBank database using BLAST.

population of bacterial strains in these samples and this observation is consistent with the information recorded on the datasheet of the respective products. Also consistent with the manufacturer's claim, Brand B sample only showed one type of colony morphology. All bacteria counts were lower than that claimed by the respective manufacturers although surpassing the minimum threshold of 10^6 CFU/capsule (Shah, 2000) and this finding seems to agree with earlier reports concerning mislabeling and overestimation of probiotics in various products (Reuter, 1997; Holzapfel *et al.,* 1998; Hamilton-Miller *et al.,* 1999; Temmerman *et al.,* 2002). The counts varied from 10^7 to 10^9 CFU/g for capsule samples and CFU/mL for liquid samples, and with only slight variations (i.e. error bars < 1%) in the viable count (Figure 1A).

This suggests a fairly consistent amount of viable cells between samples from the same batch and from different batches of the tested products. Brand B samples yielded viable bacteria count which are closest to that claimed by the manufacturer, thus indicating a better recovery compared to the other tested products. It must be noted that Brand B is in liquid form and contains only one probiotic strain i.e. *L. reuteri*, while Brand A and C contain a consortium of probiotic bacteria of which, only *Lactobacilli* strains were enumerated on the De Man Rogosa and Sharpe (MRS) selective medium. We detected tetracycline-resistant probiotic bacteria in all three brands of probiotic supplements except for the 2nd batch of Brand A and the 1st batch of Brand C which did not grow at 4 µg/mL of tetracycline. The inhibition concentration of tetracycline varies slightly among isolates from different brands and between batches of the same brand (Table 1). Although there is no report of antibiotic resistant study on health supplements, previous studies have reported the presence of tetracycline-resistant *Lactobacillus* strains in human, silage and food sources (Stsepetova *et al.,* 2008), and tetracycline-resistant *L. reuteri* from dairy origins (Egervarn *et al.,* 2007).

Table 1. The antibiotic minimum inhibitory concentrations for probiotic isolates

	Inhibition concentration (µg/mL)		
Source	Tetracycline	Kanamycin	Erythromycin
Brand A			
(1st batch)	42	>100	44
(2nd batch)	4†	>100	>100
Brand B	28	>100	44
Brand C			
(1st batch)	4†	4†	>100
(2nd batch)	38	4†	>100
(3rd batch)	32	>100	4†

An aliquot of 100 µL overnight culture of bacteria isolate was transferred into 5 mL of antibiotic-MRS broth (Difco, USA) and incubated at 37 °C for 24 hrs in an aerobic orbital shaker (Infors AG, Switzerland). (†) indicates no bacteria colonies detected at 4 µg/mL of the respective antibiotics.

Sources and origins of the probiotic strain may influence the type and composition of tetracycline resistant gene(s) due to the exposure and interactions with different consortium of bacteria present in their original habitats. In comparison, high level of resistance (>100 µg/mL) to kanamycin was observed for isolates from all brands of probiotic supplements except for the 1st and 2nd batches of Brand C. Previous studies have also reported kanamycin resistant *Lactobacillus* from various sources such as the European food and dairy products (Temmerman *et al.,* 2002), the new *Lactobacillus* isolates from Fonterra Research Centre Culture Collection (Zhou *et al.,* 2004), the starter cultures of dairy and pharmaceutical products (D'Aimmo *et al.,* 2006) and the isolates of human vagina (Ocana *et al.,* 2006). Erythromycin-resistant isolates with different inhibition concentrations were detected in all brands of probiotic health supplements except for the 3rd batch of Brand C. Erythromycin-resistant *Lactobacillus* has also been reported in human gastro-intestinal tract (Klaenhammer and Muller, 1999) while erythromycin-resistant *L. reuteri* isolates were found to be present in samples from dairy origins (Egervarn *et al.,* 2007).

Further, we attempt to identify known antibiotic resistant genes from the resistant isolates using a PCR approach. To this end, we detected the presence of *erm(C)* gene in the genomic DNA of resistant isolates from Brand A (1st batch), B and C (1st and 2nd batches) and all gave PCR products of approximately 295 bp in size with sequences of > 90% identity to the *erm(C)* gene of *Staphylococcus aureus* (GenBank Accession: AF466409.1) (Figure 1B). Therefore, it is likely that the *erm(C)* gene is responsible for conferring resistance towards erythromycin in the respective isolates. Although the *erm(C)* gene was originally isolated from *S. aureus*, this gene is also present in *L. reuteri* (GenBank Accession: FJ489650.1). Interestingly, since both Brand A and C did not contain *L. reuteri* and since *erm(C)* is not known to be present in the probiotic strains of these samples (as claimed by the manufacturer), the detection of *erm(C)* in their resistant isolates therefore implies gene acquisition by transfer events (Volokhov *et al.,* 2003; Egervarn *et al.,* 2007).

Currently, there is no widely accepted regulation and validation of manufacturers' claims and labels of functional foods including probiotic health supplements (Berner and O'Donnell, 1998; Przyrembel, 2001). As such, this study provides useful preliminary data that sheds light on the antibiotic resistant profile of probiotics in health supplements. Curative strategies could be applied to probiotic strains to remove plasmids carrying unwanted antibiotic resistance genes. For example, the commercial probiotic *L. reuteri* strain DSM 17938 was derived from *L. reuteri* (ATCC 55730) by removing two resistant plasmids without losing any probiotic characteristics (Rosander *et al.,* 2008). Additionally, the use of efficient diagnostic tools such as microarray chips for rapid and convenient antibiotic susceptibility screening in food production process can also be performed. In summary, resistance towards antibiotics was detected in the tested probiotic health supplements although the antibiotic resistant profile varies from batch-to-batch. Taken together, these results support our hypothesis that antibiotic resistance exists in probiotic strains isolated from health supplements and highlight the accompanying concerns and the broader implications that may follow.

Acknowledgement

RL conceived and supervised the project, AW performed the experiments and data analysis, and both RL and AW wrote the manuscript. We thank Mr. Pakiraji @ Pakiraju a/l S. Ramaya, Ms. Chua Lee Hui, and Mr. Tai Biing Huei for the technical support, and Ms. Amanda Ooi Siok Lee for reading and formatting the manuscript.

Competing Interests

The authors declare that they have no competing interests.

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