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Investigating the transmissibility of *tet*(W) in bifidobacteria exposed to acid and bile stress

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Transfer of antibiotic resistance genes from probiotic bacteria to pathogens poses a safety concern. Orally administered probiotics are exposed to stressful conditions during gastrointestinal transit. In this study, filter mating experiments were performed to investigate the potential role of exposure of *Bifidobacterium* isolates to acid and bile stress on the transfer of a tetracycline resistance gene, *tet*(W), to *Enterococcus faecalis* ATCC 51299. No *E. faecalis* transconjugants were obtained after mating with either stressed or unstressed *Bifidobacterium*, thereby suggesting that *tet*(W) could not be transferred as a result of exposure to gastrointestinal stresses.

Key words: *Bifidobacterium*, gastrointestinal stress, probiotics, *tet*(W), antimicrobial resistance

Bifidobacteria are commonly incorporated into foods as probiotics because of their associated health benefits [1]. Intrinsic resistance to antibiotics is desirable in probiotics when they are co-administered with antibiotics for the prevention of antibiotic-associated diarrhea. However, it becomes a safety issue if there is a risk of horizontal transfer of resistance genes to pathogens [2]. The addition of probiotic bacteria to various products makes them a potential source for the spread of antibiotic resistance genes [3].

Tetracycline resistance is common in bifidobacteria due to *tet* genes, especially *tet*(W), located on the bacterial chromosome [4]. Although integrated in the chromosome, the *tet*(W) gene of *Bifidobacterium* may be surrounded by genes coding for transposases that catalyze gene movement. This may suggest that *tet*(W) can be transferred to other bacteria under certain conditions. However, there is currently no evidence to demonstrate this [5].

Orally administered probiotic bifidobacteria encounter stressful conditions in the gastrointestinal tract, such as acid in the stomach and bile in the small intestine [6]. Such conditions may modify their physiological properties, leading to various consequences, which may include antibiotic resistance gene transfer [7]. Therefore, the aim of this study was to investigate the effect of exposure to acid and bile stresses on the transmissibility of *tet*(W) from *Bifidobacterium* isolates to *Enterococcus faecalis* ATCC 51299.

Products containing *Bifidobacterium* were purchased from stores in Coventry, UK (Table 1). In addition, a freeze-dried commercial culture, Nu-Trish *Bifidobacterium* BB-12, was kindly provided by Chr. Hansen (Berkshire, UK). Bifidobacteria were isolated on Reinforced Clostridial Agar (RCA) (CM0151, Oxoid, Hampshire, UK) containing 50 mg/l of lithium mupirocin (69732, Sigma-Aldrich, Switzerland) (RCA-MUP). Cultures of *Escherichia coli* K12 and *E. faecalis* ATCC 51299 were provided by Coventry University. Prior to experiments, cultures of *Bifidobacterium* were prepared on RCA incubated under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) in an anaerobic workstation (Don Whitley Scientific, Shipley, UK) for 48 hr at 37°C. *Escherichia coli* K12 *and E. faecalis* ATCC 51299 were prepared on nutrient agar (CM0309, Oxoid) incubated under aerobic conditions for 24 hr at 37°C.

The minimum inhibitory concentration (MIC) of tetracycline for each culture was determined by broth microdilution. Inocula were prepared by suspending colonies obtained from each solid medium in normal saline (8.5 g/l NaCl). A stock solution of tetracycline (tetracycline hydrochloride, T-3383, Sigma) at a concentration of 1,280 µg/ml was prepared in sterile distilled water. From this stock, a twofold dilution series (128–0.25 µg/ml) of tetracycline in 150 µl of Reinforced Clostridial Medium (RCM, CM0149, Oxoid) or Iso-Sensitest Broth (ISB, CM0473, Oxoid) was prepared in round-bottomed 96-well microtiter plates for *Bifidobacterium* and *E. faecalis*

Table 1. Sources of bifidobacteria used in this study

Isolate code	Product type	*Bifidobacterium* description on product
1	Yogurt	*B. longum*
2	Yogurt	*B. lactis*
3	Yogurt	*Bifidobacterium*
4	Yogurt	*Bifidobacterium*
5	Capsule	*B. animalis* ssp. *lactis*
6	Freeze-dried BB-12	*B. animalis* ssp. *lactis*

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Table 2. Primer sequences used in this study

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
16S-23S rDNA ITS	GTCGTAACAAGGTAGCCGTA	CAAGGCATCCACCGT	55
tet(W)	TGGAATTCTTGCCCATGTAGACG	GAACATATGGCGCACCTTGTCC	64
trp-tet(W)*	ATTCAGCGACGAACTGGCACAG	CGCTTGAATGGTAATCCCACG	63

*Primers amplify *trp* gene with 5′ end of *tet*(W).

51299/*E. coli* K12, respectively. Wells were inoculated with 10 µl of bacterial suspension. Negative controls were 150 µl of uninoculated RCM or ISB, and positive controls were inoculated broths without tetracycline. Plates were incubated with lids under appropriate conditions for each culture. The MIC was identified as the lowest antibiotic concentration at which no growth (turbidity) was observed. The bacteria were classified as resistant or susceptible to tetracycline based on defined break points [8].

Bifidobacterium isolates were phenotypically characterized by Gram staining and catalase reaction. All cultures were genotypically characterized by a polymerase chain reaction based on 16S-23S rDNA internal transcribed spacer region (ITS-PCR), according to the method described by Xu *et al.* [9], with modifications. Extraction of genomic DNA was carried out using InstaGene Matrix (7326030, Bio-Rad, Hertfordshire, UK) according to the manufacturer's instructions.

The 25 µl reaction mixture contained 2.5 µl of 10x PCR buffer including 20 mM $MgCl_2$ (B9004S, New England Biolabs, Hertfordshire, UK), 4 µl of 1.25 mM dNTP mix (N0447, New England Biolabs), 0.1 µl of 5U Taq DNA polymerase (M0267X, New England Biolabs), 1 µl each of 10 pmol/µl forward and reverse primer (Table 2), 15.4 µl of sterile Milli-Q water, and 1 µl of DNA template. PCR amplification was carried out in an Eppendorf Master Cycler (22331, Eppendorf, Germany) using the following temperature program: initial denaturation (1 min, 94°C) followed by 35 cycles of denaturation (1 min, 94°C), annealing (1 min, 55°C), and elongation (1 min, 72°C) and then final extension (7 min, 72°C).

Cultures were then screened for the presence of *tet*(W) and *trp* coding for the tetracycline resistance gene and the flanking transposase gene, respectively, by a PCR using primers in Table 2 [4], according to the method described by Masco *et al.* [10], with modifications. The 50 µl PCR assay mix contained 32.8 µl of sterile Milli-Q water, 1 µl of 1.25 mM dNTP mix (N0447, New England Biolabs), 5 µL of 10x PCR buffer including 20 mM $MgCl_2$ (B9004S, New England Biolabs), 3 µl each of 10 pmol/µl forward and reverse primer (Table 2), 0.2 µl of 5U Taq DNA polymerase (M0267X, New England Biolabs), and 5 µl of DNA template. PCR amplification was carried out using the following temperature program: initial denaturation (5 min, 95°C) followed by 25 cycles of denaturation (45 sec, 94°C), annealing (1 min, at primer-specific temperature [Table 2]), and elongation (1 min, 72°C) and then final extension (10 min, 72°C).

All PCR amplicons were visualized by electrophoresis on 1% (1.2% for ITS-PCR) agarose gels containing 2 µl GelRed stain (41003, Biotium, Fremont, CA, USA); wells were loaded with 10 µl of PCR product + 2 µl of loading dye (R0611, Thermo Fisher Scientific, Loughborough, UK). The gels were run in 1x Tris-Borate-EDTA (TBE) buffer for 1 hr at 120 V. The sizes of the PCR products were estimated using DNA molecular size markers (SM1113, Thermo Fisher Scientific; N3234, New England Biolabs). Gel images were taken under UV light in a UV transilluminator (Gel Doc EZ Imager, Bio-Rad).

Stress conditions for three *Bifidobacterium* isolates (isolates 1, 2, and 6) were determined according to the method described by Amund *et al.* [7], with modifications. RCM was acidified with 1 M hydrochloric acid (HCl) to pH values of 3 and 4, supplemented with bovine bile (B3883, Sigma-Aldrich) to 0.5% and 1% (w/v), or unadjusted as the control. Suspensions of 48-hr cultures in normal saline (approximately 10^8 cfu/ml) were prepared, from which 1 ml volumes were inoculated into 9 ml each of RCM and incubated anaerobically at 37°C. Enumeration on RCA was carried out immediately after inoculation (Time 0) and at predetermined intervals (1, 2, 3, and 24 hr). Stressful conditions were determined as those where there was no growth after 24 hr of incubation. Whereas unadjusted controls showed growth after 24 hr, adjusted RCM (acid, bile) resulted in a decline or no growth after 24 hr. Furthermore, after 1 hr of incubation, there was no significant change in numbers observed in any of the stress treatments (results not shown). Therefore, the conditions chosen to induce stress in the bifidobacteria for the conjugation experiments were pH 3 and 1% (w/v) bovine bile for 1 hr.

Fresh colonies of donor bacteria (*Bifidobacterium*) were suspended in 5 ml of RCM that had been acidified to pH 3 with 1 M HCl, supplemented with 1% (w/v) bovine bile, or unadjusted, until a turbid suspension was reached. Suspensions were incubated anaerobically at 37°C for 1 hr in order to achieve acid-stressed, bile-stressed, and unstressed (control) treatments, respectively [7]. After incubation, cells were harvested by centrifugation at 4,400 rpm for 5 min. The supernatants were discarded, and the remaining pellets were resuspended in normal saline to achieve a turbidity equivalent to a 2 McFarland standard (approximately 6×10^8 cfu/ml).

Conjugation experiments were carried out between the bifidobacteria and *E. faecalis* ATCC 51299 by the filter mating method described by Ouoba *et al.* [11], with modifications. Fresh colonies of recipient bacteria (*E.*

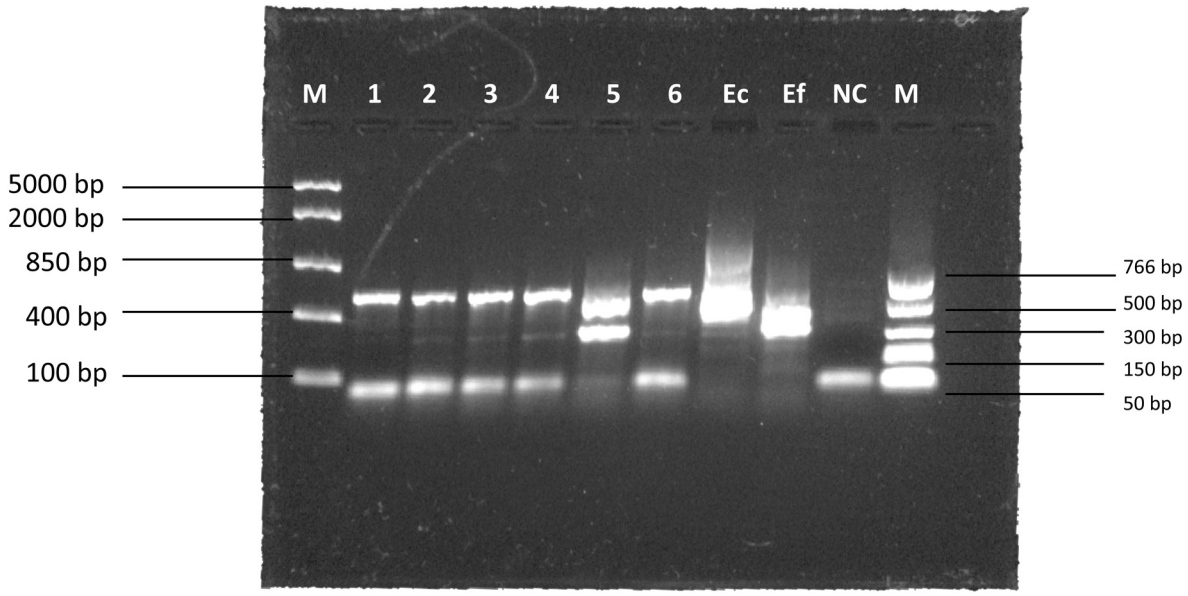

Fig. 1. ITS-PCR banding patterns. M: marker; Ec: *E. coli* K12; Ef: *E. faecalis* ATCC 51299; NC: negative control.

Table 3. Tetracycline MICs and detection of *tet*(W) and *trp* in *Bifidobacterium* isolates and recipient bacteria

Bacteria	Break point (μg/ml) [8]	MIC (μg/ml)	R or S*	*tet*(W)	*trp*
1	8	32	R	+	+
2	8	16	R	+	+
3	8	16	R	+	+
4	8	16	R	+	+
5	8	>128	R	+	+
6	8	32	R	+	+
E. coli K12	8	4	S	+	–
E. faecalis ATCC 51299	4	0.5	S	–	–

*R: resistant; S: susceptible.

faecalis ATCC 51299) were suspended in normal saline until a similar turbidity to the donor suspensions was reached (2 McFarland standard). Subsequently, a 1:10 dilution of the recipient suspension was made (approximately 10^7 cfu/ml). One milliliter of each donor suspension was mixed with 1 ml of diluted recipient suspension (thereby achieving an approximate donor-recipient ratio of 10:1). The mixtures were each filtered through a membrane filter (pore size 0.45 μm, diameter 47 mm) (63069, GN-6 Metricel Membrane, Pall Life Sciences, Portsmouth, UK), using a filter holder and vacuum pump. To trap the bacteria in the membrane, the filters were washed with 2 ml of normal saline. Finally, the filters with donor and recipient bacteria were placed on Brain Heart Infusion (BHI) agar (CM1135, Oxoid) and incubated overnight under aerobic conditions at 37°C. Afterwards, each filter was transferred into a 15 ml tube containing 5 ml of normal saline and vortexed to resuspend bacteria. To detect potential *E. faecalis* transconjugants, 100 μl of suspension was spread on MacConkey agar (CM0007, Oxoid) supplemented with 10 μg/ml of tetracycline and incubated under aerobic conditions. Suspensions were also plated on RCA-MUP and MacConkey agar to confirm viability of donors and recipients (results not shown).

All *Bifidobacterium* isolates were phenotypically characterized as Gram-positive, catalase-negative rods. Genotypic characterization by ITS-PCR showed all isolates except one exhibited similar banding patterns, with a primary band around 500 bp in size (Fig. 1).

Bifidobacterium isolates were confirmed to be resistant to tetracycline (Table 3), and *tet*(W) and *trp* were detected (Fig. 2 and Fig. 3). PCR amplicons had sizes of approximately 1,888 bp and 1,474 bp, respectively, which is consistent with Gueimonde *et al.* [4]. *E. coli* K12 was found to be sensitive to tetracycline (Table 3) but positive for *tet*(W) (Fig. 2). *E.*

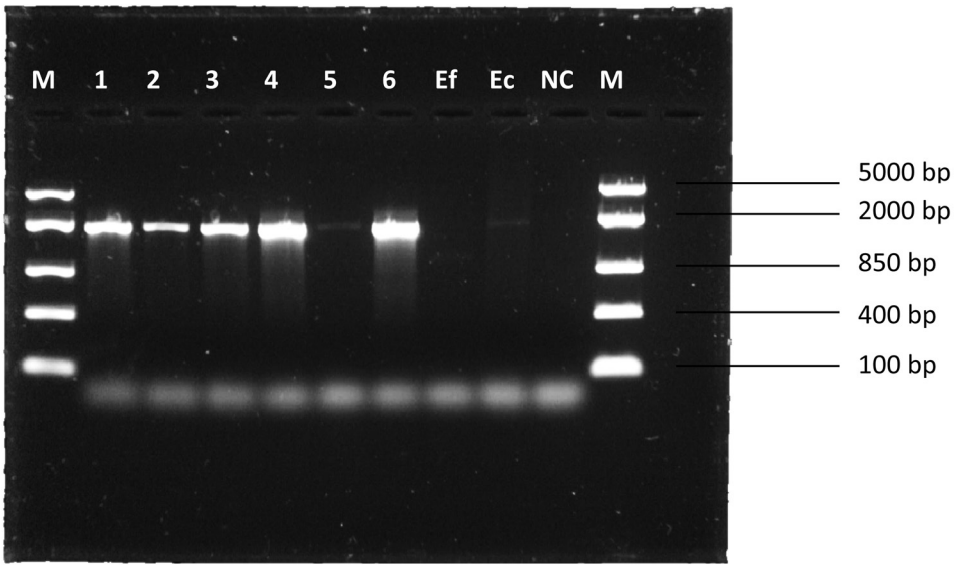

Fig. 2. PCR amplicons of *tet*(W). M: marker; Ef: *E. faecalis* ATCC 51299; Ec: *E. coli* K12; NC: negative control.

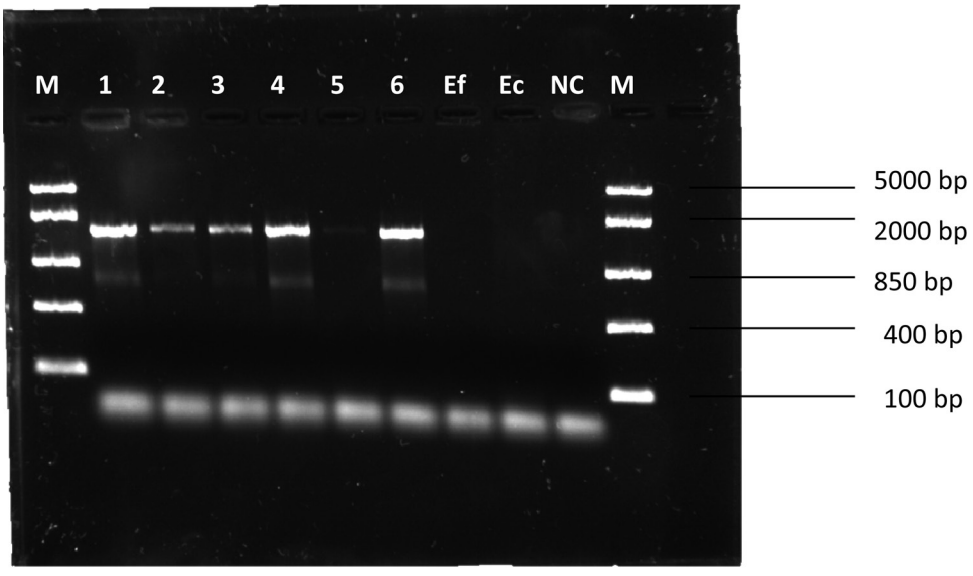

Fig. 3. PCR amplicons of *trp-tet*(W). M: marker; Ef: *E. faecalis* ATCC 51299; Ec: *E. coli* K12; NC: negative control.

faecalis ATCC 51299 was found to be sensitive to tetracycline (Table 3) and lacked *tet*(W) (Fig. 2). *E. coli* K12 was therefore excluded from subsequent conjugation experiments due to the detection of *tet*(W).

Filter mating of *E. faecalis* ATCC 51299 with stressed or unstressed *Bifidobacterium* isolates resulted in no tetracycline resistant transconjugants, thereby suggesting that exposure to gastrointestinal stress conditions such as acid and bile may not lead to mobilization and subsequent transfer of the *tet*(W) gene to other enteric bacteria.

Sublethal food preservation stress conditions (pH, osmotic, high/low temperature) have been demonstrated to significantly increase plasmid transmission rates between plasmid-bearing *E. coli* donor cultures and recipient *E. coli* and *Salmonella* Typhimurium strains *in vitro* [12]. Transposon movement and transposition in *E. coli* has also been shown to be influenced by nutritional stress [13]. The aforementioned may justify the need to evaluate candidate probiotic bacteria for not just the presence and transferability of antibiotic resistance genes but also the presence and transferability of antibiotic resistance genes with the added consideration of environmental stress, including gastrointestinal stress.

Based on the ITS-PCR banding patterns and the *Bifidobacterium* BB-12 commercial culture as the reference strain, the *Bifidobacterium* isolates (donors) in the experiments could be considered to be *B. animalis* ssp. *lactis*. While low frequency transfer of *tet*(W) between *Bifidobacterium* strains has been demonstrated *in vitro* [14], to our knowledge, no studies have been able to demonstrate transfer of *tet*(W) from *B. animalis* ssp. *lactis* to other bacteria or other bifidobacteria. Strains of *B. animalis* ssp. *lactis* are the most commonly used bifidobacteria commercially [15] and are considered to be safe [16].

It should be noted that there appeared to be some instances of mismatches between the ITS-PCR banding pattern and the *Bifidobacterium* description on the product label (i.e., isolates 1 and 5) (Fig. 1). Such findings have been reported in other studies on probiotic products [1, 15, 17] and have been suggested to be due to misidentification or undeclared bacterial cultures. Correct identification and labelling are crucial in ensuring safety and functionality [15].

It has been highlighted in previous research that many *in vitro* experimental conditions do not fully represent the complex conditions of the gut, and the impact of these conditions on the transfer of antibiotic resistance is still unclear [4]. Furthermore, standardized conjugation protocols for *in vitro* experiments with bifidobacteria have yet to be developed [18]. To our knowledge, this report represents the first attempt at examining the relationship between gastrointestinal conditions and antibiotic resistance gene transfer by bifidobacteria. Further investigations should examine a wider range of *Bifidobacterium* strains and recipient bacteria and include variations in incubation conditions (aerobic, anaerobic) and donor-recipient ratios, among others.

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