Horizontal transfer of \textit{vanA} between probiotic \textit{Enterococcus faecium} and \textit{Enterococcus faecalis} in fermented soybean meal and in digestive tract of growing pigs

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Abstract

\textbf{Background:} The aim of this study was to investigate the intergeneric transfer of vancomycin resistance gene \textit{vanA} between probiotic enterococci in the fermentation progress of soybean meal and in the digestive tract of growing pigs. One \textit{vanA} genotype vancomycin resistant \textit{E. faecium} strain, \textit{Efm4}, and one chloramphenicol-resistant \textit{E. faecalis} strain, \textit{Efs2}, were isolated from twenty-nine probiotic basis feed material / additive samples. For \textit{in vitro} conjugation, \textit{Efm4} and \textit{Efs2} were used as starter to ferment soybean meal. For \textit{in vivo} conjugation, thirty growing pigs were randomly assigned to five groups (\(n=6\)), treated with a basic diet, or supplemented with 10% fermented soybean meal, 1% \textit{Efm4}, 5% \textit{Efs2} or a combination of 1% \textit{Efm4} + 5% \textit{Efs2} for 7 d, respectively. Fecal samples of pigs in each group were collected daily for the isolation and dynamic analysis of \textit{Efm4}, \textit{Efs2} and transconjugants. The sequence types (STs) of \textit{Efm4}, \textit{Efs2} and transconjugants were analyzed by multilocus sequence typing (MLST). The \textit{vanA} harboring plasmid in \textit{Efm4} and transconjugants was analyzed by S1-pulsed field gel electrophoresis (PFGE) and further verified by multiple alignments.

\textbf{Results:} The results showed that, in FSBM, transconjugants were detected 1 h after the fermentation, with a conjugation frequency of \(\sim 10^{-3}\) transconjugants / recipient. Transconjugants proliferated with \textit{Efm4} and \textit{Efs2} in the first 8 h and maintained steadily for 10 d till the end of the experiment. Additionally, \textit{in vivo} experiment showed that transconjugants were recovered in one of six pigs in both FSBM and \textit{Efm4} + \textit{Efs2} groups, with conjugation frequency of \(\sim 10^{-5}\) and \(\sim 10^{-4}\), respectively. MLST revealed the ST of \textit{Efm4}, \textit{Efs2} and transconjugants was ST1014, ST69 and ST69, respectively. S1-PFGE confirmed the existence of the \textit{vanA}-harboring, 142,988-bp plasmid, which was also a multi-drug resistant plasmid containing \textit{Tn1546}-like transposon.

\textbf{Conclusions:} The findings revealed the potential safety hazard existing in the commercial probiotic enterococci in China, because the horizontal transfer from farm to fork could potentially pose a safety risk to the public.

\textbf{Keywords:} Chloramphenicol, Digestive tract, Enterococci, Fermented soybean meal, Growing pigs, Probiotics, Transconjugants, \textit{vanA}, Vancomycin

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Background
Enterococci species are widely distributed in the environment and enable to colonize in the gastrointestinal tract in humans and most animals [1]. For decades, some enterococci strains like Enterococcus faecium and Enterococcus faecalis are used as probiotics in human and farm animals as a kind of lactic acid bacteria [2]. Previous studies have proven that enterococci can maintain a balance among the intestinal flora [3], promote the absorption of nutrients [4–6] and improve the host immunity [7–9]. In spite of those probiotic properties, however, enterococci have also been known as one of the multiresistant pathogen, ranking among the leading causes of hospital-acquired infections worldwide [10–12]. Vancomycin therapy is one of the most efficient treatments for enterococci infections. While the emergence and rapid spread of vancomycin-resistant enterococci (VRE) represents a particular challenge [13, 14], as there are few remaining therapeutic options for infections caused by VRE [15, 16]. Glycopeptide resistance genes vanA is proven to be the major vancomycin resistant mechanism known in the enterococci. Within all the nine van gene types [17], vanA is the most frequently indentified one among enterococci isolates from clinical settings [18, 19]. The ability of enterococci to acquire and exchange plasmids and mobile genetic elements that carry antimicrobial resistance gens (like vanA) has contributed to their role as multiresistant pathogens within both human and animals.

The spread of VRE as well as vanA is not restricted only clinically but they appear also in the community and natural environment as well as animal sectors. For instance, the emergence of VRE in food animal in Europe is proven to have a high association with the use of avoparcin, a glycopeptide antibiotic as growth promoting agent in animal feed [20]. Avoparcin was never approved as a feed additive in China, and worldwide reports on VRE in the livestock industry is limited.

Recently, growing attention has been focused on the worrisome situation of VRE in hospitals or community care centers, which might be traced back to the consumed food, such as yogurt and pork, and eventually to the diet that farm animals intake [21]. Therefore, the aim of this study was to determine the VRE strains in commercial available probiotic enterococci in Beijing, China. Moreover, to assess the potential security issue of these bacteria as an accumulator of antibiotic resistance genes (ARGs), horizontal dissemination of vanA between E. faecium and E. faecalis in fermented soybean meal (FSBM) and the digestive tract of growing pigs were also analyzed.

Materials and methods
Bacterial isolates
Enterococci based FSBM and silage corn samples, as well as microbial feed additive samples were purchased in swine farms and local husbandry and veterinary stations in Beijing, China, during June to December, 2016. For FSBM and silage corn samples, enterococci enrichment was performed by adding 25 g sample into 225 mL of Buffered Peptone Water (Beijing Land Bridge, China), vortexed, and then incubated at 37 °C for 24 h. Then 1 mL of the enrichment was added into 9 mL Bile Esculin Azide (BEA; Beijing Land Bridge, China) broth and then incubated 37 °C for 24 h. The changing color of BEA broth from transparent dark brown to opaque black was a sign of the existence of enterococci. A loop of enrichment was streaked on BEA agar plate, incubated 37 °C for 24 h. For feed additive samples, 1 g or 1 mL of the sample was dissolved in 9 mL normal saline, then a loop of the solution was streaked on BEA agar plate, incubated 37 °C for 24 h.

The identification of suspected strains were performed by VITEK 2 Compact Automatic Bacterial Identification and Drug Sensitivity Analysis System (bioMerieux, France) using specific Gram-positive bacteria identification cards (bioMerieux, France), followed by further confirmation of 16S rDNA-based polymerase chain reaction (PCR) screen using universal primers 27-F and 1,492-R, and specific primers for E. faecium and E. faecalis (Additional file 1: Table S1).

Antimicrobial susceptibility tests
Isolates confirmed as E. faecium or E. faecalis were subjected to antimicrobial susceptibility testing to determine their resistance to twenty common antibiotics of nine categories (Additional file 1: Table S2) in accordance with the Clinical and Laboratory Standards Institute (CLSI) [22]. Each isolate was inoculated into 1 mL of sterilized normal saline by picking 3 to 5 colonies using cotton swab from an overnight culture on BHI agar to visually match a McFarland turbidity standard of 0.5. The bacteria liquid was evenly coated onto the surface of un-supplemented Mueller-Hinton (Oxoid, UK) agar plates using cotton swab. Each plate was pased with five different antimicrobial disks (Beijing TianTan, China), and then incubated at 37 °C for 24 h. The diameter of inhibition zones was measured to the nearest millimeter and interpreted according to the CLSI standards and previous studies [22, 23]. The E. faecalis reference strain ATCC 29212 was used as the quality control.

Choice of donor and recipient strains
The only VRE strain, E. faecium strain, Ef4, isolated from a FSBM sample, resistant to vancomycin but susceptible to chloramphenicol, was chosen as donor strain. While another E. faecalis strain, Ef52, isolated from a microbial feed additive sample, with opposite vancomycin and chloramphenicol resistant phenotype to Ef4, was chosen as recipient strain. The genomic DNA of Ef4
and Efs2 were extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer’s protocol and further evaluated by PCR for the detection of van and chl genes. (Additional file 1: Table S1).

Amplified PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Cleaned fragments were submitted to Sangon Biotech (Beijing, China) and sequenced from both the forward and reverse stands. The sequence was then analyzed using BLAST.

Freeze-dried powder of Efm4 and Efs2 were produced by National Feed Engineering Technology Research Center (Beijing, China), with initial bacteria counts ~ 10^{10} CFU/g, respectively, then stored at −20 ºC. Before use, the bacterial activity would be detected by measuring bacterial colony-forming ability after incubation for 24 h at 37 ºC on BHI agar plates.

\[ \text{(Conjugation) frequency} = \frac{\text{Clones of Transconjugants (CFU/plate)}}{\text{Clones of Recipients (CFU/plate)}} \]

**In vivo conjugation: animals, feeding and sample collection**

All procedures used in these experiments were conducted in accordance with the Chinese Guidelines for Animal Welfare and were approved by the China Agricultural University Institutional Animal Care and Use Committee (Beijing, China) [29]. The pig cages, pigpens and feeding appliances were pre-disinfected, then confirmed the absence of enterococci as well as van and chl genes using methods described previously [30].

A total of thirty barrows (Duroc × Landrace × Yorkshire) at the age of 60 d, with initial body weight of 15.38 ± 2.8 kg were individually housed and provided with a non-medicated corn-soybean meal basal diet and sterilized water *ad libitum* for 10 d. Fresh fecal samples were collected daily to confirm the absence of enterococci as well as vanA. After acclimatization, pigs were randomly assigned to one control group (n = 6), fed with the same basal diet previously described; and four treatment groups (n = 6), fed with basal diet supplemented with 10% FSBM, 1% Efmp4, 5% Efs2 or a combination of 1% Efmp4 + 5% Efs2 for 7 d, respectively.

The estimation of FSBM consumption was about 5 kg/d, so 5 bags of FSBM were performed daily, from 1 d to 6 d of the experiment as described above. The fermentation process lasted for 2 d. At this time point, the bacteria concentrate of Efmp4 and Efs2 was both ~10^9 CFU/g FSBM. Then 5 kg fresh FSBM were uniformly dispersed into 45 kg basal diet to feed FSBM group barrows. Meanwhile, the initial bacteria concentrate of Efmp4 in Efs2 group and Efmp4 + Efs2 group was ~1.0 × 10^8 CFU/g feed, and the initial bacteria concentrate of Efs2 in Efmp4 group and Efmp4 + Efs2 group was ~5.0 × 10^8 CFU/g feed, respectively.

Fresh rectal feces sample of each pig was collected once every 24 h for serial plating and counting of Efmp4, Efs2, and transconjugants using selective BHI agar plates. Donor, recipient and transconjugant strains on selective plates and then resistant genes were identified as described above.

**Multilocus sequence typing**

To investigate the genetic heterogeneity of the donor, recipient and transconjugants, the MLST analysis was performed. 7 housekeeping genes were amplified using the primers and protocol specified by MLST website (https://pubmlst.org/efaecium/ and https://pubmlst.org/efaecalisis/), respectively. Allele and sequence types (STs) assignments were made at the publicly accessible MLST database described above [2, 31].
S1-PFGE and southern hybridization

S1-PFGE was performed as described previously [2]. Overnight incubated cells of Efm4, Efs2 and transconjugants were embedded in InCert Agarose (Lonza, USA), respectively, followed by digestion with S1 nuclease (TaKaRa, Japan). The DNA restriction fragment were separated in 1% SeaKem Gold agarose (Lonza, USA) using a pulse gel electrophoresis apparatus (Bio-Rad, USA). Genomic DNA of Salmonella serovar Braenderup strain H9812, digested with XbaI (TaKaRa, Japan), was used as a molecular standard.

Southern hybridization was performed on the S1-PFGE gel using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) with digoxin-labeled DNA probes specific for vanA, followed by manufacturer’s protocol.

Plasmid sequencing

The whole genome of Efm4 including vanA-harboring plasmid was sequenced using the Pacific Biosciences RS II (Pacific Biosciences, Menlo Park, CA, USA) sequencing platform. De novo assemblies of PacBio Reads were performed by SMRT Analysis pipeline v2.3.0 in conjunction with RS_HGAP_Assembly. Three protocols and additional assemblies were performed by minimus 2 (Pacific Biosciences, Menlo Park, CA, USA) sequenced (~ 13,000 bp) to verify their transfer into Efs2.

By targeting the regions adjacent vanA, primers (Additional file 2: Supplemental data 1) were designed based on the sequence of vanA-carrying plasmid in Efm4. Multiple alignment of acquire sequence was conducted using SnapGene (Version 4.2.6) against the sequence of vanA-carrying plasmid Efm4.

Results

Antimicrobial susceptibility

Among twenty-nine enterococci isolates, two strains (6.90%, 2/29) were susceptible to all the twenty antibiotics tested; the remaining strains (93.10%, 27/29) showing resistance to at least one antibiotic (Table 1). An E. faecium strain, Efm4, exhibited a high MIC to vancomycin (> 1,024 μg/mL) harboring vanA, while an E. faecalis strain, Efs2, resistant to chloramphenicol (128 μg/mL) carrying catA1 on genome were selected as donor and recipient strains (Table 2).

In vitro conjugation

During the fermentation process, the donor strain Efm4 and the recipient strain Efs2 both grew well and had similar growth kinetics. Concentrations of Efm4 reached a maximum of 1.25 × 10⁷ CFU/g FSBM at hour 10, with the concentrations of Efs2 reached a maximum of 6.0 × 10⁸ CFU/g FSBM as well (Fig. 1). Transconjugants were detected at the first detection point of fermentation (1 h), with the cell count at 0.94 × 10⁵ CFU/g FSBM, and the conjugation frequency being 1.2 × 10⁻³ (0.94 × 10⁵ CFU/g FSBM / 7.8 × 10⁷ CFU/g FSBM, transconjugants/Efs2). The number of transconjugants increased continuously and then peaked at day 3, at 5.8 × 10⁶ CFU/g FSBM. Moreover, the transconjugants remained consistently at ~ 10⁶ CFU/g FSBM during day 1 (from hour 6) to day 10 (Fig. 1).

In vivo conjugation

No pigs were colonized by vancomycin or chloramphenicol-resistant enterococci prior to the study (day –10 to –1, data not shown). Besides, during the test period, no VRE strain was detected in the fecal samples collected from the control group fed with basil diet.

The donor, recipient, and transconjugants exhibited similar growth kinetics, indicating that these two strains could stably reside the digestive tract of growing pigs, and therefore there was no growth defect that could have affected the results (Fig. 2a and b). In the fecal samples obtained from Efm4 + Efs2 group, the concentration of Efm4 and Efs2 reached a maximum of 6.44 × 10⁷ CFU/g fecal and 9.28 × 10⁷ CFU/g fecal at day 4, respectively (Fig. 2a). Similarly, the concentrations of Efm4 and Efs2 peaked at 4.88 × 10⁷ CFU/g and 7.28 × 10⁷ CFU/g on day 3 and day 5 in the FSBM group, respectively (Fig. 2b). Notably, transconjugants were observed in both two groups, although only positively detected in one pig of six for each group. In Efm4 + Efs2 group, the conjugation frequency reached to 1.97 × 10⁻⁴ and 6.25 × 10⁻⁴ at day 6 and day 7 (Fig. 2c, Table 3), respectively.

In contrast, the transconjugant was more frequently occurred, with a conjugation frequency of 9.4 × 10⁻⁵, 4.60 × 10⁻⁴, 2.75 × 10⁻⁵, 5.0 × 10⁻⁵ and 9.4 × 10⁻⁵, from day 3 to day 7, respectively (Fig. 2b, Table 3).

The presence vanA and catA1 in transconjugants was confirmed by further PCR bothly in vitro and in vivo experiments.

Molecular typing of probiotic enterococci isolates

The MLST analysis identified that the ST of Efm4, Efs2 and transconjugants was ST1014, ST69 and ST69, respectively (Table 1).

S1-PFGE and southern hybridization

S1-PFGE analysis exhibited one visible plasmid bands (~ 140 kb) in the donor strain Efm4 and another bands (~ 60 kb) in the recipient strain Efs2. It could be observed clearly that the transconjugants harbored two visible plasmids from both Efm4 and Efs2 (Fig. 3).
Southern hybridization confirmed that vanA hybridized on the visible plasmid of Efm4 with a size of ~140 kb, which was subsequently named pQZ67 in this study.

**Analysis of the plasmid pQZ67**

The complete DNA sequence of plasmid pQZ67 was obtained by whole-genome sequencing (Fig. 4). 142,988-bp pQZ67 consisted of 38,000 amino acid residues and containing 185 potential encoding sequences (CDSs), with a G + C content of 34.03%. The vanRSHAX gene cluster was located between two insertion sequences which were IS21 and IS256. It was a Tn1546-like compound transposon, which played a crucial role in horizontal transmission and might assist in the horizontal transfer of vanA in Enterococcaceae.

Multiple alignment showing the vanA-carrying fragments (~13,780 bp) from the FSBM as well as Efm4 + Efs2 group were identical to the corresponding region of

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**Table 1** Antimicrobial susceptibilities and sequence types of enterococci isolated from feed/feed additives

| Strain | Origin | Antibiotics | VA | TCL | RA | C | AM | PIP | CZ | P | MPN | AMX | OFL | CIP | GTF | GM | TE | MNO | E | KIA | FT | FU | ST |
|--------|--------|-------------|----|-----|----|---|----|-----|----|---|-----|-----|-----|-----|-----|----|----|-----|---|-----|-----|-----|----|
| Efm1   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST94|
| Efm2   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST40|
| Efm3   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST296|
| Efm4   | FSBM   | R           | R  | R   | R  | R | R  | R   | R  | R | R   | R   | R   | R   | R   | R  | R  | R   | R | R   | R   | ST1014|
| Efm5   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST296|
| Efm6   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST94|
| Efm7   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST6 |
| Efm8   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST5 |
| Efm9   | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST6 |
| Efm10  | FSBM   |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     |     | ST6 |
| Efm11  | Silage corn |           |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST6 |
| Efm12  | Silage corn |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST178 |
| Efm13  | Silage corn |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST5 |
| Efm14  | Silage corn |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST160 |
| Efm15  | Silage corn |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST6 |
| Efm16  | Silage corn |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST726 |
| Efm17  | Feed additive |           |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST812 |
| Efm18  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST812 |
| Efm19  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST5 |
| Efm20  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST5 |
| Efm21  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST361 |
| Efm22  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST21 |
| Efm23  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST21 |
| Efm24  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST695 |
| Efm25  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST24 |
| Efm26  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST24 |
| Efm27  | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST21 |
| Efs1   | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST6 |
| Efs2   | Feed additive |             |    |     |    |   |    |     |    |   |     |     |     |     |     |    |    |     |   |     |     | R   | ST69 |

Efm, _E. faecium_ isolates; Efs, _E. faecalis_ isolates; \( \), susceptible; R, resistant; I, intermediate; ST, sequence type; VA, Vancomycin; TCL, Teicoplanin; RA, Rifampicin; C, Chloramphenicol; AM, Ampicillin; PIP, Piperacillin; CZ, Cefamedin; P, Penicillin; MPN, Meropenem; AMX, Amoxicillin; OFL, Ofloxacin; CIP, Ciprofloxacin; GTF, Gatifloxacin; GM, Gentamicin; TE, Tetracycline; MNO, Minocycline; E, Erythromycin; GI, Kitasamycin; FT, Nitrofurantoin; FU, Furazolidone
vanA-carrying plasmid in Efm4, expect single base mutation within vanA gene in transconjugants (Additional file 2: Supplemental data 1).

**Discussion**

To the best of our knowledge, this is the first time of the observation on the transfer of vanA of probiotic origin occurred in the process of FSBM and in the digestive tract of growing pigs. Over the past few decades, with the extensive emergence of antibiotic-resistant strains, probiotics are increasingly used in human and food animals as suitable replacements of antibiotics [33, 34]. As traditional lactic acid bacteria, E. faecium and E. faecalis have been wildly used in foods, drugs, dietary supplements, microbial feed and feed additives [35]. However, some prospective studies showed that these bacteria can serve as an accumulator of antibiotic resistance genes (ARGs) potentially providing them to pathogens, raising the problem of pathogen resistance [36, 37]. For the past two decades, the production and consumption of microbial products associated with the increase in intensive livestock farming, and the safety of these products is important for food safety. Previous studies have shown concern on probiotics applied in feed containing transferable ARGs [38, 39]. Using filter mating experiments, Nawaz et al. [40] found that ermB and tetM from lactobacillus strains could be successfully transferred to E. faecalis, with conjugation frequency ranged from $10^{-5}$ to $10^{-6}$. Moreover, Ma et al. [41] used Lactobacillus delbrueckii strain harboring mcrC as donor strain, and E. faecalis ATCC 29212 as recipient strain, resulting in a conjugation frequency as high as $2.2 \times 10^{-2}$. Similarly, our study found out that, during the production of FSBM, the vanA could be transferred easily at the early stage (detected only 1 h after the fermentation began), and the transconjugations increased steadily during the whole fermentation process of FSBM. Nevertheless, the conjugation frequency ranged from $10^{-3}$ to $10^{-4}$, which was about 10 to 100 fold higher than primary studies [42, 43]. It might be because that FSBM provided a suitable environment for conjugations to happen, where three necessary conditions were need: full contact, absolute stationary and sufficient nutrition [44, 45].

Most of the previous experiments of resistance genes transfer in vivo were performed on specific pathogen

**Table 2** Antimicrobial susceptibilities and sequence type of donor strain Efm4 and recipient strain Efs2

| Strain | Origin           | VA | TCL | RA | C   | AM | PIP | CZ | P  | MPN | AMX | OFL | CIP | GTF | GM | TE | MNO | E  | KIA | FT | FU |
|--------|------------------|----|-----|----|-----|----|-----|----|----|-----|-----|-----|----|-----|----|----|-----|----|-----|----|----|
| Efm4   | FSBM             | R  | R   | R  | R   | R  | R   | R  | R  | R   | R   | R   | R  | R   | R  | R  | R   | R  | R   | R  | ST1014 |
| Efs2   | Feed additive    | R  | \_  | R  | \_  | R  | \_  | R  | \_ | R   | \_  | R   | \_ | R   | \_ | R  | \_  | R  | \_  | ST69 |

Efm, E. faecium isolates; Efs, E. faecalis isolates; \_, susceptible; R, resistant; I, intermediate; ST, sequence type; VA, Vacomycin; TCL, Teicoplanin; RA, Rifampicin; C, Chloramphenicol; AM, Ampicillin; PIP, Piperacillin; CZ, Cefamedin; P, Penicillin; MPN, Meropenem; AMX, Amoxicillin; OFL, Ofloxacin; CIP, Ciprofloxacin; GTF, Gatifloxacin; GM, Gentamicin; TE, Tetracycline; MNO, Minocycline; E, Erythromycin; GI, Kitasamycin; FT, Nitrofurantoin; FU, Furazolidone

**Fig. 1** Growth kinetics of Efm4, Efs2 and transconjugants during the fermentation process of soybean meal. The solid circular represents the growth kinetics of the donor strain Efm4. The solid square represents the growth kinetics of the donor strain Efm4; the solid minute triangle represents the growth kinetics of the transconjugants. Efm4, vanA positive strain Enterococcus faecium Efm4; Efs2, chloramphenicol-resistant strain Enterococcus faecalis Efs2
free (SPF) or gnotobiotic animals. Bourgeois- Nicolaos et al. [46] revealed that vanA could transfer from pig derived E. faecium to human derived E. faecalis in germ-free mice intestines, but only 2 colonies of transconjugants were detected after administration donor and receiver strains intragastrically for 14 d. Moreover, the transfer of vanA-harboring plasmid from poultry and pig derived strain to human fecal E. faecium strains in digestive tract of germ-free mice was observed by Dahl et al. [47], indicating that even transient colonization strains might provide a significant reservoir to transfer resistance genes to permanent commensal bacteria. A study by Lester et al. [48] demonstrated that conjugation could occur in the human gastrointestinal tract. In this study, three of six volunteers who ingested ~10^9 CFU vanA-harboring E. faecium bacteria in 250 mL milk had transconjugant bacteria in their feces at a mean conjugation frequency of ~10^-7 transconjugants/recipient. This was in line with the observations in our results, in particular for the Efm4+Efs2 group, as the

Table 3 Colony count of Efs2 and transconjugants from fecal samples of in vivo conjugation

| Day | Efs2, x 10^6 CFU/g fecal | Efm4+Efs2, x 10^6 CFU/g fecal | Transconjugants, x 10^3 CFU/g fecal |
|-----|--------------------------|-------------------------------|---------------------------------|
| 1   | Efs2 7.60                | Efm4+Efs2 3.70               | Efs2 1.50 Efm4+Efs2 0.20        |
|     | FSBM 0.55                |                               | FSBM 2.85 Efm4+Efs2 4.90        |
| 2   | Efs2 12.50               | Efm4+Efs2 15.00              | Efs2 8.20 Efm4+Efs2 16.50       |
|     | FSBM 12.30               |                               | FSBM 15.00 Efm4+Efs2 11.30      |
| 3   | Efs2 22.50               | Efm4+Efs2 33.00              | Efs2 8.50 Efm4+Efs2 42.00       |
|     | FSBM 41.00               |                               | FSBM 36.00 Efm4+Efs2 14.50      |
| 4   | Efs2 68.00               | Efm4+Efs2 175.00             | Efs2 77.00 Efm4+Efs2 27.00      |
|     | FSBM 20.50               |                               | FSBM 10.15 Efm4+Efs2 11.40      |
| 5   | Efs2 55.00               | Efm4+Efs2 101.50             | Efs2 51.00 Efm4+Efs2 41.00      |
|     | FSBM 43.00               |                               | FSBM 87.00 Efm4+Efs2 35.5       |
| 6   | Efs2 65.00               | Efm4+Efs2 3.75               | Efs2 65.00 Efm4+Efs2 18.00      |
|     | FSBM 55.00               |                               | FSBM 18.00 Efm4+Efs2 14.00      |
| 7   | Efs2 5.00                | Efm4+Efs2 6.55               | Efs2 7.00 Efm4+Efs2 12.10      |
|     | FSBM 10.00               |                               | FSBM 12.00 Efm4+Efs2 27.00      |

Efm, E. faecium isolates; Efs, E. faecalis isolates; FSBM, fermented soybean meal
transconjugants were obtained 5 d after ingesting Efm4 together with Efs2, and the conjugation frequency was $\sim 10^{-4}$ to $\sim 10^{-5}$.

It was noteworthy that transconjugants in the FSBM passed through the digestive tract of growing pigs successfully and could be detected after 3 d of feeding. Moreover, this cycle was 2 d sooner than feeding Efm4 and Efs2 directly. In addition, in commercial production facility, the SBM fermentation process usually lasted only for 2 to 5 d before further processing, and the inoculation amount of bacteria was much more than we used in the current study [49]. This emphasized the possibility that the resistance genes harbored by commercial probiotics used in feed and feed additives can be disseminated in digestive tracts of animal, which may cause further infections under extreme conditions. Many researchers explored the transferability of resistance genes from farm to fork [50, 51]. Nawaz et al. [40] analyzed the antibiotic resistance in lactic acid bacteria from retail fermented foods in Xi’an, China. Not only did they identify tetS gene from Lactobacillus brevis and Lactobacillus kefiri for the first time, but they also observe the ermB gene from L. fermentum and L. salivarius. Furthermore, the tetM gene from L. plantarum and L. brevis was also observed to be successfully transferred to E. faecalis by filter mating, with a conjugation frequency as $\sim 10^{-6}$ to $\sim 10^{-5}$. In addition, He et al. [52] analyzed the oxazolidinone / phenicol resistance gene optrA from 17 non-related E. faecalis isolates from human and animal origin, and the IS1216E elements on the plasmid were also found to contribute the most in the dissemination of the optrA gene. The IS 1216E elements were also detected on pQZ67 plasmid in our study, demonstrating the likelihood of in vitro and in vivo transfer of vanA between enterococci and other Gram-positive bacteria from food or feed to animal or human via food chain in vitro and in vivo.

In this study, the diversity of STs showed that the commercial probiotic enterococci strains were from multiple sources. Additionally, it should be noted that the sequence type of VRE strain Efm4 was ST1014 (Fig. 3). ST1014 was evolved out of ST78, which was the dominant clone complex (CC) in most cities in China, causing the prevalence and spread of VRE [53].

![Fig. 3 S1-PFGE analysis of Efm4, Efs2 and transconjugations and southern hybridization. Line M, molecular weight marker Salmonella braenderup H9812; Line 1 and 6, donor strain E. faecium Efm4; Line 2 and 7, recipient strain E. faecalis Efs2; Line 3 and 8, transconjugants obtained from FSBM; Line 4 and 9, transconjugants obtained from Efm4+ Efs2 group; Line 5 and 10, transconjugants obtained from FSBM group. FSBM, fermented soybean meal; PFGE, pulsed-field gel electrophoresis; Efm4, vanA positive strain Enterococcus faecium Efm4; Efs2, chloramphenicol-resistant strain Enterococcus faecalis Efs2.](image)
Furthermore, another reported vancomycin resistant E. faecium strain with the same ST1014 was isolated in a hospital in Shandong Province, China, in 2013 [53]. Although there was no evidence showing the direct link between that isolate and Ef4, the possible affiliation between ST1014 and ST78 (CC17) still rang the alarms of the safety of probiotic enterococci applied in feed and food.

Ideally, probiotics used in food and feed production should harbor none of the transferable resistance genes and should be sensitive to pathogen related antibiotics [54]. European Food Safety Authority (EFSA) recommended that bacterial strains harboring virulence factors or transferable ARGs should not be used in animal feeds, probiotic and fermented foods for human [55, 56]. The transmission of ARGs in digestive tracts is a major health concern related to the probiotic application. Unfortunately, until now, ARGs are not included in the standard screening assays before production and application in foods and feeds. Most of the fermentation starter probiotic strains that used for food and animal feed processing were lacking of thorough and rigorous assessments. The donor strain Ef4 used in this study was isolated from a retail FSBM product, revealing the risk of dissemination multiple resistant plasmids was not only through the ingestion of food animals, but also via further feed processing like SBM fermentation. In addition to vanA, the macrolide-resistant gene erm and two aminoglycoside resistant genes aadK and aphA were also identified in pQZ67 (Fig. 4), explained the resistant of Ef4 to erythromycin, kitasamycin and gentamicin. This also indicated that pQZ67 was a multidrug resistance plasmid.

Fig. 4 Genetic map of the vanA harboring plasmid pQZ67. The circles display (from the outside to inside): (i) size in bp; (ii) positions of the predicted coding sequences transcribed in clockwise orientation; (iii) position of predicted coding sequences transcribed in counterclockwise orientation; (iv) GC content plotted against 50%, with brown indicating > 50% and purple indicating < 50%; and (v) GC skew [(G + C)/(G-C)] in a window of 300 bp
In the past decade, the consumption of FSBM in Asian countries has increased sharply accompanied with the rapid development of animal husbandry [57]. To date, although there has been no report of VRE or other antibiotic-resistance probiotics in FSBM and in the intestines of growing pigs, the potential risk of horizontal transfer of resistance genes is still hanging in cliff, because the consumption is huge while the surveillance is lacked.

Conclusion
In conclusion, our study illustrated that vanA could successfully transfer among enterococci during the fermentation process of soybean meal and also in the digestive tract of growing pigs. Thus, it is suggested that when considering an Enterococcus strain as a starter for probiotics, each specific strain should be carefully evaluated to determine the presence of all known virulence factors.

Additional files

Additional file 1: Table S1. Primers used for PCR and DNA sequencing in this study and size of the PCR-targeted products. Table S2. Name, abbreviation and drug concentration of 20 antibiotics. (DOCX 19 kb)

Additional file 2: Primers used for partially sequencing of transconjugations and size of the PCR-targeted products. (DOCX 96 kb)

Abbreviations
ARGs: Antibiotic resistance genes; BEA: Bile Esculin Azide; BHI: Brain heart infusion; CC: Clonal Complex; Ef/F: Enterococcus faecium/Ef: Enterococcus faecalis; FSBM: Fermented soybean meal; MIC: Minimum inhibitory concentration; PCR: Polymerase chain reaction; PFGE: Pulsed-field gel electrophoresis; SMB: Soybean meal; VRE: Vancomycin-resistant enterococci

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Accession number(s)
The sequence of the 142,988-bp sequence of the van-carrying plasmid pQZ67 has been deposited in the GenBank database under accession No.CP025427.

Authors’ contributions
SQ: Designed the experiments; NL and HY: Performed the experiments; NL, HY and HL: Analyzed the data; NL: wrote the paper, which was edited by XM, CW and SQ; ZW, CHS: Contributed reagents and analysis tools; SQ: Resourced the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All procedures used in experiments of our lab were performed in accordance with the China Agricultural University Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-003).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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