*Haryana Vet.* (December, 2023) **62(2),** 1-5 ISSN 0033-4359 (P) **Research Article**

# DETECTION OF ANTIMICROBIAL RESISTANCE AMONG THE PROBIOTIC BACTERIA OF DIETARY SUPPLEMENTS USED IN LIVESTOCK AND POULTRY

PRABHU M.*[1,3], MALMARUGAN S.[2], RAMYA K.[3], PONNUSAMY P.[3], ARTHANARI ESWARAN M.[1] and THANGAVELU A.[3]

[1]Department of Veterinary Microbiology, Veterinary College and Research Institute (VCRI), Udumalpet, [2]Department of Veterinary Microbiology, VCRI, Tirunelveli, [3]Department of Veterinary Microbiology, VCRI, Namakkal-637 002, TANUVAS, Tamil Nadu, India

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## ABSTRACT

Antimicrobial resistance (AMR) is reported to be an emerging problem worldwide and the possibility of transfer of genes responsible for AMR from a probiotic organism to commensal gut microbes and coinfecting pathogenic bacteria has gained much research attention to ensure the safety of the host species. The present study was aimed to detect the AMR genes present in the *Lactobacillus* spp. in the commercially available therapeutic and dietary probiotic supplements. The viable *Lactobacillus* spp. were selectively isolated on MRS agar and tested for susceptibility of antimicrobial agents by Kirby-Bauer agar disk diffusion test. PCR amplification targeting nine different AMR genes (viz., *ermB*, *ermC*, *msrC*, *tet(M)*, *tet(W)*, *tet(L)*, *vanX*, *dfrA*, *aac(3)-II* genes), in four products including one livestock probiotic product (L1) and three poultry supplements (P2, P4 and P6) revealed the presence of *msrC* gene which encodes an erythromycin efflux pump. This confirms the presence of erythromycin resistance gene in probiotic supplements and warrants research efforts to reveal the transferability of this gene to other gut microflora and associated pathogens.

**Keywords:** Antimicrobial resistance, AMR genes, Erythromycin, *Lactobacillus* spp., Probiotic bacteria

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Probiotic microorganisms are primarily live bacteria or yeast, if fed in adequate quantity, usually confer health-promoting benefits (Hill *et al.*, 2014) to the host species, ranging from improving gut health and immune response to the control of antibiotic induced diarrhea and cancer (Kechagia *et al.*, 2013; Wong *et al.*, 2015). These probiotics have gained popularity and global acceptance and are extensively used in the field of medicine, food, dairy and poultry farming. They are commonly used in animals for better feed conversion ratio and weight gain (Pratishtha, 2008). Though many commercially available probiotics are generally regarded as safe (GRAS) according to the US Food and Drug Administration guidelines, nowadays researchers devote key attention to resolve their safety issues. Notably few authors reported about the adverse effects of some probiotics on the host such as immunocompromise, lactic acidosis, brain fogginess, bacteremia and antimicrobial resistance (AMR) gene transfer (Li *et al.*, 2020). Of which, AMR is considered as an emerging global problem and the extensive use of many probiotics in association with antibiotic usage or irrational antibiotic usage may gradually create a reservoir of AMR genes in probiotic organisms (Mathur and Singh, 2005). Though the intrinsic AMR can be regarded as beneficial attribute as the probiotics help to restore the gut microflora of the host during antibiotic therapy, but the transfer of genes responsible for drug-resistance to the commensal and infective microorganisms poses a serious clinical threat (Broaders *et al.*, 2013). Hence, the probability of transfer of these AMR genes draws the attention of researchers to ensure the safety of probiotic microflora used in therapeutic or dietary supplements. Therefore, the current study was planned to assess the AMR in probiotic organisms particularly the *Lactobacillus* spp., isolated from commercially marketed dietary products used in livestock and poultry.

*Corresponding author: drprabhuvirol@gmail.com

## MATERIALS AND METHODS

### Isolation of the probiotic bacteria

A total of 10 probiotic supplements containing *Lactobacillus* spp. were collected from the field and pharmacy as listed in Table 1. The probiotic *Lactobacillus* spp. present in the commercial supplements were selectively isolated by using *Lactobacillus*-selective De Man, Rogosa and Sharpe agar (MRS) media. In brief, bacteria from the dietary supplements were initially grown in anaerobic conditions in liquid MRS broth overnight at 37° C. The subsequent plating was done onto the MRS agar (HiMedia, Mumbai, India), and incubated in anaerobic jar overnight at 37° C. The single, pure isolated colonies were used for further antibiotic susceptibility testing and nucleic acid extraction.

### Antimicrobial susceptibility test

Antibiotic susceptibility testing was done as per

Kirby-Bauer agar disc diffusion test (Bauer *et al.*, 1966) with commercially procured antibiotic discs (Himedia, Mumbai, India). The pure colonies isolated on MRS agar were suspended in saline to achieve 0.5 McFarland standard and the same was spread on Muller Hinton agar (MHA) plates with a sterile cotton swab. The antibiotics discs were placed firmly and incubated. *Staphylococcus aureus* isolate available in the laboratory was used as reference strain. The antibiotics used in the current study include ampicillin (AMP-10µg), amoxiclav (amoxicillin/ clavulanic acid) (AMC-30µg), methicillin (MET-5µg), ceftriaxone (CTR-30 µg), tetracycline (TET-30µg), oxytetracycline (O-30µg), co-trimoxazole (sulpha/ trimethoprim) (COT-25µg), amikacin (AK-30µg), erythromycin (E-15µg), gentamicin (GEN-10µg), enrofloxacin (EX-10µg), ofloxacin (OF-5µg), levofloxacin (LE-5µg) and tylosine (TL-15µg). The diameter of the zone of inhibition was measured and the results were read as susceptible (S), resistant (R) or moderately susceptible (MS) as described by Bruslik *et al.* (2015).

**Nucleic acid extraction**

The bacterial DNA was extracted from the isolated pure colonies cultivated overnight at 37°C on MRS agar by thermal lysis method. From the cultures, one or two colonies were taken and suspended in 50 µl of Nuclease free water contained in a micro centrifuge tube. This tube was placed in a hot boiling water bath for 10 min, followed by snap chilling at 4º C and centrifugation at 13000 rpm for 5 min (Ponnusamy *et al.*, 2017). The supernatant was removed without disturbing the bacterial pellet, and stored at -20º C till use.

**Detection of antimicrobial resistance (AMR) genes by PCR**

PCR amplification was done in 25 µl volumes by individually (uniplex) targeting the AMR genes related with resistance to macrolides [*ermB*, *ermC*, *msrC* (encoding an erythromycin efflux pump) genes], tetracycline [ribosomal protection proteins *tet(M)* and *tet(W)* or efflux protein *tet(L)*], vancomycin (*vanX* gene that encodes D-ala-D-aladipeptidase), trimethoprim (*dfrA* gene encoding drug-resistant dihydrofolate reductase (DHFR) enzyme) and aminoglycoside (*aac(3)-II*). The PCR reaction (25 µl) mix included 2x Taq DNA polymerase Master Mix RED (Ampliqon, Denmark) with 1.5mM $MgCl_2$ (12.5 µl), 1 µl of each forward and reverse primers (10 pmol), 1 µl of template DNA from the colonies and nuclease-free water (9.5 µl). The list of primers used and the thermal cycling conditions are given in Table 2.

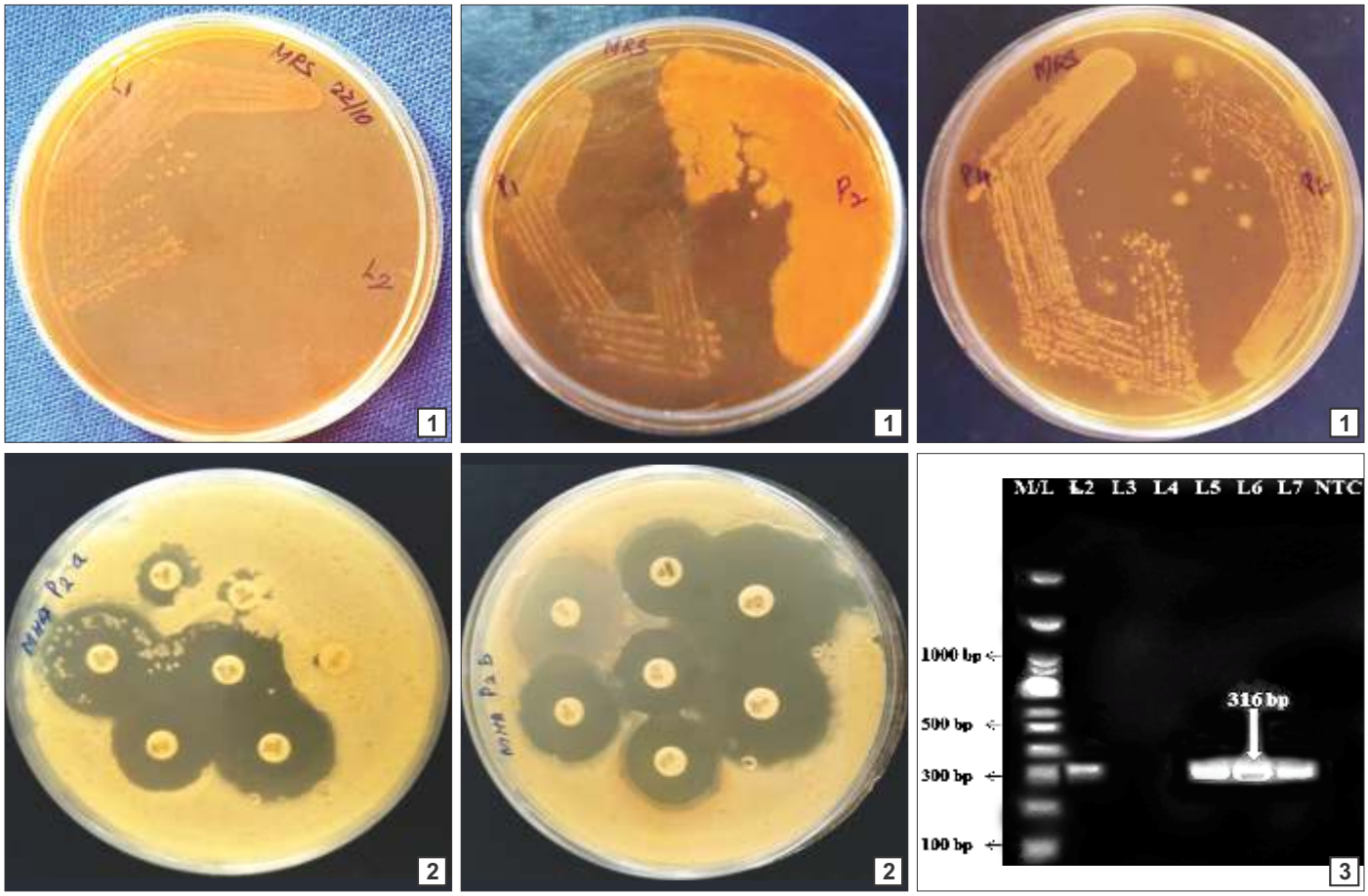
**Agarose Gel electrophoresis**

The individual PCR amplicons of the AMR genes were subjected to agarose gel electrophoresis (1.5% agarose), visualized by using ethidium bromide (1 µg/ml) and the results were documented by using the Gel Documentation system.

## RESULTS AND DISCUSSION

The antimicrobial resistance (AMR) has become an emerging global problem and the probiotics are now considered for study about the possible involvement in AMR (Selvin *et al.*, 2020). The widespread and irrational use of antimicrobial drugs has led to the emergence of AMR in probiotic organisms, which is a major concern worldwide because these probiotics can transfer the resistance AMR genes to other pathogenic organisms (Sharma *et al.*, 2014). Further, during the combination therapy in which probiotics are fed with antibiotics, the development of various resistance mechanisms can offset the bactericidal effects of any given antibiotic agents. The transfer of AMR genes from these probiotic organisms to commensal microflora and pathogenic organisms in the gut speculates the safety of probiotics (Toomey *et al.*, 2009) and this warrants better research efforts to ensure the safety of probiotic supplements. The European Food Safety Authority (EFSA) recommends the limited use of microbes that carry the transferrable AMR genes in food products (EFSA, 2007) and the nature of AMR gens in the incorporated candidate bacteria must be studied before approval of EFSA's Qualified Safety Presumption (QPS) status (EFSA, 2008). Several authors have reported the resistance among the lactic acid bacteria to different classes of antibiotic agents such as macrolides, -lactamase inhibitors and aminoglycosides (Devirgiliis *et al.*, 2013; Sharma *et al.*, 2014). Therefore, this study is designed to identify and evaluate the AMR in probiotic organisms incorporated in the commercially available therapeutic and dietary supplements.

Out of 10 samples tested, only two livestock supplements (L1 and L2) and four poultry probiotic supplements (P1, P2, P4 and P6) have shown viable growth of *Lactobacillus* spp. in MRS agar (Fig. 1) and the colonies were also observed by Gram staining [Gram positive, spore forming (mostly *L. sporogenes*), short or long rods seen individually, in pairs or as short chains]. These six isolated cultures were tested for antibiotic susceptibility using Muller-Hinton Agar (MHA) by using Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). However, L2 and P1 samples did not show any growth on MHA and hence were subjected to antibiotic susceptibility




Figs. 1 to 3. **(1)** Isolation of *Lactobacillus* spp. on MRS agar; **(2)** Antibiotic Sensitivity Testing of *Lactobacillus* spp isolated from probiotic supplement; **(3)** PCR amplification of *msrC* gene encoding erythromycin efflux pump (Lane M/L1: 100 bp DNA ladder; Lane 2: sample L1; Lane 3: sample L2; Lane 4: sample P1; Lane 5: sample P2; Lane 6: sample P4; Lane 7: sample P6; NTC: Non-template control)

**Table 1. List of probiotic supplements tested**

| S.No | Sample ID | Probiotic bacteria present | Intended for use in |
|---|---|---|---|
| 1. | L1 | *Lactobacillus sporogenes* – $10 \times 10^7$ cfu | Livestock |
| 2. | L2 | *Lactobacillus sporogenes* – $20 \times 10^8$ cfu | |
| 3. | L3 | *Lactobacillus sporogenes* – 2 million cfu<br>*L. acidophilus* – 1.5 million cfu | |
| 4. | L4 | *Lactobacillus sporogenes* – $25 \times 10^7$ cfu | |
| 5. | L5 | *Lactobacillus sporogenes* – $20 \times 10^6$ cfu | |
| 6. | L6 | *Lactobacillus* sp. – $15 \times 10^{10}$ cfu | |
| 7. | P1 | *Lactobacillus sporogenes* – 100 million cfu/gm | Poultry |
| 8. | P2 | *Lactobacillus rhamnosus* – 3 billion cfu/gm | |
| 9. | P4 | *Lactobacillus acidophilus*, *L. casei*,<br>*L. bulgaricus Bifidobacterium lactis* | |
| 10. | P6 | *Lactobacillus sporogenes* | |

test on MRS agar under anaerobic conditions at 37º C. The sensitivity pattern is recorded based on the zone of inhibition by the selected antibiotics. Out of 14 antibiotics used in the study, the product P2 showed resistance to oxytetracycline, ampicillin, erythromycin and methicillin (Fig. 2); the sample P4 showed resistance towards oxytetracycline and erythromycin; whilst P1 and P6 revealed resistance to ampicillin, oxytetracycline and erythromycin. Among the livestock products tested the product L1 showed resistance to gentamicin, amikacin and erythromycin. L2 showed intermediate resistance to gentamicin and erythromycin. They were sensitive to other antibiotics tested in the study.

The antimicrobial susceptibility profiles of many

**Table 2. List of primers used and PCR amplification conditions**

| S. No | AMR gene | Primer | Primer Sequence (5'-3') | Amplification conditions | Product size (bp) | Reference |
|---|---|---|---|---|---|---|
| 1. | *aac*(3)-II | F | ATATCGCGATGCATACGCGG | 94°C for 5 m, 94°C 1 m, 55°C 1 m, 72°C 1 m; 35x; 72°C for 10 m | 877 | Arpin *et al.*, 2003 |
| | | R | GACGGCCTCTAACCGGAAGG | | | |
| 2. | *tetM* | F | GTGGACAAAGGTACAACGAG | 93°C for 3 m, 93°C 1 m, 62°C 1 m, | 406 | Malhotra-Kumar *et al.*, 2005 |
| | | R | CGGTAAAGTTCGTCACACAC | | | |
| 3. | *tetL* | F | TGGTGGAATGATAGCCCATT | 65°C 4 m; 30x; | 229 | |
| | | R | CAGGAATGACAGCACGCTAA | 65°C 3 m | | |
| 4. | *ermB* | F | TGGTATTCCAAATGCGTAATG | | 745 | |
| | | R | CTGTGGTATGGCGGGTAAGT | | | |
| 5. | *tetW* | F | GAGAGCCTGCTATATGCCAGC | 95°C for 5 m, 94°C, | 168 | Masco *et al.*, 2006 |
| | | R | GGGCGTATCCACAATGTTAAC | 45 s, 64ºC 1 m, 72ºC 1 m; 25x; 72ºC 10 m | | |
| 6. | *ermC* | F | AATCGTCAATTCCTGCATGT | 95ºC for 1 m; 94°C | 299 | Klare *et al.*, 2007 |
| | | R | TAATCGTGGAATACGGGTTTG | 30 s, 55°C 30 s, 72°C 30 s; 30x; 72°C 4 m | | |
| 7. | *vanX* | F | TCGCGGTAGTCCCACCATTCGTT | 95ºC for 30 s, 55ºC | 454 | Liu *et al.*, 2009 |
| | | R | AAATCATCGTTGACCTGCGTTAT | 45 s, 72ºC 2 m; 30× | | |
| 8. | *msrC* | F | TATTGGAACATATCCGCAAACAAG | 95ºC for 30 s, 52ºC | 316 | |
| | | R | GTTGCCATATCAATGAAATTAGTCG | 45 s, 72°C 2 m; 30× | | |
| 9. | *dfrA* | F | CTTTTCTACGCACTAAATGTAAG | 95ºC for 30 s, 50ºC | 474 | |
| | | R | CATTATCAATAATTGTCGCTCAC | 45 s, 72ºC 2 m; 30× | | |

*Lactobacillus* spp., have been reported in many countries. It has been revealed that *Lactobacillus* spp. is susceptible to erythromycin, tetracycline and chloramphenicol (D'Aimmo *et al.*, 2007) and are found to be intrinsically resistant to fluoroquinolones, aminoglycosides and glycopeptides (Liu *et al.*, 2009). However, among the nine antibiotic resistance genes screened (Table 2), four isolates namely L1, P2, P4 and P6 showed positive PCR results (316 bp amplicon) for *msrC* gene which encodes an erythromycin efflux pump (Fig. 3) and negative for all other genes screened in the study.

The erythromycin antibiotic is a macrolide that binds in the tunnel of the 50S ribosomal subunit to inhibit RNA dependent bacterial protein synthesis (Lovmar *et al.*, 2006). The *Enterococcus faecium* strains contain *msrC* gene, endogenously present either in the chromosome or on an epidemic plasmid that play a role in macrolide resistance (Portillo, 2000). Earlier, the lactic acid bacteria isolated from a sausage (Brazilian artisanal calabrese) also revealed resistance to erythromycin which was shown to be mediated through *ermA*, B and C genes (de Castilho *et al.*, 2019). Previous report revealed that the *msrC* gene in general is not equally dispersed in all *Enterococcus faecium* isolates and its deactivation leads to two-eight fold decrease in the macrolide-lincosamide streptogramin B (MLSB) resistance. It was also reported that *E. faecium* isolates does not always carry *msrC* gene, while higher incidence was reported in *Staphylococcus aureus* leading to erythromycin resistance. Liu *et al.* (2009) found the presence of *msrC* gene in some *E. faecium* isolates while no other genes responsible for erythromycin genes were reported indicating that *msrC* gene might play an essential role in development of resistance to erythromycin. Hence, it was suggested not to use the strains of *E. faecium* in commercial foods or drugs (Liu *et al.*, 2009). However, the detailed mechanism of transfer of *msrC* resistant gene from the *Lactobacillus* spp. to the gut microflora and concomitant pathogenic microorganism should be explored in near future. Further, it is recommended that safety of probiotic and dietary supplement products must be ensured by conducting extensive studies to explore the presence and transferability of AMR genes before approving the product for commercial use as per the standard guidelines. There must be a statutory body or agency to regulate and monitor the approval of such products for animal use and the products not qualified with the standards have to be banned

for use in poultry and livestock.

## CONCLUSIONS

In conclusion, the drug resistant bacteria continue to pose a serious risk to the food industry. The probiotic organisms are anticipated to be involved in transfer of drug-resistant AMR genes to pathogenic organisms or commensal microflora of the gut system of animals and human beings. The probability of this drug-resistant gene transfer warrants the necessity to study the safety of organisms used in probiotic products. The present study revealed the presence of *msrC* gene encoding erythromycin efflux pump from isolates of four probiotic products used in livestock and poultry field. However, the transferability of this resistant gene to the pathogenic isolates has to be explored by further studies. Thereby, evaluation of the safety of probiotic bacteria used as supplements in livestock/poultry field could be guided. So that, the potential risk can be reduced in future by adopting appropriate counter measures such as alteration in formulation, dosage, appropriate combination therapy of antibiotics and probiotics etc. for safety of animal health.

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