Effect of Four Tibetan Veterinary Medicinal Plants Combinated with Ethyl Acetate Extract of *Enterococcus faecium* on Biofilm Formation of *Escherichia coli* E6

Yin Xiangyu[1#], Zhang Min[2#], Li Ping[2], Gao Wenwu[2], Feng Weidong[2], Ye Zhongmin[2], Yang Danjiao[2*], Chen Chaoxi[1*]

1. Southwest Minzu University, Chengdu 610045, China; 2. Institute of Animal Husbandry, Ganzi Tibetan Autonomous Prefecture of Sichuan Province, Kangding 626000, China

Abstract **[Objective]** To screen sensitive drugs and their combinations against biofilm formation of *Escherichia coli* isolated from Tibetan pigs and Tibetan chickens. **[Method]** Semi-quantitative modified crystal violet staining and the micro broth dilution method were employed to assess the biofilm-forming capacities of 152 *E. coli*, and their susceptibility to 12 commonly used antibiotics. Meanwhile, the checkerboard testing method was employed to evaluate the combined antibacterial activity of the sequential solvent fractions (methanol, dichloromethane, ethyl acetate, and petroleum ether) from four Tibetan veterinary medicinal plants (*Spenceria ramalana* Trimen, *Thalictrum delavayi* Franch., *Gentiana sino-ornata* Balf. f., *Lonicera rupicola* Hook.f. & Thomson), the ethyl acetate extract of two *Enterococcus faecium* and antibiotics with high resistance rates [chloramphenicol (CHL), oxytetracycline (OTC), ampicillin (AMP), and sulfamethoxazole (SMZ)] against ten selected multi-drug resistant *E. coli* (designated E1-E10). Lastly, the biofilm eradication of drug combinations on biofilms of *E. coli* E6 was examined. **[Result]** Most of the 152 *E. coli* exhibited moderate and no biofilm-forming capacities. The resistance rates to CHL, sulfamethazine (SM2), OTC, AMP, and SMZ exceeded 90%. In contrast, the resistance rates to difloxacin (DIF), ciprofloxacin (CIP), amikacin (AMK), ceftiofur (EFT), tobramycin (TOB), and ceftriaxone (CTR) were below 50%. Notably, the strains demonstrated a higher susceptibility to amikacin, with a drug resistance rate of only 19.90%. The MIC values for the *E. coli* E1-E10 were observed to range from 3.93 to 15.63 mg/mL for the methanol extract of *S. ramalana*, 3.93 to 31.52 mg/mL for the ethyl acetate extract of *T. delavayi*, 7.81 to 15.63 mg/mL for the methanol extract of *G. sino-ornata*, and 7.81 to 31.52 mg/mL for the ethyl acetate extract of *L. rupicola*. The ethyl acetate extracts from *E. aecium* S16 and S17 exhibited MIC values ranging from 0.42 to 13.38 mg/mL and from 0.45 to 3.63 mg/mL, respectively. The combinations of sequential solvent fractions derived from four Tibetan veterinary medicinal plants, the ethyl acetate extract of *E. faecium*, and antibiotics exhibiting high resistance rates demonstrated varying effects. At the MIC, all drug combinations showed a significant biofilm eradication effect compared to their single applications. The ethyl acetate extracts of *L. rupicola* and *T. delavayi*, combined with OTC and AMP, demonstrated a stronger inhibitory effect on *E. coli* E6 biofilm compared to other combinations. Specifically, the ethyl acetate extracts of *L. rupicola* and *T. delavayi* inhibited *E. coli* E6 biofilm by 48.92% and 42.58% in the presence of OTC, and by 48.58% and 47.84% in the presence of AMP, respectively. **[Conclusion]** The combined application of four selected Tibetan medicinal plants, probiotics, and antibacterial agents may offer a potential solution to address drug resistance and biofilm formation in *E. coli* isolated from Tibetan pigs and Tibetan chickens.

Keywords Tibetan veterinary medicinal plants; Biofilm-forming capacity; Multidrug resistance; Drug susceptibility

Escherichia coli, a prevalent conditional intestinal pathogen in livestock and poultry, is responsible for various health issues, including diarrhea, acute enteritis, septicemia, and other persistent infections. These prolonged infections are frequently associated with biofilm formation, facilitating extensive and sustained drug resistance and pathogenicity. The unique living environment within the *E. coli* biofilm offers essential nutrients while protecting against environmental stress, such as antibiotics and chemical disinfectants [1–3]. In recent years, researchers both domestically and internationally have explored a variety of therapeutic options. Physical[4–7], chemical[8–10], and biological [11–12] methods have demonstrated the ability to prevent and inhibit biofilm formation to some extent; however, each of these approaches possesses inherent limitations. The emergence of super-resistant bacteria, along with the growing diversity and prevalence of multidrug-resistant strains, has prompted both domestic and international researchers to focus on the research and development of novel antibacterial agents. This response is essential to address the escalating issue of bacterial drug resistance. Botanical drugs and probiotics represent some of the most

Received: 2024–11–05 Accepted: 2024–11–22

Supported by Project of Sichuan Provincial Department of Science and Technology (2016KZ0007) & Science and Technology Project of Sichuan Province (2022JDRC0121) & Innovative Research Projects for Postgraduate at Southwest Minzu University (CX2020SZ49) & Sichuan Provincial Department of Science and Technology (2023NSFSC0179).

#These authors contributed equally to this work.

*Corresponding author. E-mail: 2468157293@qq.com; chaoxi8832@163.com

dynamic and rapidly developing fields in contemporary research. Probiotics, including *Enterococcus faecalis*, are extensively utilized in animal husbandry. To some degree, they exhibit preventive and therapeutic effects against diseases caused by pathogenic bacteria, while also offering advantages related to safety and environmental sustainability[13-14]. Numerous studies have indicated that compounds found in medicinal plants, including alkaloids[15-17], chlorogenic acids[18], and phenolic acids[19], exhibit significant antibacterial activity. However, the antibacterial effects of medicinal plants frequently utilized in Tibetan veterinary clinics have yet to be documented.

Based on the references, we selected four commonly utilized Tibetan veterinary medicinal plants, *Spenceria ramalana* Trimen, *Thalictrum delavayi* Franch., *Gentiana sino-ornata* Balf. f., *Lonicera rupicola* Hook.f. & Thomson, for the preparation of various solvent extracts in the study. This selection was motivated by the prevalent incidence of bacterial infectious diseases affecting Tibetan pigs and Tibetan chickens in the plateau region of Northwest Sichuan Province. Furthermore, we conducted a series of drug susceptibility tests involving two strains of *E. faecalis* (S16 and S17), which were identified in a prior phase of the research, alongside commonly used antibiotics. The objective of this investigation was to provide empirical data to facilitate the identification of effective drugs and their combinations against the biofilm formation of *E. coli* isolated from Tibetan pigs and Tibetan chickens, thereby establishing a foundation for the development and application of novel antibacterial agents.

1 Materials and Methods

1.1 Strains and medicinal plants A total of 152 strains of *E. coli* were collected from large-scale farms of Tibetan pigs and Tibetan chickens located in Heishui County, within the Aba Tibetan and Qiang Autonomous Prefecture of Sichuan Province, as well as from free-range farms of Tibetan pigs and Tibetan chickens in Moxi Town, Luding County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province. The collected strains comprised 38 from Tibetan pigs and 35 from Tibetan chickens in Heishui County, with 1 sampled from environmental soil, 2 from pig urine, and 2 from eggs. Additionally, 25 from Tibetan pigs and 49 from Tibetan chickens were isolated from Hailuogou Valley, Luding County. The reference strain ATCC25922 was maintained by the Veterinary Pharmacology Laboratory at Southwest Minzu University, while *E. faecalis* S16 and S17 were generously provided by Professor Zhang Huanrong from the same institution.

L. rupicola and *G. sino-ornata* were collected from Danqing Yaowang Mountain, located in Seda County, Ganzi Tibetan Autonomous Prefecture. *T. delavayi* was sourced from Hailuogou Valley in Moxi Town, Luding County, Sichuan Province. Additionally, *S. ramalana* was isolated from Re'er grassland in Ruoergai County, Aba Tibetan and Qiang Autonomous Prefecture.

1.2 Antibacteri al drugs and culture media A total of twelve antibacterial agents, including chloramphenicol (CHL), ciprofloxacin (CIP), difloxacin (DIF), sulfamethazine (SM2), sulfamethoxazole (SMZ), oxytetracycline (OTC), doxycycline (DOX), ampicillin (AMP), ceftriaxone (CTR), ceftiofur (EFT), amikacin (AMK), and tobramycin (TOB), were procured from Shanghai Yuanye Bio-tech Co., Ltd. and Aladdin Biochemical Technology Co., Ltd. Tryptone soya brot h (TSB) was isolated from Guangdong Huankai Bio-tech Co., Ltd., MacConkey agar was sourced from Beijing Aoboxing Bio-tech Co., Ltd., and Mueller-Hinton (MH) broth, MH agar, and *E. faecalis* broth were acquired from Qingdao Hope Bio-tech Co., Ltd.

1.3 Drug susceptibility test The drug susceptibility test was conducted using the micro broth dilution method. Specifically, 180 μL of MH broth was added to the first column of a 96-well plate, followed by 20 μL of the prepared anti-bacterial drug stock solution to achieve a double dilution. The final column served as a blank control. The moderate-sized colonies exhibiting robust growth were selected and subsequently incubated in MH broth at a constant temperature for 12 h. Following incubation, the bacterial suspension was adjusted to a 0.5 McFarland standard. The bacterial solution was then diluted, and 100 μL of the diluted suspension was added to each well of a 96-well plate. The plate was incubated at a constant temperature of 37 ℃ for 12 h, after which the results were observed and documented.

1.4 Determination of biofilm-forming capacity The modified semi-quantitative crystal violet staining method was employed[20]. A total of 180 μL of TSB broth was dispensed into each well of a 96-well plate, while 200 μL of TSB broth was added to columns 6 and 7 to serve as a control. The bacterial solution, which had been cultured for 12 h, was adjusted to a 0.5 McFarland standard, and 20 μL of this solution was added to columns 1-5 and columns 8-12, respectively, with four wells designated for each strain. Subsequently, the plate was incubated for 24 h at 37 ℃. All bacterial solutions were removed, and the plate was rinsed three times with sterilized normal saline. Subsequently, 200 μL of methanol was added for fixation; the methanol was discarded after 15 min, and the plate was allowed to dry naturally. Following this, 200 μL of a 2% crystal violet staining solution was applied, discarded after 10 min. The plate was again allowed to dry naturally before adding 200 μL of 33% glacial acetic acid. The optical density (OD) at 570 nm was measured after incubation at 37 ℃ in a constant temperature chamber for 30 min. Blank broth served as the control, with the average value recorded as ODc. The results were evaluated according to the fol-

lowing criteria: strong film-forming capacity was defined as OD>4 ODc; moderate film-forming capacity was indicated by 2 ODc<OD ≤4 ODc; weak film-forming capacity was characterized by ODc<OD≤2 ODc; and no film-forming capacity was identified when OD≤ODc.

1.5 Screening for *E. coli* with multidrug resistance and strong biofilm-forming capacity A total of 10 multidrug-resistant strains exhibiting significant biofilm-forming capacities were identified from 152 *E. coli*, as outlined in Sections **1.3** and **1.4**.

1.6 Antibacterial activity of sequential solvent fractions derived from four Tibetan veterinary medicinal plants and ethyl acetate extracts of *E. faecalis* The naturally dried specimens of *G. sino-ornata*, *L. rupicola*, *T. delavayi*, and *S. ramalana* were subjected to crushing and sieving through a 40-mesh sieve. Subsequently, 1 kg of the resulting powder was extracted using petroleum ether, dichloromethane, ethyl acetate, and methanol. The combined filtrates from the three extraction processes were concentrated through rotary evaporation of the solvents, yielding lyophilized powders of sequential solvent fractions. An appropriate quantity of lyophilized powder was dissolved in dimethyl sulfoxide, and the solution was then adjusted to a constant volume using sterilized water. Subsequently, the initial concentrations of sequential solvent fractions for each drug were determined. The solution was filtered through a 0.22 μm microporous filter membrane to eliminate bacterial contamination for subsequent use[34]. The sequential solvent fractions of methanol, dichloromethane, ethyl acetate, and petroleum ether from *G. sino-ornata*, *L. rupicola*, *T. delavayi*, and *S. ramalana* were designated as follows: GSMT, GMDCM, GMEAC, GMPET for *G. sino-ornata*; LRMT, LRDCM, LREAC, LRPET for *L. rupicola*; TDMT, TDDCM, TDEAC, TDPET for *T. delavayi*; and SRMT, SRDCM, SREAC, SRPET for *S. ramalana*.

The supernatant of the culture broth of *E. faecalis* S16 and S17, which were incubated stationary for 12 h, was extracted using an equal volume of ethyl acetate through three repetitions to produce a lyophilized powder [21]. The resulting extracts were designated as EFS16 and EFS17, respectively. An appropriate quantity of lyophilized powder of the ethyl acetate extract of *E. faecalis* was dissolved in dimethyl sulfoxide (DMSO), and adjusted to a constant volume with sterilized water to convert its initial concentration. The resulting solution was subsequently filtered through a 0.22 μm microporous filter membrane to eliminate bacterial contamination for future applications.

The antibacterial activity of sequential solvent fractions derived from four Tibetan veterinary medicinal plants, as well as the ethyl acetate extract of *E. faecalis*, against the strains identified in Section **1.5** was assessed using the micro broth dilution method.

1.7 Combined drug susceptibility test The sequential solvent fractions with potent antibacterial activity and the ethyl acetate extract of *E. faecalis*, as screened in Section **1.6**, along with four antibacterial agents exhibiting a high resistance rate identified in Section **1.3**, were subjected to a combined drug susceptibility test utilizing the checkerboard dilution method. The combined mode of action against the *E. coli* screened in Section **1.5** was subsequently determined based on the fractional inhibitory concentration (FIC) value[22].

1.8 Biofilm eradication test Based on the experimental results presented in Section **1.7**, combinations of drugs that demonstrated either synergistic or additive effects on *E. coli* were selected. The effects of various drugs administered individually and in combination at their minimum inhibitory concentration (MIC) on the eradication of biofilm from strains eradication by strong and moderate film-forming capacities were investigated, utilizing methodologies referenced in the reference[20].

2 Results and Analysis

2.1 Analysis of drug resistance and resistance spectra of *E. coli* isolated from Tibetan pigs and Tibetan chickens The resistance spectra of 152 strains of *E. coli* to 12 antibacterial agents are presented in Tab.1. The resistance rates of the isolated strains to CHL, SM2, OTC, AMP and SMZ were notably high, with the resistance rate for AMP reaching 100%. In contrast, the resistance rates for DIF, CIP, AMK, EFT, TOB, and CTR were all below 50%. The highest sensitivity rate was observed for AMK, which was recorded at 80.10%.

The statistical analysis of the resistance spectra of 152 *E. coli* revealed a relatively diverse distribution of resistance spectra, encompassing a total of 50 distinct spectra. The predominant resistance spectra identified were CHL/DIF/SM2/OTC/DOX/AMP/SMZ, which constituted 13.16% of the total. Furthermore, 134 strains exhibited resistance to more than six antibacterial agents, representing 93.41% of the total strains analyzed. Among these, 12 strains demonstrated resistance to 12 different agents, accounting for 7.89%, while 18 strains exhibited resistance to 11 agents, comprising 11.84% of the total (Tab.2).

2.2 Analysis of the biofilm-forming capacity of *E. coli* derived from various sources The summary analysis of the biofilm-forming capacities of *E. coli* isolated from various sources in Heishui and Luding counties is presented. Among the *E. coli* derived from Tibetan chickens in Heishui, the percentages exhibiting strong, moderate, weak, and no biofilm-forming capacity were 11.43%, 34.29%, 42.86%, and 11.43%, respectively. In contrast, the *E. coli* isocated from Tibetan pigs displayed percentages of 2.63%, 55.26%, 26.31%, and 15.79% for strong, moderate, weak, and no biofilm-forming capacity, respectively. Notably, one strain of *E. coli* isolated from chicken farm soil demonstrated a strong biofilm-forming ca-

pacity phenotype. Additionally, two strains of *E. coli* derived from Tibetan pig urine exhibited a weak biofilm-forming capacity phenotype, while two strains from Tibetan eggs displayed weak and no biofilm-forming capacity phenotypes, respectively. Among the *E. coli* isolated from Tibetan chickens in Luding, the percentages demonstrating strong, moderate, weak, and no biofilm-for-ming capacity were 0%, 64.00%, 28.00%, and 8.00%, respectively. In contrast, the strains derived from Tibetan pigs exhibited percentages of 6.12%, 24.49%, 46.94%, and 22.45% for strong, moderate, weak, and no biofilm-forming capacity, respectively. Furthermore, the data presented in the table indicated that the biofilm of 152 strains predominantly exhibited two phenotypes: moderate and weak biofilm-forming capacity, which comprised 40.13% and 38.16% of the total, respectively. In contrast, the phenotypes characterized by strong biofilm-forming capacity and no biofilm-forming capacity represented smaller percentages, with only 9 strains demonstrating strong biofilm-forming capacity, accounting for 5.92% of the total (Tab.3).

Tab.1 Drug susceptibility testing of 152 *E. coli* to 12 antibacterial agents

Drug	Drug resistancerate %	Drug mediate rate %	Drug susceptibilityrate %	MIC range μg/mL	MIC_{50} μg/mL	MIC_{90} μg/mL
AMP	100	0	0	8.00–256	256	256
OTC	98.03	1.32	0.66	0.25–256	64	256
SMZ	97.37	0	2.63	0.25–256	256	256
CHL	95.39	3.29	1.32	4.00–256	256	256
SM2	92.76	0	7.24	0.25–256	256	256
DOX	83.55	8.55	7.89	2.00–256	16	128
CTR	46.71	0	53.29	0.25–256	0.5	64
DIF	46.71	26.32	26.97	0.25–256	2	256
CIP	42.11	0	57.90	0.25–256	0.25	8
EFT	41.45	3.95	54.61	0.25–256	2	64
TOB	35.53	0	64.47	0.25–256	2	256
AMK	19.74	0	80.26	0.25–256	8	256

Tab.2 Distribution of drug resistance spectra of 152 *E. coli*

Antibacterial agents	Number of strains	Percentage//%
CHL/DIF/SM2/OTC/DOX/AMP/SMZ	20	13.16
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/EFT/TOB/CTR	16	10.52
CHL/DIF/SM2/OTC/DOX/AMP/CIP/AMK/SMZ/EFT/TOB/CTR	12	7.89
CHL/SM2/OTC/DOX/AMP/SMZ	12	7.89
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/EFT/CTR	8	5.26
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/CTR	5	3.29
CHL/SM2/OTC/AMP/SMZ	5	3.29
CHL/SM2/OTC/DOX/AMP/CIP/SMZ	4	2.63
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/EFT/CTR	4	2.63
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ	4	2.63
CHL/DIF/SM2/OTC/DOX/AMP/AMK/SMZ	3	1.97
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/EFT	3	1.97
CHL/SM2/OTC/DOX/AMP/SMZ/EFT/CTR	3	1.97
CHL/DIF/SM2/OTC/DOX/AMP/CIP/AMK/SMZ/TOB/CTR	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/EFT/TOB	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/EFT/TOB/CTR	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/EFT	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/CIP/SMZ/TOB	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/EFT/TOB	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/TOB	2	1.32
CHL/DIF/SM2/OTC/DOX/AMP/SMZ/CTR	2	1.32
CHL/SM2/OTC/DOX/AMP/SMZ/TOB	2	1.32
CHL/DIF/OTC/DOX/AMP/SMZ	2	1.32

Note: Less than 1% of the drug resistance spectra are not included in the table.

2.3 Antibacterial activity of sequential solvent fractions derived from four Tibetan veterinary medicinal plants and ethyl acetate extracts of *E. faecalis* A total of 10 multidrug-resistant strains were identified from 152 *E. coli*, designated E1 to E10. Exception for strain E6, which exhibited moderate biofilm-forming capacity, the remaining 9 strains demonstrated strong biofilm-forming capacity and exhibited high resistance to CHL, AMP, OTC, and SMZ. The antibacterial activities of sequential solvent fractions derived from four Tibetan veterinary medicinal plants, as well as ethyl acetate extracts from two strains of *E. faecalis*, against 10 strains of *E. coli*, are presented in Tab.4.

2.4 Combined drug susceptibility test The findings of the combined drug susceptibility test involving four extracts from Tibetan veterinary medicinal plants, two ethyl acetate extracts of *E. faecalis*, and four highly resistant antibacterial agents against 10 strains of *E. coli* are presented in Tab.5. (1) GSMT exhibited indifferent or antagonistic interactions when combined with CHL, SMZ, and AMP, while primarily demonstrating antagonistic effects in conjunction with OTC. LREAC displayed synergistic or additive interactions with OTC and AMP, synergistic or indifferent effects with CHL and SMZ. TDEAC revealed synergistic or indifferent

Tab.3 Biofilm-forming capacity of *E. coli* derived from various sources

Origins	Biofilm-forming capacity				Sub total
	Strong	Moderate	Weak	Absent	
Tibetan chickens (Heishui)	4 (11.43%)	12 (34.29%)	15 (42.86%)	4 (11.43%)	35
Tibetan pigs (Heishui)	1 (2.63%)	21 (55.26%)	10 (26.31%)	6 (15.79%)	38
Chicken farm soil (Heishui)	1 (100%)	0	0	0	1
Tibetan pig urine (Heishui)	0	0	2 (100%)	0	2
Tibetan chicken eggs (Heishui)	0	0	1 (50.00%)	1 (50.00%)	2
Tibetan chickens (Luding)	0	16 (64.00%)	7 (28.00%)	2 (8.00%)	25
Tibetan pigs (Luding)	3 (6.12%)	12 (24.49%)	23 (46.94%)	11 (22.45%)	49
Total	9 (5.92%)	61 (40.13%)	58 (38.16%)	24 (15.79%)	152

Tab.4 MIC range, MIC_{50} and MIC_{90} of sequential solvent fractions derived from four Tibetan veterinary medicinal plants and ethyl acetate extracts of *E. faecalis* against E1-E10

Drug	MIC range mg/mL	MIC_{50} mg/mL	MIC_{90} mg/mL	Drug	MIC range mg/mL	MIC_{50} mg/mL	MIC_{90} mg/mL
GSMT	7.81–15.6	7.81	15.63	TDMT	15.63– >62.50	31.25	>62.50
GMDCM	15.63–>62.50	31.25	>62.50	TDDCM	7.81–31.25	31.25	31.25
GMEAC	15.63–>62.50	31.25	>62.50	TDEAC	3.93–31.25	7.81	15.63
GMPET	31.25–15.63	>62.50	>62.50	TDPET	15.63–>62.50	31.25	>62.50
LRMT	15.63–>62.50	31.25	>62.50	SRMT	3.93–15.63	7.81	15.63
LRDCM	15.63–>62.50	31.25	>62.50	SRDCM	15.63–>62.50	>62.50	>62.50
LREAC	7.81–31.25	15.63	31.25	SREAC	15.63–>62.50	31.25	>62.50
LRPET	15.63–>62.50	31.25	>62.50	SRPET	15.63–>62.50	15.63	31.25
EFS16	0.45–3.63	1.81	3.63	EFS17	0.42–13.38	0.83	3.34

effects with SMZ, and synergistic or additive interactions with CHL, AMP, and OTC. SRMT exhibited additive or indifferent interactions with CHL and OTC, and indifferent or antagonistic effects with AMP and SMZ. (2) In addition to the additive or indifferent effects observed with the combination of GSMT and EFS16 and EFS17, the combination of GSMT with EFS16 demonstrated synergistic effects against two strains of *E. coli*. Furthermore, LREAC exhibited synergistic or additive effects when combined with EFS16 and EFS17. TDEAC displayed synergistic or additive effects in conjunction with EFS16, additive effects when paired with EFS17. Lastly, the combination of SRMT and EFS16 resulted in either additive or indifferent effects with EFS16, while also showing synergistic or additive effects with EFS17. (3) In conjunction with CHL, both EFS16 and EFS17 exhibited additive and indifferent effects. When combined with OTC, EFS16 demonstrated either synergistic or additive effects, while EFS17 displayed synergistic, additive, or indifferent effects. In the presence of AMP, EFS16 again showed either synergistic or additive effects, whereas EFS17 demonstrated either additive or indifferent effects. Finally, when combined with SMZ, both EFS16 and EFS17 exhibited additive and indifferent effects.

2.5 Effect of single and combined applications on the eradication of *E. coli* E6 biofilms The inhibition of *E. coli* E6 biofilm by the drugs, both individually and in combination at MIC, indicated that when administered alone, the ethyl acetate extracts of *L. rupicola* and *T. delavayi*, as well as the ethyl acetate extracts of *E. faecalis*, exhibited a higher eradication rate of E6 biofilm compared to AMP and OTC. Notably, the extract EF17 demonstrated the highest eradication rate, achieving an inhibition rate of 28.34%. The application of drugs in combination showed that the eradication of *E. coli* E6 biofilm was more effective when utilizing the ethyl acetate extracts of *L. rupicola* and *T. delavayi* in conjunction with the antibacterial agents AMP and OTC, compared to the combination of ethyl acetate extracts of *E. faecalis* with the same antibacterial agents or Tibetan veterinary medicinal plants (*L. rupicola* and *T. delavayi*). Specifically, the inhibition rates of *E. coli* E6 biofilm when using the combination of ethyl acetate extracts of *L. rupicola* and *T. delavayi* with OTC were recorded at 48.92% and 42.58%, respectively. In contrast, the inhibition rates of the E6 biofilm with the combination of ethyl acetate extracts of *L. rupicola* and *T. delavayi* with AMP were 48.58% and 47.84%, respectively (Tab.6).

3 Discussion

3.1 Isolation and identification of *E. coli* from Tibetan pigs and Tibetan chickens and analysis of their drug resistance levels Currently, there are relatively few domestic issues related to drug resistance in *E. coli* derived from Tibetan pigs and Tibetan chickens when compared to similar sources from other regions. Li Peng *et al.*[23] identified a prevalence of 55.6% of *E. coli* in fecal samples from Tibetan pigs raised in the mountainous forests near Juemu Valley, Linzhi City, Tibet. Notably, the resistance rate observed in their study was lower than that reported in the present investigation. The resistance rate of *E. coli* isolated from the feces of free-range Tibetan chickens, as reported by Gongga *et al.*[24], exhibited significant differences compared to the findings of the present study. This discrepancy is primarily attributed to variations in the sample size utilized in the respective analyses. In addition, a study conducted by Wang Runjin *et al.*[25] reported resistance rates of *E. coli* to penicillin, gentamicin, norfloxacin, levofloxacin, and amoxicillin at 77.13%, 19.47%, 18.10%, 17.77%, and 15.39% , respectively. These rates were

Tab.5 Combined drug susceptibility test of sequential solvent fractions derived from four Tibetan veterinary medicinal plants, ethyl acetate extracts of *E. faecium* and antibiotics with high resistance rate against E1-E10

Drug combinations	Synergistic effect	Additive effect	Indifferent effect	Antagonistic effect
GSMT+CHL	0	0	8	2
GSMT+OTC	0	1	9	0
GSMT+AMP	0	0	8	2
GSMT+SMZ	0	0	3	7
LREAC+CHL	0	7	3	0
LREAC+OTC	6	4	0	0
LREAC+AMP	8	2	0	0
LREAC+SMZ	0	3	7	0
TDEAC+CHL	6	4	0	0
TDEAC+OTC	5	5	0	0
TDEAC+AMP	1	9	0	0
TDEAC+SMZ	0	7	3	0
SRMT+CHL	0	8	2	0
SRMT+OTC	0	9	1	0
SRMT+AMP	0	0	7	3
SRMT+ SMZ	0	0	9	1
EFS16+GSMT	2	7	1	0
EFS17+GSMT	0	9	1	0
EFS16+LREAC	3	7	0	0
EFS17+LREAC	6	4	0	0
EFS16+TDEAC	2	8	0	0
EFS17+TDEAC	0	10	0	0
EFS16+SRMT	0	2	8	0
EFS17+SRMT	1	9	0	0
EFS16+CHL	0	2	8	0
EFS17+CHL	0	4	6	0
EFS16+OTC	7	3	0	0
EFS17+OTC	6	3	1	0
EFS16+AMP	8	2	0	0
EFS17+AMP	0	9	1	0
EFS16+SMZ	0	1	9	0
EFS17+SMZ	0	7	3	0

Tab.6 Effect of single and combined applications on *E. coli* E6 biofilm eradication at MIC concentration

Drugs (administered alone or in combination)	Inhibition rate %	Drugs (administered alone or in combination)	Inhibition rate %
LREAC	24.39	TDEAC	21.55
OTC	14.24	AMP	17.54
EFS16	25.49	EFS17	28.34
LREAC+OTC	48.92	LREAC+AMP	48.58
TDEAC+OTC	42.58	TDEAC+AMP	47.84
EFS16+OTC	41.53	EFS16+AMP	33.88
EFS17+OTC	32.37	EFS17+AMP	34.26
EFS16+LREAC	37.33	EFS16+TDEAC	33.50
EFS17+LREAC	34.51	EFS17+TDEAC	35.61

lower than those observed in the present study. The findings of a drug susceptibility test conducted on 2 954 strains of *E. coli* isolated from pigs and chickens in Sichuan Province between 2009 and 2014, as reported by Yue Xiuying *et al.*[26], were largely consistent with the results obtained in the current study. The isolation rate of *E. coli* from captive Tibetan pigs, Tibetan chickens, and the surrounding environment of farms in the Northwest Sichuan plateau region was 74.9%. This finding indicates a potential risk of *E. coli* incidence within the farms and their vicinity in the sampled area. Consequently, it is essential to improve feeding management practices and adopt a systematic approach to medication usage to address this issue scientifically. The findings of this study offer empirical support regarding the resistance of *E. coli* in livestock and poultry within the Northwest Sichuan plateau. This information is essential for the appropriate selection of antibacterial agents and their rational application, grounded in a more comprehensive understanding of the prevalence of *E. coli* in local agricultural settings.

3.2 Inhibition of *E. coli* by medicinal plant extracts and ethyl acetate extracts of *E. faecalis* Numerous relevant studies have demonstrated that various active constituents found in medicinal plants, including flavonoids, alkaloids, terpenoids, and phenolics, exhibit significant antibacterial activity. In the current study, four Tibetan veterinary medicinal plants were evaluated for their antibacterial activity in sequential solvent fractions. The methanol extracts of *G. sino-ornata* and *S. ramalana*, as well as the ethyl acetate extracts of *L. rupicola* and *T. delavayi*, exhibited significant antibacterial activity against the isolated strains. This activity may be attributed to the presence of alkaloids, chlorogenic acid, and anthraquinones in these extracts[15-17]. Furthermore, *E. faecalis*, a widely utilized probiotic in the domain of animal husbandry, plays a

crucial role in the prevention and treatment of animal diseases. The ethyl acetate extracts from the two strains of *E. faecalis* selected for this study demonstrated a significant inhibitory effect on E1-E10, which aligns closely with the findings reported by Zheng Wei *et al.*[13]. The findings from the combined drug susceptibility tests indicated that the four extracts derived from Tibetan veterinary medicinal plants, along with the ethyl acetate extracts from the two probiotic strains, exhibited a synergistic effect. This observation is consistent with the results reported by Zhu Yanhua *et al.*[14], which demonstrated that the combination of traditional Chinese medicine and *Bacillus subtilis* was more effective than the application of a single agent. The ethyl acetate extract of *E. faecalis* S16 demonstrated a synergistic effect when combined with OTC and AMP. It is hypothesized that this phenomenon may be attributed to the acidic chemical properties of the active component[27-28].

3.3 Eradication of *E. coli* biofilm by medicinal plant extracts and ethyl acetate extracts of *E. faecalis* Numerous studies have indicated that bacteriocin-producing strains of *Lactobacillus* and *Bifidobacterium* possess the ability to eliminate dental plaque. Furthermore, *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. reuteri* have been shown to inhibit the biofilm formation of *Streptococcus* species [29-30]. The findings indicated that the two strains of *E. faecalis* exhibited the most significant inhibitory effect on *E. coli* E6 biofilm formation when the antibacterial agents were administered individually. Conversely, when the agents were administrated in combination, the ethyl acetate extracts of *L. rupicola* and *T. delavayi*, along with OTC and AMP, resulted in an enhanced inhibitory effect on biofilm formation. This phenomenon may be attributed to the disruption of the extracellular structure of the biofilm by the two extracts, which potentially facilitates the penetration of the antibiotics into the biofilm´s interior, thereby allowing them to exert an antibacterial effect[31]. The findings regarding biofilm eradication indicate that the combination of ethyl acetate extracts from *L. rupicola* and *T. delavayi*, in conjunction with two strains of *E. faecalis*, exhibited a greater inhibitory effect on biofilm formation than either agent administrated individually. This may be attributed to the disruption of the cell wall and increased permeability of the cell membrane[32-34]. Consequently, future research should investigate and clarify the mechanisms by which the combination of medicinal plant extracts and ethyl acetate extracts of *E. faecalis* function as antibacterial agents in the eradication of biofilms formed by *E. coli*.

References

[1] Hall SD, Coterton JW, Stoodley P. Bacterrial biofilm:from the natural environment to infectious diseases [J]. Nat Rev Microbiol, 2004, 2 (2): 95–108.

[2] Parsek MR, Sinqh PK. Bacterial biofilms: an emerging link to disease pathogenesis[J]. Annu Rev Microbiol, 2003, 5(7): 677–701.

[3] Szewzyk U, Szewzyk R, Manz W, *et al.* Microbiological safety of drinking water [J]. Annu Rev Microbiol, 2000, 5(4): 81–127.

[4] Poortinga AT, Bos R, Busscher HJ. Controlled electrophoretic deposition of bacteria to surfaces for the design of biofilms [J]. Biotechnol Bioeng, 2000, 67(1): 117–120.

[5] Gartenmann SJ, Thurnheer T, Attin T, *et al.* Influence of ultrasonic tip distance and orientation on biofilm removal [J]. Clin Oral Investig, 2007, 21(4): 1029–1036.

[6] Soghomonyan D, Trchounian K, Trchounian A. Millimeter waves or extremely high frequency electromagnetic fields in the environment: what are their effects on bacteria[J]. Appl Microbiol Biotechnol, 2016, 100(11): 4761–4771.

[7] Li C, Lesnik KL, Fan Y, *et al.* Millimeter scale electron conduction through exoelectrogenic mixed species biofilms [J]. FEMS Microbiol Lett, 2016, 363(15): 153.

[8] Dryden M, Dickinson A, Brooks J, *et al.* A multicentre clinical evaluation of reactive oxygen topical wound gel in 114 wounds [J]. J Wound Care, 2016, 25(3): 140, 142–146.

[9] Carrillo U, Diaz-Villanueva V, Modenutti B. Sustained effects of volcanic ash on biofilm stoichiometry, enzyme activity and community composition in North-Patagonia streams[J]. Sci Total Environ, 2018, 15(621): 235–244.

[10] Lellouche J, Friedman A, Lahmi R, *et al.* Antibiofilm surface functionalization of catheters by magnesium fluoride nanoparticles[J]. Int J Nanomedicine, 2012(7): 1175–1188.

[11] Godeke J, Paul K, Lassak J, *et al.* Phage- induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1[J]. SMEJ, 2011, 5(4): 613–626.

[12] Resch A, Fehrenbacher B, Eisele K, *et al.* Phage release from biofilm and planktonic *Staphylococcus aureus* cells[J]. FEMS Microbiol Lett, 2005, 252(1): 89– 96.

[13] Zheng Wei. Study on the antibacterial activity and safety of *Escherichia coli* KQ2.6[D]. Hangzhou:Hangzhou Normal University, 2016.

[14] Zhu Yanhua, Zhang Denghui, Zheng Hongxing, *et al.* Study on the inhibitory effect of compound traditional Chinese medicine probiotic preparation on *Escherichia coli* and *Salmonella* in chicks[J]. Foreign Animal Husbandry Studies on Pigs and Poultry, 2016, 36 (5): 84–85.

[15] Yang Weixia, Zhou Le, Geng Huiling, *et al.* A survey of study of chemical components of medicinal plants of Gentianaceae [J]. Acta Botanica Boreali-Occidentalia Sinica, 2003, 23(12): 2235–2240.

[16] Zhang Yueyue, Wang Junming, Cui Ying, *et al.* Research progress of anxiolytic and antidepressant bioactive ingredients of Chinese medicine alkaloids and the pharmacological effect of Coptidis *Rhizoma alkaloids*[J]. China Journal of Traditional Chinese Medicine and Pharmacy2015, 30(4): 1184–1187.

[17] Pan Zheng, Gao Yunling, Cai Yingfan, *et al.* Determination of berberine hydrochloride and total alkaloids in Thalictrum [J]. Chinese Archives of Traditional Chinese Medicine, 2007, 25(8): 1643–1645.

[18] Li Ruiguo, Wang Caijun.Extraction of chlorogenic acid from different organs of *Lonicera japonica* and its antibacterial effect [J]. Lishizhen Medicine and Materia Medica Research, 2012, 23(5): 1196– 1197.

[19] Guan Kuikui. Exploration of the therapeutic effect of total phenols in horseshoe yellow on ulcerative colitis in C57BL/6 mice [D]. Chengdu: Southwest University for Nationalities, 2021.

[20] Stepanovic S, Cirkovic I, Ranin L, *et al.* Biofilm formation by *Salmonella* spp. and *Listeria*

monocytogenes on plastic surface[J]. Lett Appl Microbiol, 2004, 38(5): 428–432.

[21] Qiao Zipeng, Wang Qizhi, Liu Jianfu, *et al.* Study on the antioxidant and antibacterial activities of ethyl acetate extract from endophytic fungi of *Cirsium grandiflorum*[J]. Chinese Pharmacy, 2021, 30(5): 421–433.

[22] Tian Shuqin, Deng Xiaoting, Chen Chaoxi. Guidelines for veterinary pharmacology experiments[M]. Beijing: China Agricultural Press, 2010.

[23] Li Peng. Investigation of drug resistance and mechanisms of *Escherichia coli* and *Enterococcus* from tibetan pigs [D]. Beijing: China Agricultural University, 2015.

[24] Gongga, Yixicuom, Zaxilam, *et al.* Isolation, identification and drug resistance study of *Escherichia coli* from Tibetan chickens [J]. Chinese Journal of Animal Husbandry and Veterinary Medicine, 2016, 32(7): 55–56.

[25] Wang Runjin, Chen Xi, Ma Jinying.Serotype investigation and drug resistance testing of *Escherichia coli* in Tibetan pigs along the Lhasa Nyingchi section of the Sichuan Tibet highway[J]. Swine Production, 2018(1): 105–106.

[26] Yue Xiuying, Ge Rong, Wu Xiaolan, *et al.* Study on antibiotic resistance of *Escherichia coli* from pigs and chickens in Sichuan province[J]. Chinese Journal of Veterinary Medicine, 2017, 53(1): 93–95.

[27] Sengelov G, Halling-Sorensen B, Aarestrup FM. Susceptibility of *Escherichia coli*, and *Enterococcus faecium*, isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in *Escherichia coli* from food animals[J]. Vet Microbiol, 2013, 95 (1–2): 91.

[28] Zhu Erqun, Wang Erzhu, Chang Juan, *et al.* Study on the inhibition of complex probiotics combined with aureomycin on the growth of *Escherichia coli* [J]. Journal of Anhui Agricultural Sciences, 2015, 43(6): 161–163.

[29] Lakhtin VM, Lakhtin MV, Pospelova VV, *et al.* Lectins of *lactobacilli* and *bifidobacteria*. II. Probiotic lectins of *lactobacilli* and *bifidobacteria* as possible signal molecules regulating inter- and intrapopulation relationships between bacteria and between bacteria and the host [J]. Microbial Ecology in Health & Disease, 2009, 19(3): 153–157.

[30] Lin X, Chen X, Chen Y, *et al.* The effect of five probiotic lactobacilli strains on the growth and biofilm formation of *Streptococcus mutans*[J]. Oral Dis, 2015, 21(1): 128–134.

[31] Eaton TJ, Gasson MJ. A variant enterococcal surface protein Esp (fm) in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates[J]. FEMS Microbiol Lett, 2002, 216(2): 269.

[32] Peng Zixin, Hu Ting, Wang Anru, *et al.* Research on the antibacterial mechanism and application of bacteriocins[J]. Chinese Journal of Veterinary Medicine, 2011, 47(7): 50–52.

[33] Zhou Zhie, Luo Qiushui, Xiong Jianhua, *et al.* Antimicrobial mechanisms of 3-O-caffeoyl quinic acid and 3, 5-di-O-caffeoyl quinic acid against *Escherichia coli*[J]. Food Science and Technology, 2014, 39(3): 228–232.

[34] Zhang Zhixuan, Cui Shuting, Zhu Zhongbo, *et al.* Research progress in application of extraction technology and screening method of effective parts of Chinese materia medica[J]. Chinese Journal of Information on Traditional Chinese Medicine, 2021, 28(5): 132–136.