AJAB

Asian J Agric & Biol. xxxx(x).
DOI: 10.35495/ajab.2023.327

Original Article

Isolation, identification and biological characteristics of pathogenic *Enterococcus faecalis* from Tibetan sheep

Wang Dongjing[1*], Su Zhonghua[2], Yuan Zhenjie[1], Mikhlid H. Almutairi[3]

[1]Institute of Animal Husbandry and Veterinary, Tibet Autonomous Regional Academy of Agricultural Sciences, Lhasa 850009, China

[2]Tibet Autonomous Region Animal Disease prevention and Control Center, Lhasa 850009, China

[3]Zoology Department, College of Science, King Saud University, P.O. Box: 2455, 11451, Riyadh, Saudi Arabia

Received:
November 24, 2023

Accepted:
December 29, 2023

Published Online:
February 15, 2024

**Corresponding author email:*
17808745806@163.com

Abstract

This study was conducted to identify the biological characteristics of pathogen responsible for the death of Tibetan sheep in Nagarze County, Tibet, China. For this purpose, samples were collected from diseased animals and for bacterial culture and isolation. The isolated strains were subjected to several tests which included gram staining, biochemical identification, PCR amplification, genetic evolution analysis, in-vitro drug sensitivity and in-vivo pathogenicity tests. The results revealed that 7 strains of gram-positive cocci were isolated from Tibetan sheep, named TS-1, TS-2, TS-3, TS-4, TS-5, TS-6, and TS-7. These strains exhibited specific biochemical characteristics consistent with *Enterococcus faecalis*. Whereas, PCR amplification results were consistent with the expected outcomes on target band of approximately 1500 bp. Genetic evolutionary analysis revealed a significant homology (96.0%-99.9%) between the isolates and *Enterococcus faecium*. In-vitro drug sensitivity tests demonstrated that all the isolates exhibiting multiple drug resistance. Furthermore, the isolated strains displayed varying degrees of pathogenicity in mice. This study confirms that *Enterococcus faecium* is the causative agent for the deaths of Tibetan sheep. These findings enhance our understanding of the disease and suggest valuable insights for its prevention, control, and future research.

Keywords: Tibetan sheep, *Enterococcus faecium*, Isolation, Identification, Drug resistance, Pathogenicity

How to cite this:

Dongjing W, Zhonghua S, Zhenjie Y and Almutairi MH. Isolation, identification and biological characteristics of pathogenic *Enterococcus faecalis* from Tibetan sheep. Asian J. Agric. Biol. xxxx(x): 2023327. DOI: https://doi.org/10.35495/ajab.2023.327

Introduction

Enterococcus is a widespread bacterium which is found in the intestines of humans and animals. Currently, there are 46 known species of *Enterococcus*, including *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus lactis*, *Enterococcus mongolicus*, *Enterococcus saccharolyticus* and *Enterococcus avis*, etc (Wang, 2019; Ennahar and Cai, 2005; Sagor et al., 2022). *Enterococcus* is an opportunistic pathogen and is ranked as the second most common pathogen causing

hospital infections by the National Institute of Social Sciences (Routsi et al., 2003). It is a reservoir for drug-resistant genes and is related to a transmission mechanism within the intestines by which drug resistant genes are transferred to other bacteria. Numerous clinical infections are associated with *Enterococcus faecalis* (Peng and Xu, 2018; Wang et al., 2022). *Enterococcus* causes various infections such as urinary tract infections, skin and soft tissue infections, abdominal infections, sepsis, endocarditis, and meningitis (Olesen and Møller, 2002). Moreover, it can lead to outbreaks within hospital settings (Kuzucu et al., 2005). Previous studies have indicated that *Enterococcus faecalis* can be transmitted from animals to humans and posing serious threats to human health (Zhou, 2015a; Jessica et al., 2009).

Recently, increase in the number of infection reports related to animals by *Enterococcus faecalis* has gained significant attention of scientists. Gong et al. (2019) isolated a strain of *Enterococcus faecalis* from the visceral tissue of deceased forest musk deer. This particular strain carried a multidrug-resistant genome island PR11 (Gong et al., 2019). Moreover, Wei et al. (2021) isolated a strain of *Enterococcus faecalis* from the lungs of deceased foxes. This strain caused death in experimental mice and was preliminarily identified as the main cause of death in foxes (Wei et al., 2021). Bai et al. (2021) isolated a strain of *Enterococcus* faecalis from the brain tissue of sheep that had been died for unknown reasons at a sheep farm in Ordos. The identification of suitable medicine through drug sensitivity testing effectively controlled further spread of disease at sheep farm (Bai et al., 2021; Al-Shammari and Sadoon, 2022).

Tibetan sheep is a primitive sheep breed in China that inhabits at high altitudes of over 3500 meters. It is the main livestock breed at the plateau areas of China and plays a crucial role in local economic development (Alberfkani et al., 2022; Ali et al., 2023; Ding et al., 2023; Fathy et al., 2023; Li, 2020; Wojtasiak et al., 2023). In 2022, various outbreaks were observed among Tibetan sheep in Nagarze County, Tibet, China. These reports have gained much attention for identifying the pathogens due to sudden outbreaks in Tibetan sheep. Therefore, this study was aimed to isolate and identify the pathogenic *Enterococcus faecalis* strains that could be the reason of deaths in Tibetan sheep at Nagarze County, Tibet, China.

Material and Methods

Sample collection

The anal swabs, viscera and intestinal contents were collected from Tibetan sheep exhibiting symptoms such as anorexia and diarrhea at Nagarze County, Tibet, China.

Bacterial isolation and cultivation

Tibetan sheep feces, viscera, ascites fluid, intestinal contents and other samples were inoculated into Tryptic Soy Broth (TSB) culture medium supplemented with 5% fetal bovine serum. The mixture was then incubated overnight at a constant temperature of 37 ℃. Subsequently, the mixture was streaked onto TSA solid culture medium also supplemented with 5% fetal bovine serum. Single colonies were purified and cultivated until pure Gram-positive cocci colonies were obtained. For Gram staining and microscopic examination one third portion of a single colony was taken.

Biochemical identification

Fifteen micro biochemical reaction tubes including melitriose, galactose, xylose, esculin, mannitol, starch, sorbitol, urease, salicin, xylose-gelatin, glucose, maltose, rhamnose, sulfureted hydrogen and simoncitrate were selected for testing. The isolated bacterial solution was inoculated in the micro biochemical reaction tubes and incubated at 37 ℃ for duration specified in the user manual. After incubation the results were observed and analyzed.

Molecular biology assay

For extracting the bacterial genome 2 mL of isolated bacterial liquid was used and water boiling method was applied. The 16S rRNA universal primer was used for PCR identification using the extracted genome as a template (Gao et al., 2022). The PCR positive products were sent to Qingke Biological Company for sequencing. The sequencing results were analyzed using NCBI Blast and DNA Star MegAlign was used for homology and phylogenetic analysis. The reference sequence information is provided in Table 1.

Table-1. 16S rRNA reference strains.

Species	Strain	accession number	source	country
Enterococcus faecalis	JCM 5803	NR_040789.1	feces	Japan
Cryptococcus neoformans	GAL7	NR_181755.1	The intestinal tract of the wax moth	UK
Enterococcus supranolol	ATCC 43076	NR_041707.1	unspecified	America
Streptococcus agalactiae	ATCC 13813	NR_040821.1	milk	Japan
Escherichia coli	U 5/41	NR_024570.1	urine	France
Bacillus licheniformis	AD6	OL757865.1	mine	Armenia
Bacillus cereus	ATCC14579	AF290547.1	soil	America
Enterococcus faecalis	CAU8318	MF424835.1	milk	China
Enterococcus faecalis	20	JN560898.1	unspecified	China
Enterococcus faecalis	STN12	MW391810.1	unspecified	China
Enterococcus faecalis	DU-1	MH249092.1	cheese	China
Enterococcus faecalis	gp34	KM495939.1	cheese	Iran
Enterococcus faecalis	IMAU98388	MW135205.1	unspecified	China
Enterococcus faecalis	IMAU98389	MW135206.1	unspecified	China
Enterococcus faecalis	IMAU98395	MW135209.1	unspecified	China
Enterococcus faecalis	MLG20-28	MT457705.1	unspecified	China
Enterococcus faecalis	zhy44	KC422716.1	unspecified	China
Enterococcus faecalis	ABRIINW.N7	MK367697.1	ewes' colostrum	Iran
Enterococcus faecalis	E50	MW386399.1	unspecified	China
Enterococcus faecalis	KMJC41	OP764046.1	cheese	Iran
Enterococcus faecalis	LZM23	OQ255840.1	unspecified	China
Enterococcus faecalis	BMT-1-3-1	CP063593.1	Immunosuppressive patients	Germany

Drug resistance test

The drug resistance test of isolated strains was conducted using the Kirly Baue method, in accordance with the regulations of the American Clinical Laboratory Standardization Association (CLSI) (Xu and Zhang, 2005) and user manual.

Animal pathogenicity test

The bacterial solution was diluted to a concentration of 10^8 CFU/mL using sterile physiological saline. Moreover, a total of 40 Kunming mice (approximately 18 g) were divided into 8 groups with 5 mice in each group. Experimental groups 1 to 7 were the infection groups whereas, the eighth group served as the control group. Exactly 500 µL of the isolated strains TS-1, TS-2, TS-3, TS-4, TS-5, TS-6, and TS-7 were injected into the mice of experimental groups 1 to 7, respectively. The control group was injected with 500 µL of sterile physiological saline. The mental state, eating habits and mortality of the mice was observed regularly, while feeding them normally. In case of death, the mice were promptly dissected and their hearts, liver, spleen, lungs, kidneys, intestines were collected in a sterile manner for bacterial isolation.

Results

Bacterial isolation and cultivation

Seven bacterial strains were isolated from diseased tissues of Tibetan sheep in Nagarze County, Tibet, China. These strains included one strain which isolated from lungs, two strains which were isolated from ascites and four isolated from anal swabs. The isolated bacteria were Gram-positive, spherical, blue-purple and scattered (Figure 1). They were named TS-1, TS-2, TS-3, TS-4, TS-5, TS-6 and TS-7 respectively.

Biochemical identification

Biochemical tests were conducted on the isolated strains and the results are shown in Table 2. The biochemical test results of the 7 isolated strains are

consistent: they can decompose lactose, aescin, mannitol, sorbitol, salicylic acid, glucose and maltose but do not decompose raffinose, xylose, starch and rhamnose. The urease reaction and Simmons citrate reaction are negative, and do not produce hydrogen sulfide. The biochemical characteristics of the isolated strains are consistent with those of *Enterococcus faecalis*.

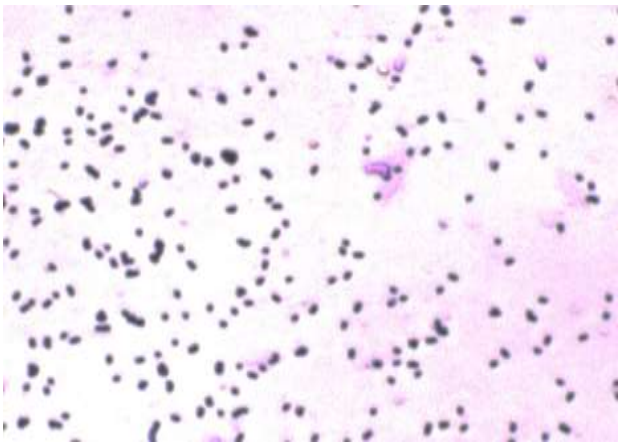

Figure-1. Gram staining of isolated strains.

Table-2. Biochemical Identification Results

Project	Strain						
	TS-1	TS-2	TS-3	TS-4	TS-5	TS-6	TS-7
Melitriose	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+
Xylose-gelatin	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-
Sulfureted hydrogen	-	-	-	-	-	-	-
Simoncitrate	-	-	-	-	-	-	-

16S rRNA PCR Identification Results

Genomic DNA was extracted from seven bacterial strains that were preliminarily isolated. The DNA was then amplified using 16S rRNA universal primers through PCR. The obtained results showed a target band of approximately 1500 base pairs in size (Figure 2).

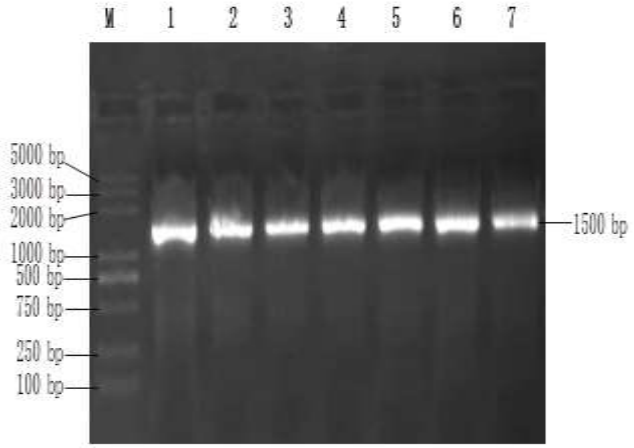

Figure-2. PCR amplification results of 16S rRNA gene. M indicates Trans2K Plus DNA Ladder; while 1, 2, 3, 4, 5, 6, and 7 indicates TS-1, TS-2, TS-3, TS-4, TS-5, TS-6, TS-7, respectively.

Sequence analysis

The sequencing results were compared and analyzed using NCBI's Blast online alignment function, and the reference sequences were downloaded for homology and phylogenetic analysis using DNA Star software. It can be seen in Figure 3 that the homology between the 7 isolates is 96.0%~99.9%, 96.2%~99.9% with *Enterococcus faecalis* isolates, 70.87%~76.4% with *Escherichia coli* U 541, 85.6%~89.1% with *Bacillus cereus* ATCC14579, 82.2%~86.8% with *Streptococcus lactis* ATCC 13813, 85.1%~90.3% with *Bacillus licheniformis* AD6 and 92.7%~96.0% with *Enterococcus faecalis* JCM 5803, The homology with *Cryptococcus* GAL7 is 93.9%~97.4% and with *Enterococcus glycolyticus* ATCC 43076 is 92.4%~96.4%.

The 16S rRNA nucleic acid sequence evolution tree depicted (Figure 4) that seven isolates share the same branch as *Enterococcus faecium*. Specifically, TS-1, TS-4, TS-6 and *Enterococcus faecium* isolate KMJC41 are clustered together, TS-5 and *Enterococcus faecium* isolate E50 form another cluster, TS-3 and *Enterococcus faecium* isolate BMT-1-3-1 are grouped together, and TS-2 and TS-7 are located on relatively independent small branches. It's worth noting that the isolated strains are distinctly separated from *Enterococcus faecalis*, *Cryptococcus*, *Enterococcus saccharifying*, *Escherichia coli*, *Streptococcus agalactis*, *Bacillus licheniformis* and *Bacillus cereus* as they are placed in different branches.

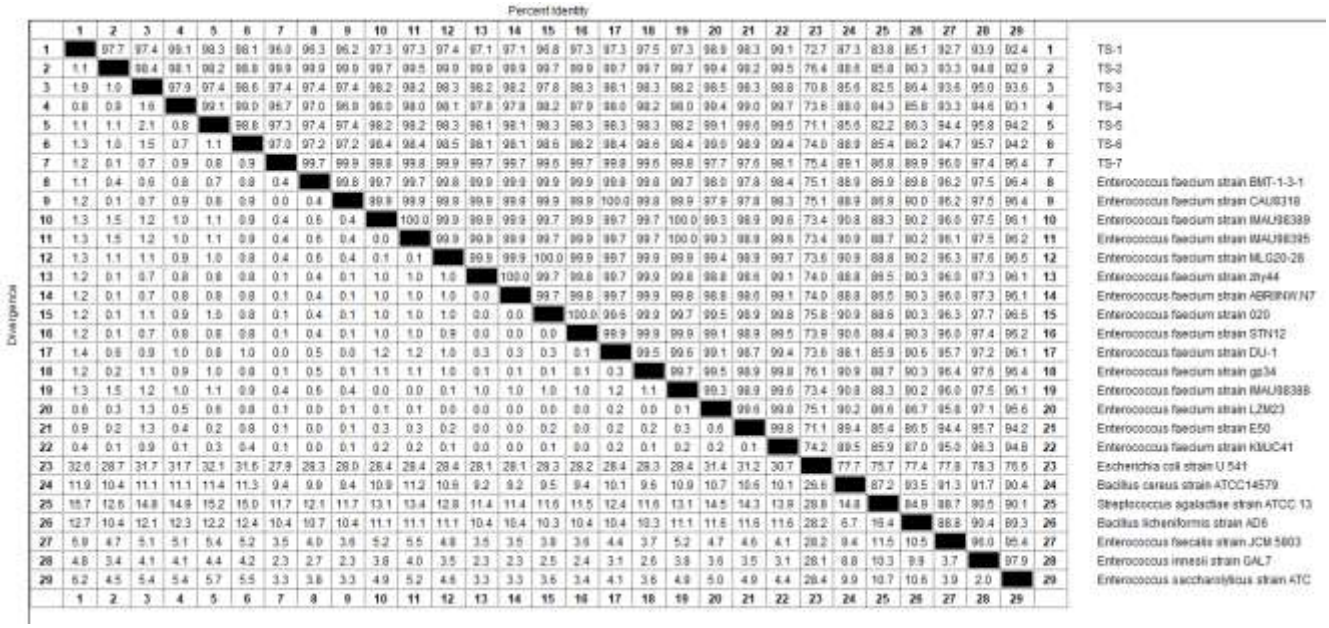

Figure-3. Homology analysis results of isolated strains.

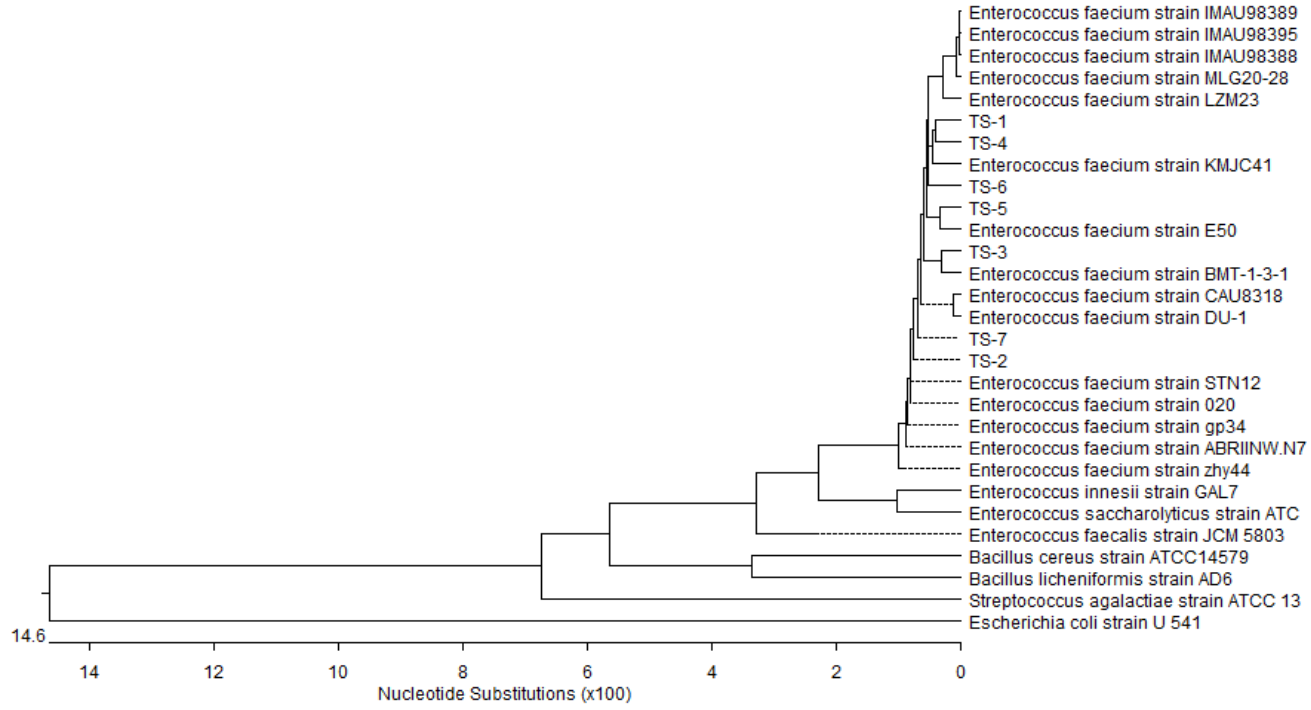

Figure-4. 16S rRNA gene evolution tree.

Drug resistance test

The results of the drug resistance test are shown in Table 4. The resistance rate of the isolated strains to ciprofloxacin and cephalosporin is 57.14%, to penicillin and clindamycin is 42.86%, to oxacillin, kanamycin, amikacin, gentamicin, compound sulfamethoxazole and erythromycin is 28.57%, to ampicillin, ofloxacin, and chloramphenicol is 14.29% and to amoxicillin, norfloxacin, vancomycin, neomycin Ceftriaxone, tetracycline, doxycycline and minocycline are all sensitive. The results of multiple drug resistance are shown in Figure 5 and all 7 isolated strains exhibit multiple drug resistance. Among them, three or more resistant strains account for 85.71%, and four or more resistant strains account for 57.14%.

Table-4. Results of in vitro drug sensitivity test

Drug classification	Drug Name	Strain							Drug resistance rate/ (%)
		1	2	3	4	5	6	7	
penicillins	Ampicillin	R	S	S	S	S	S	I	14.29
penicillins quinolones	Oxacillin	R	R	I	S	S	I	S	28.57
	amoxicillin	S	S	S	S	S	S	S	0.00
	Penicillin	S	I	R	R	S	I	R	42.86
	piperacillin	S	S	S	I	S	S	S	0.00
	ciprofloxacin	S	R	R	S	I	R	R	57.14
quinolones glycopeptides	Norfloxacin	S	S	I	S	S	S	S	0.00
	Ofloxacin	S	S	I	R	I	S	S	14.29
	vancomycin	S	S	S	S	S	S	S	0.00
Chloramphenicol	chloramghenicol	S	S	I	I	S	S	R	14.29
aminoglycoside	kantrex	R	R	I	S	S	I	S	28.57
aminoglycoside Lincoamide class	Amikacin	I	R	I	S	R	S	S	28.57
	Gentamycin	I	S	S	R	S	R	S	28.57
	Neomycin	S	S	S	S	I	S	I	0.00
	clindamycin	R	S	S	I	R	R	I	42.86
cephalosporins	cefoperazone	S	S	S	S	S	S	S	0.00
cephalosporins sulfanilamide grous	Cephalexin	R	R	I	R	R	S	S	57.14
	Ceftriaxone	S	I	S	S	S	S	S	0.00
	SMZ-TMP	R	R	I	S	S	S	S	28.57
Tetracyclines	tetracycline	S	S	I	S	S	I	I	0.00
Tetracyclines Macrolides	Doxycycline	S	S	S	I	S	S	S	0.00
	minocycline	S	S	S	S	I	S	S	0.00
	erythromycin	I	R	I	S	I	S	R	28.57
Macrolides	midecamycin	S	S	I	S	S	S	S	0.00

Note: S. Sensitivity; I. Intermediary; R. Drug resistance; 1. 2, 3, 4, 5, 6, and 7 represent TS-1, TS-2, TS-3, TS-4, TS-5, TS-6, and TS-7, respectively.

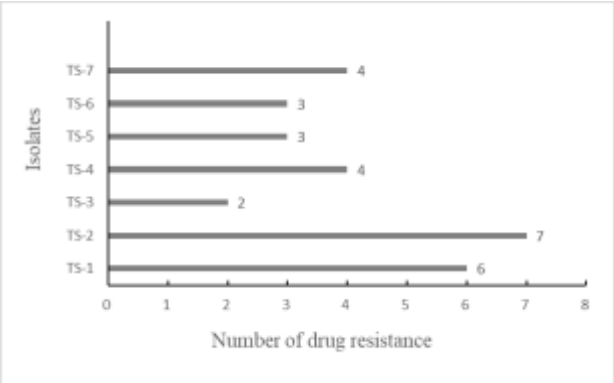

Figure-5. Result chart of drug resistance quantity of isolates.

Animal pathogenicity test

The isolated strains exhibited different levels of pathogenicity in mice. Mice in the experimental groups displayed symptoms such as clustering, mental depression, reduced feed intake, diarrhea and even death. In contrast, mice in the control group did not experience mortality and had normal appetite and mental state (Table 5). Furthermore, mice of the experimental group showed varying degrees of bleeding and congestion in their internal organs whereas, the mice of control group did not exhibit any significant pathological changes. *Enterococcus faecalis* was once again isolated from the visceral tissues of the mice from experimental group whereas, no bacterial growth was observed in the visceral tissues of mice of control group.

Table-5. Results of Animal Pathogenicity Test

Group	Animal pathogenicity test results
Experimental Group 1	Two mice died, with remaining mice experiencing mental depression, clustering, diarrhea and reduced feed intake
Experimental Group 2	Two mice died, with remaining mice experiencing mental depression, clustering, diarrhea and reduced feed intake
Experimental Group 3	No death occurred, the mice were mentally depressed, clustered, had diarrhea and had reduced feed intake
Experimental Group 4	No death occurred, the mice were mentally depressed, clustered, had diarrhea and had reduced feed intake
Experimental Group 5	One mouse died, with remaining mice experiencing mental depression, clustering, diarrhea and reduced feed intake
Experimental Group 6	No death occurred, the mice were mentally depressed, clustered, had diarrhea and had reduced feed intake
Experimental Group 7	One mouse died, with remaining mice experiencing mental depression, clustering, diarrhea and reduced feed intake
Control Group	No death occurred, the mice had normal appetite and normal mental state

Discussion

Enterococcus is a facultative anaerobic gram-positive bacterium that inhabits the intestines of humans and animals as a symbiotic bacterium. However, it can also act as a major opportunistic pathogen (Wang,

2019) due to which *Enterococcus* is not considered as 'generally recognized safe' and there is an escalating discussion regarding its potential as a beneficial bacterium (Marion et al., 2008).

The widespread and irrational use of antibiotics has led to the emergence of a large number of drug-resistant bacteria and genes. This poses a serious threat to human health and has developed as a globally prioritized issue (Jiang et al., 2019). Recent reports have highlighted the increasing prominence of *Enterococcus* resistance, particularly in relation to yak fecal samples collected from Hongyuan, Sichuan, China (Xu et al., 2017). Zhou et al. (2015b) revealed that out of 33 strains of *Enterococcus faecalis* derived from yaks in Yushu, Qinghai, 19 exhibited resistances to 10 commonly used antibiotics. Wang et al. (2020) conducted drug gene testing and found that 41 strains of *Enterococcus faecalis* exhibited 100% resistance to gentamicin, naphthoic acid and kanamycin. The resistance rates to other antibiotics were as follows: norfloxacin (31.7%), tetracycline (26.83%), chloramphenicol (7.31%), vancomycin and amoxicillin (4.88%), and ampicillin (0%). Earlier, there were no reports on the pathogenesis and drug resistance of *Enterococcus faecalis* from Tibetan sheep. The study revealed that the isolated strains had a resistance rate of 57.14% to ciprofloxacin and cephalosporin, 42.86% to penicillin and clindamycin, 28.57% to oxacillin, kanamycin, amikacin, gentamicin, compound sulfamethoxazole and erythromycin, 14.29% to ampicillin, ofloxacin and chloramphenicol and were sensitive to piperacillin, norfloxacin, vancomycin, neomycin, ceftriaxone, tetracycline, doxycycline, and minocycline. These research findings are of significant importance for selecting appropriate drugs for disease prevention and control in affected areas. Moreover, a scientific and rational approach for medication can help to reduce the occurrence of drug resistance.

Enterococcus faecium has emerged as a significant opportunistic pathogen in humans and animals with increasing infection and incidence rates in recent years (Liu et al., 2014; Zheng, 2016). However, there is limited research on the pathogenic mechanism and virulence factors of *Enterococcus faecalis* and its inherent resistance presents challenges in clinical treatment. Therefore, it is important to prevent *Enterococcus faecalis* infection (Liu, 2010). While the relationship between bacterial resistance genes and virulence factors has been confirmed that further investigation is needed to understand the specific mechanism (Cai et al., 2018). Consequently, it is crucial to explore the resistance mechanism and pathogenesis of *Enterococcus faecalis* infection to effectively control the associated diseases (Zhou et al., 2011). Lu et al. (2014) demonstrated that 60% of the tested strains of *Enterococcus faecalis* from pigs were pathogenic to mice, some with high lethality. Furthermore, Chen et al. (2015) isolated pathogenic *Enterococcus faecalis* from gray kangaroos suffering from purulent pneumonia, providing evidence of *Enterococcus faecalis* infection as the cause of the disease. Lu et al. (2003) conducted a study to investigate the correlation between an outbreak of human and pig sepsis caused by *Enterococcus faecalis*. These findings indicated that the epidemic was caused by *Enterococcus faecalis* transmitted by pigs, suggesting a potential co-infection relationship between humans and animals (Hua et al., 2000; Lu et al., 2003). The isolated strains in this study caused illness and death in Tibetan sheep in Langjia County, exhibiting varying degrees of pathogenicity to mice. Among the different drug resistance results, the strains TS-1, TS-2 and TS-7, which exhibited a higher number of drug resistances also demonstrated relatively strong pathogenicity, supporting the viewpoint of Cai et al. (2018). The pathogenic *Enterococcus faecalis* not only causes illness and death in Tibetan sheep in Langjia County but also poses a certain threat to the breeders' health.

Enterococcus is a normal inhabitant of biome in the gastrointestinal tract of humans and animals. However, it can also cause serious infections as a pathogenic bacterium (Patrick and Kimberly, 2019) which makes it an important public health issue (Michael et al., 2013). This study identified *Enterococcus faecium* as the pathogen responsible for the death of Tibetan sheep at Nagarze County, Tibet. The study investigated its biochemical characteristics, drug resistance and pathogenicity providing valuable references for clinical drug usage and serving as a foundation for further research on the pathogenesis and drug resistance mechanisms. This research is also significant in terms of public health.

Conclusion

This study isolated and identified 7 strains of pathogenic bacteria from Tibetan sheep samples at Nagarze County, Tibet. These strains were found to be *Enterococcus faecium* based on morphological,

biochemical and molecular biological analysis. The isolated strain showed multiple drug resistance to various drugs and also exhibited pathogenicity in mice. This research provides a foundation related to the prevention, control and further investigation of this disease in Nagarze County, Tibet.

Acknowledgment

We would like to express our gratitude to the Key Research and Development Projects in Xizang Autonomous Region for funding this study (XZ202301ZY0016N-1). Additionally, we would like to acknowledge YUAN Zhenjie and SU Zhonghua for their valuable support throughout the research process. Their contributions were instrumental in the successful completion of this study. This study received permission from persons whose contribution to the work is acknowledged in the manuscript. The authors extend their appreciation to the Researchers Supporting Project number (RSP2024R191), King Saud University, Riyadh, Saudi Arabia.

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: This study was financially supported by the key research and development project of Xizang Autonomous Region (XZ202301ZY0016N-1).

Contribution of Authors

Dongjing W & Zhonghua S: Planned the study, performed the experiments, wrote the first draft, handled the revision, read and approved the final manuscript

Zhenjie Y & Almutairi MH: Reviewed literature, data analysis, wrote, edited and revised the manuscript.

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