



RESEARCH ARTICLE

Isolation and Identification of Potential *Bacillus* Probiotics from Free Ranging Yaks of Tibetan Plateau, China

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ABSTRACT

The present study was planned to isolate and identify the *Bacillus* probiotics from free ranging yaks of Tibet in order to prevent the epidemic calf diseases through beneficial strains. For this purpose, three *Bacillus subtilis* strains (named BS1, BS2, and BS3) and one *Bacillus velezensis* strain (named BV1) were isolated from the intestinal contents of Tibetan yaks. Meanwhile, *Staphylococcus aureus* (*S. aureus* ATCC 26112), *Escherichia coli* (*E. coli* ATCC 25922) and *Salmonella enteritidis* (*S. enteritidis* NCTC 13349) were used as reference strains to test the antibacterial activity of the isolates. All the strains had strongest bacteriostatic ability against the reference strains with perfect bile and acid tolerance, especially BV1 exhibiting the highest antibacterial activity. In addition, antibiotic resistance, resistance gene, oral testing and hemolytic analysis were also performed to confirm the safety of these isolates. Furthermore, experimental group showed a better growth trend as compared to the control group. In conclusion, in present study four isolated strains of *Bacillus* prove to be safe, possess growth promoting activity and promote the healthy balance of gut microflora.

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INTRODUCTION

The outbreak of bacterial diseases can cause tremendous economic losses to the yaks, which has become a major limitation to health and production of yaks (Liet *et al.*, 2014). At present, the use of antibiotics is still one of the famous traditional methods to treat these infections. However, the inappropriate use of antibiotics in the livestock leads a greater impact on the health status of humans via food supply chain (Phillipset *et al.*, 2004). In addition, this also leads to the emergence of bacterial resistance, which has caused a great threat to public health and food safety in the recent years (Wang *et al.*, 2018). Commensal bacteria can evolve antibiotic resistant phenotype under frequent antibiotic exposure and transfer to humans at last. So, it is entire need to develop new

antibiotic alternatives. To date, probiotics have been considered as the perfect alternative to antibiotics (Janczarek *et al.*, 2016).

Probiotics are live microorganisms, which could benefit the host when it is colonized in the body, as defined by the World Health Organization (WHO) (FAO/WHO, 2006). It is well acknowledged that probiotics could reduce and inhibit the growth of pathogens when it is taken into the body (Wang *et al.*, 2018a). The benefits of probiotic include improving the intestinal microbial balance of the host and reducing the risk of gastrointestinal diseases (Li *et al.*, 2019). The benefits of probiotic include the alleviation of inflammation, the improvement of immunity and prevention of the prevalence of allergies in susceptible individuals (Zhang *et al.*, 2016). In addition, many reports highlights that probiotics have antimutagenic, anticarcinogenic, and antiosteoporosis effects and can reduce the risk of colon, liver and breast cancer (Shioiri *et*

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al., 2006). To date, a large amount of microorganisms has been reported as probiotics in medicine, food processing, and livestock production. The most commonly used probiotics include lactic acid bacteria, *Bacillus* species and bifidobacterium species (Argyri *et al.*, 2013). All of them are gram-positive bacteria. The typical characteristic of the bacillus is the formation of endospores, quick growth, and endure high temperature and extreme stress. The bacillus has more obvious inherent advantages as compared to the other non-spore formers.

Tibet is high altitude region having low atmospheric pressure. It is recognized as the hometown of yaks (Zhang *et al.*, 2017) and yaks have the natural ability to survive in this hypoxic and extreme weather conditions (Han *et al.*, 2016). According to the statistics, the lives of yaks mainly in Tibet, Qinghai, Sichuan provinces of China accounts for 90% of the total yaks in the world (Li *et al.*, 2015). Yak is the important source of meat and socio-economic source of the local community and nomads. However, the information of the probiotics in yaks at such high altitude is still scarce. Therefore, the current study was designed to evaluate the probiotic potential of *Bacillus* isolated from free range yaks of Tibet, in order to provide a probiotic starter for the inhibition of the epidemic bacterial disease.

MATERIALS AND METHODS

Samples collection: Intestinal contents were collected from Nyingchi region of Tibet, China. Moreover, *S. aureus*, *E. coli* and *S. enteritidis* were used as reference strains.

Isolation and identification of *Bacillus* strains: Intestinal contents were weighed for being 2 g and then homogenized in 10 mL sterile PBS solution. Then, 100 μ L suspension was absorbed and spread on Luria-Bertani (LB) agar plates incubated at 37°C for 24h. We refer to the characteristics of *Bacillus* and selected suspected colony, which was purified three times. PCR analysis and 16S rRNA sequencing was used for the final confirmation and identification of strains. The 16S rRNA gene was amplified by the universal PCR primers, according to previous studies (Wu *et al.*, 2018). At last, the obtained nucleotide sequence was subjected to BLAST at NCBI and constructed the phylogenetic tree.

Antibacterial tests in vitro: The antibacterial activity of the isolated strains were measured, according to the method of Farida (Zhang *et al.*, 2016).

Resistance to low pH and bile salts: In order to test the tolerance of strains to acid and bile, we refer to the method of Wang *et al.* (2018b).

Resistance to heat: The isolated strains of the presented study were heated at 40, 60, 80 and 100°C for 20min. On the other hand, the control group was grown under general conditions. The numbers of the isolated strains were determined by the dilution method of plate counting after 24h of incubation at 37°C. The survival rate is the ratio of the experimental group to the control group.

Antibiotic susceptibility testing: For the purpose of antibiotic susceptibility testing, we refer to the disc agar diffusion method (Wang *et al.*, 2018b).

Haemolytic activity: Fresh bacterial cultures were streaked in triplicates on blood agar plates containing sheep blood. The haemolytic activity was evaluated after 48h of incubation and the *S. aureus* was used as positive control.

PCR detection of antimicrobial resistance genes: To identify the antibiotic resistance determinants, all the isolated strains were used to detect the tetracycline resistance genes and glycopeptides (Table 1).

Table 1: PCR primers used in this study

Target gene	Primer pair	5'-3' Sequence	Reference
tet(K)	tetK-f tetK-r	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAACTCTTGAAA	Gevers <i>et al.</i> , 2003
tet (L)	tet(L)-2-1 tet(L)-2-2	CATTTGGTCTTATTGGATCG ATTACACTCCGATTTCCGG	Aarestrup <i>et al.</i> , 2000
tet (M)	tet(M)-1 tet(M)-2	GTTAAATAGTGTCTTGGAG CTAAGATATGGCTTAACAA	Aarestrup <i>et al.</i> , 2000
tet (O)	tet(O)-1 tet(O)-2	GATGGCATAACAGGCACAGAC CAATATCACCAGAGCAGGCT	Aarestrup <i>et al.</i> , 2000
vanA	vanA 1 vanA 2	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	Dutkamalen <i>et al.</i> , 1995
vanB	vanB for vanB rev	GTGCTGCGAGATACCACAGA CGAACACCATGCAACATTC	Ramos-Trujillo <i>et al.</i> , 2003

Safety test in mice: In order to test the safety of the isolated strain, Kunming mice (10-15g) were selected as experimental animals. After 3 days of acclimation, fifty mice were randomly assigned into five groups (n=10). The rats in four experimental groups were gavaged BS1, BS2, BS3, and BV1 respectively, at 1×10^9 CFU/day for 18 consecutive days. At the same time, rats in the control group were forcibly gavaged with equal volume of vehicle. All animal operations management and sample selection were in accordance with Good Laboratory Practice Guidelines (Alatgi and Chougule, 1998). Mice were weighed after every two days and their condition (mental state activity, stress, coat, diarrhea and feed intake) was evaluated daily during treatment. On the last day of the experiment, all the animals were killed by cervical dislocation. Blood was collected by cardiac puncture in sterile conditions. Bacterial translocation was analyzed in blood. 50ul blood was cultured at 37°C for 48 h in Mann Rogosa Sharp (MRS) agar and brain-heart infusion media (BHI) agar. After 48 h of incubation, the number of bacteria in the agar plates was recorded as a result of the bacterial translocation rate (number of mice that have bacterial translocations/total number of mice). Positive growth on agar plates is defined by even one colony count. Statistical analysis of the body weight data for multiple comparisons was performed by one-way analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Isolation and identification of probiotics: All the isolated strains were rod shaped, endospores producer and gram-positive bacterium as evaluated through the microscopic examination. The biochemical tests showed that all the strains were catalase-negative. On LB agar plate, we observed white opacity and marginally cocci-

shaped colonies with 3.00-5.00 mm diameter, which were suspected bacillus strains. Therefore, the four strains were named BS1, BS2, BS3, and BV1 for further analysis. The 16S rRNA sequence was used to perform a BLAST to determine the characteristics of the isolated strains. Our results showed that BV1 had 99% identity with *Bacillus velezensis*, while BS1, BS2 and BS3 were closely related to *Bacillus subtilis*.

The neighbor-joining tree (Fig. 1) showed that BS1, BS2 and BS3 formed a distinct cluster with *Bacillus subtilis* and BV1 is integrated with *Bacillus velezensis*.

Antibacterial tests *in vitro*: As shown in Fig 2, all the isolated strains had excellent antimicrobial activity against *E. coli*, *S. aureus* and *S. enteritidis*, as compared to the control group. In addition, the diameter ranged from 16.00mm to 26.00mm and BV1 had the highest antimicrobial activity among all strains. On the other hand, BS1 and BS3 did not show significant ability to inhibit the proliferation of Staphylococcus, with inhibition range of about 16.00 mm to 18.00 mm. However, BV1 showed the highest antibacterial ability against Salmonella with 26.00 mm of the diameter.

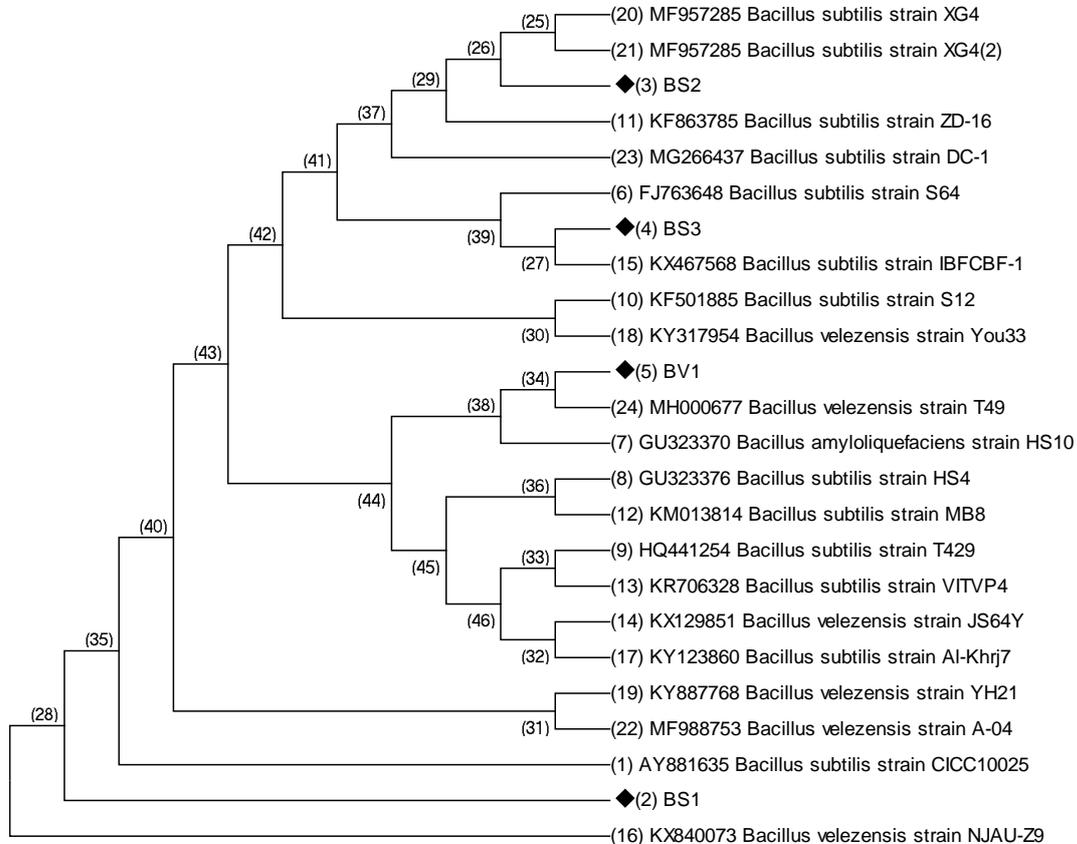


Fig. 1: The phylogenetic tree analysis of BS1, BS2, BS3 and BV1 based on the 16S rDNA and constructed by the neighbor joining method.

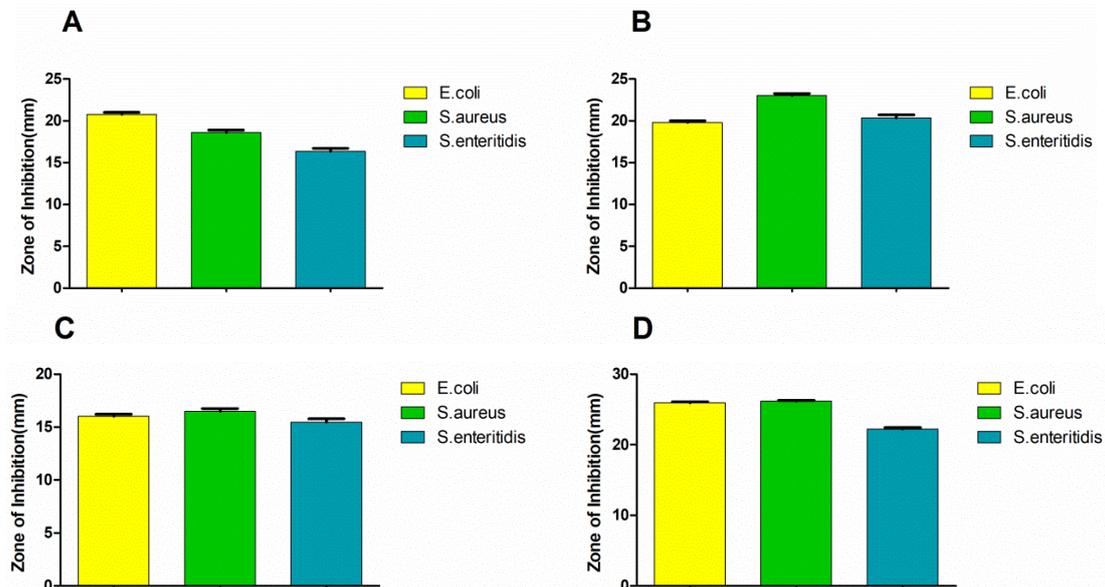


Fig. 2: The antibacterial effect of isolated strains against *S. aureus*, *E. coli* and *S. enteritidis*. A: BS1; B: BS2; C: BS3; D: BV1.

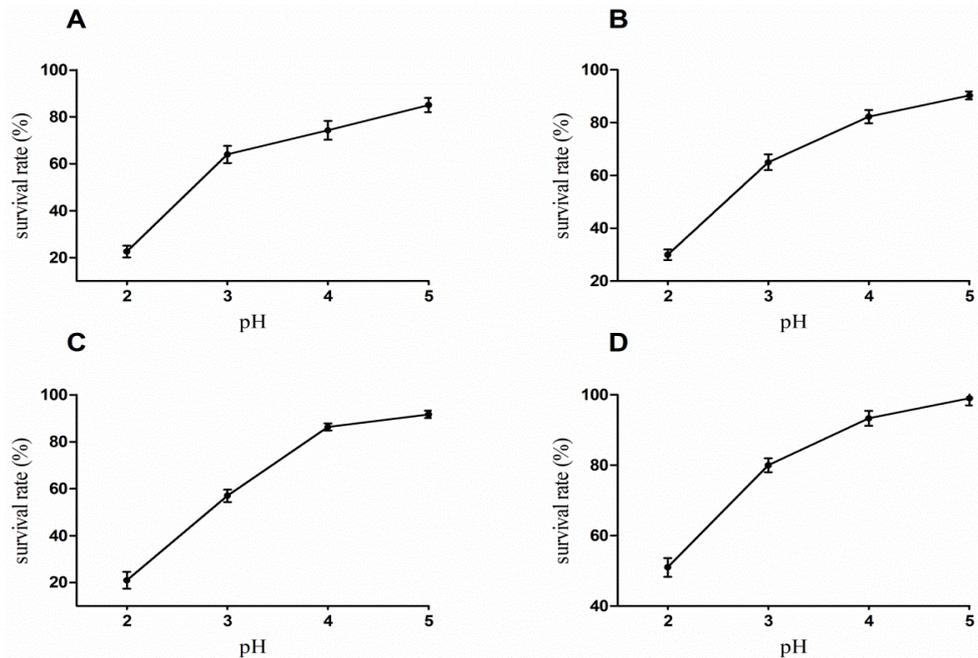


Fig. 3: Effect of the pH on the isolated strains (A: BSI; B: BS2; C: BS3; D: BV1). The data are reported as the mean \pm SD in each group (n=3).

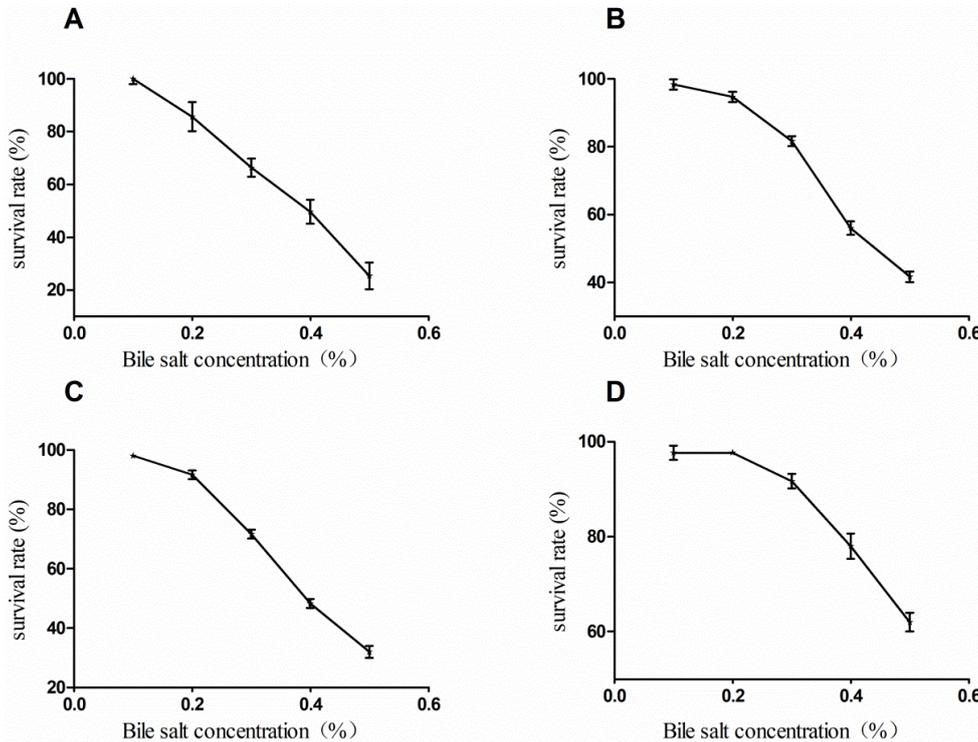


Fig. 4: The results of bile tolerance in BSI, BS2, BS3 and BV1, all data are processed with mean \pm SD (n=3).

Acid and bile salts tolerance: Survival under low pH and high bile salts is a prerequisite for probiotics to play a probiotic role. Fig. 3 and Fig. 4 show that the survival rate of the isolated strains had a tendency to decrease with the increase of acid and bile salt. BV1 showed the highest acid resistance compared to other strains, with a survival rate of approximately 80% at pH3. On the other hand, the survival rate of BV3 was only 55% at pH3 among all the strains. The majority of tested strains were found to be resistant to bile salts. In addition, the survival rate of BV1 almost unaffected at 0.3% bile salt.

Resistance to heat: Fig. 5 shows the ranges of the survival rate of all the tested strains in the heat resistance. The survival rate gradually decreases with the increase in temperature. Moreover, the survival rate of all the strains was hardly affected between 40°C to 60°C. The BS1 had the best heat resistance compare to BS2, BS3, and BV1. The survival rates of BS1 and BV1 at 100°C were 25% and 20%, respectively. However, BS2 and BS3 showed only 10% survival at 100°C.

Antibiotic resistance: The absence of antibiotic resistance and haemolytic activity is considered as a

safety and prerequisite for the selection of a probiotic strain. The resistance rate of all strains was less than 10% (Table 2). In addition, BS2 did not develop resistance to any antibiotic, which was used in this study. It was interesting that BS1, BS3, and BV1 were all resistant to Bacitracin. On the other hand, BS3 also developed resistance to Tetracycline.

Haemolytic activity: The strain of positive control exhibited β -Hemolytic activity and the isolated strains (BS1, BS2, BS3 and BV1) were γ -Hemolytic (no hemolysis) when grew in blood agar plates for 24h.

Results of resistance gene test: The tetracycline-resistance-determining genes [*tet(L)*, *tet(K)*, *tet(M)*, *tet(O)*] and vancomycin-resistance-determining genes such as *vanA* and *vanB* were not detected in all the isolated strains.

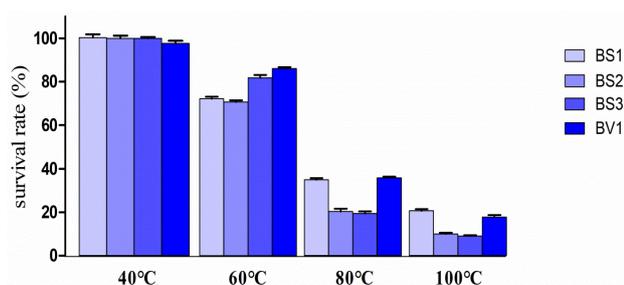


Fig. 5: The survival rate of the BS1, BS2, BS3 and BV1 after heat treatment.

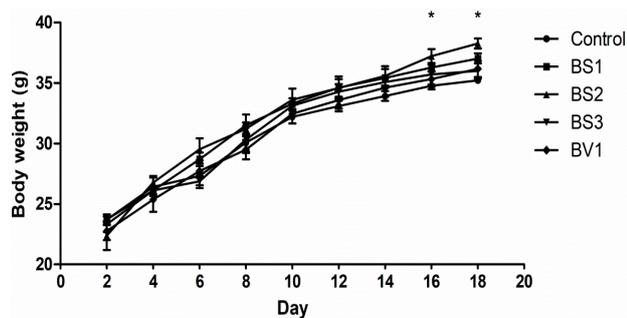


Fig. 6: Body weight evolution trends of mice in control and probiotic-treated groups.

Table 2: Antibiotic susceptibility test of BS1, BS2, BS3 and BV1

Antibiotics	BS1	BS2	BS3	BV1
Enrofloxacin	S	S	S	S
Tetracycline	S	S	R	S
Ceftriaxone	S	S	S	S
Cefixime	S	S	I	S
Norfloxacin	S	S	S	S
Teicoplanin	S	S	S	S
vancomycin	S	S	S	S
gentamicin	S	S	S	S
lincomycin	S	S	S	S
roxithromycin	S	S	S	S
Cefalexin	S	S	S	S
ciprofloxacin	S	S	S	S
erythromycin	S	S	S	I
neomycin	S	I	S	S
acetylspiramycin	S	S	S	S
novobiocin	S	S	S	S
bacitracin	R	I	R	R
azithromycin	S	S	S	S
cefuroxime	S	S	S	S
florfenicol	S	S	S	S

Note: S, sensitive; R, resistant; I, intermediate.

Results of animal's safety test: During the entire trial, no treatment-related illness or death was observed. Animals in the experimental and control groups had no obvious changes in general signs such as behavior, mental status, hair color, feed intake, aggressiveness. Oral administration of the BS1, BS2, BS3, and BV1 had no adverse effect on food intake. No significant difference in the food intake of animals in the experimental and control groups was recorded (data not shown). As shown in Fig 6, a significant differences were observed in the body weight changes between the control, BS1 and BS2 groups after 16 days ($P < 0.05$). In terms of bacterial translocation, no bacterial translocation has occurred in the blood either in the control group or in the experimental groups. Normal appearance of the internal organs was observed by macroscopic evaluation. No any ulcers and intestinal adherence in the small intestine were found in this study.

DISCUSSION

Infectious diseases are a threat for animal health and productivity (Mahmood *et al.*, 2018; Wang *et al.*, 2018a; Mehmood *et al.*, 2017). The bacterial disease causes enormous economic loss to the farm animals every year. However, the traditional methods of treatment are still dominated by the use of antibiotics. The unselective use of antibiotics could lead to drug resistance and affect public food safety (Wang *et al.*, 2018b). More importantly, it could upset the normal balance of gut flora. A number of investigators have shown that probiotics establish beneficial bacteria as soon as possible in the gut of the newborn flock and enhance the body immunity (Nguyen *et al.*, 2015). More importantly, probiotics also play an important role in reducing inflammation, cholesterol, allergies and restraining pathogenic bacteria (Dong *et al.*, 2018). Tibet is the highest and largest among the plateaus in World and known as the "roof of the world". Yak is an ancient species in Tibet, which is naturally resistant to high temperatures and hypoxic conditions (Qiu *et al.*, 2012). Therefore, yak may have a special gut flora compared to other animals at low altitudes. However, very rare reports are available on the gut microbial communities or probiotics isolated from Tibetan yaks. Previously, a high mortality due to diarrhea in yaks was reported and the presence of drug residues in meat and dairy products. Therefore, we aimed to isolate effective probiotics from yak to reduce the negative effects of antibiotics and reduce economic losses.

The fundamental characteristic of *Bacillus* includes survival and germination in the intestine, the formation of biofilms and secretion of antibiotics (Guo and Li, 2006; Hong *et al.*, 2009). In a feeding study, *Bacillus* could decrease the incidence of diarrhea in pigs, which were treated by *Bacillus toyonensis* (Kantas *et al.*, 2015). Previous research shows that *Bacillus* reduces diarrhea in piglets challenged with *E. coli* and significantly increase weight gain compare to control group (Williams *et al.*, 2009). *E. coli*, *S. aureus*, and *S. enteritidis* are common pathogens leading to diarrhea in the yak. The study shows that the BV1, BS1, BS2 and BS3 had highest antibacterial properties against pathogens. In particular, BV1 showed the highest antibacterial properties against *S. aureus* and the inhibition zones diameter was 26.00 mm, which was significantly higher than the control group. *Bacillus* could

produce spores to make them more resistant to miserable conditions and are deemed as probiotics for their beneficial qualities for human and animal health. The isolated strains showed a high survival rate at 100°C and accord with former research results (Guo and Li, 2006). *Bacillus* has already been found in feces and ileal biopsies of diverse mammals, suggesting that they could adapt the conditions of stomach and intestines (Guo *et al.*, 2006; Fakhry *et al.*, 2008). BV1, BS1, BS2, and BS3 were able to maintain a high survival rate under pH3 and 0.3% bile salts suggesting that they have the potential for colonization in the intestine.

The safety of a probiotic must be evaluated *in vitro* before its usage in the animal husbandry. The use of probiotics was supervised by the European Food Safety Authority (EFSA) and they must be proved non-toxicogenic. EFSA claims that the antibiotic resistance of probiotics is a matter of great concern (Adimpong *et al.*, 2012). It is considered an excellent feature of probiotics, which does not harbor acquired and transferable antibiotic resistance (Vizoso Pinto *et al.*, 2006). In a previous study, *Bacillus* was isolated from the intestinal tract in fish and has been given a Qualified Presumed Safety (QPS) status in animal nutrition through the acute and chronic toxicity experiments (Guo *et al.*, 2016). In this study, all strains were tested for drug sensitivity and PCR amplification of drug resistance genes and safety experiments in mice. The results showed that most of the isolates were sensitive to antibiotics. However, BV1 and BS1 were resistant to bacitracin, and BS3 resistant to bacitracin and tetracycline. In addition, drug resistance genes were not detected by PCR analysis and the mice did not show any abnormalities after feeding the isolates. In conclusion, BV1, BS1, BS2, and BV3 have good antibacterial properties and potential for probiotics and could be used as a substitute for antibiotics.

Conclusions: This study shows that BS1, BS2, BS3 and BV1 have the potential to inhibit the growth of pathogenic bacteria. In addition, they were resistant to low pH, high bile and high temperature. The safety test indicates that BS1, BS2, BS3 and BV1 are safe and could promote the increase of body weight in mice to some extent. Our findings may provide a new method to treatment the bacterial diseases in yaks.

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Authors contribution: AL, YW and JL conceived and designed the experiments; SS, KM, XJ, LZ, ZL, MW, MI contributed sample collection, reagents preparation and analysis tool; AL and YW wrote the manuscript. All the authors involved in discussing the contents of the manuscript and agreed for publication.

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