He *et al. Animal Diseases* (2024) 4:17
https://doi.org/10.1186/s44149-024-00124-z

Animal Diseases

ORIGINAL ARTICLE

Open Access

Probiotic potential of lactic acid bacteria isolated from yaks

Yuanyuan He[1†], Feiran Li[1†], Mengen Xu[1], Chao Jin[1], Yu Zhang[1], Shah Nawaz[1], Muhammad Fakhar-e-Alam Kulyar[1], Mudassar Iqbal[1], Zhen Qin[3] and Jiakui Li[1,2*]

Abstract

The prevalence of bacterial digestive diseases in plateau animals has caused considerable losses to the Tibetan livestock industry. Therefore, this study aimed to isolate safe lactic acid bacteria (LAB) with beneficial probiotic properties to protect yaks from intestinal diseases. After 16S rDNA matching, four strains of *Lactobacillus fermentum* (A4), *Pediococcus pentosaceus* (A3.4 and A1.2), and *Pediococcus acidilactici* (B1.9) were isolated from the intestinal tissues and content of healthy yaks. The results indicated that A4 was more tolerant to bile salt (0.3%), while A3.4 had better stability in an acidic (pH = 3.0) environment. The results of the antibacterial activity test suggested that the isolates inhibited most pathogenic bacteria by up to 20%, except for A3.4, which inhibited *Pasteurella* and *Staphylococcus aureus* by more than 20%. Moreover, the results of the antioxidant test demonstrated that A4 and A3.4 had potent antioxidant activity. In addition, the drug sensitivity test revealed that the isolates were susceptible to commonly used antibiotics. In terms of safety, the isolates promoted growth, enhanced intestinal development, and protected the intestinal barrier without causing any adverse effects. In conclusion, LAB isolated from yak intestinal contents are potential probiotics with excellent antibacterial properties.

Keywords *Lactobacillus*, Probiotics, Identification, Yaks, Tibet

Introduction

A variety of antibiotics have been discovered and applied in the animal husbandry industry due to their anti-infective and growth-promoting effects over the past few decades (Oliver et al. 2011; Low et al. 2021). However, the misuse of antibiotics has been reported to potentially generate environmental hazards to human health in recent years (Cabello and Godfrey 2016). In addition, the emergence of antimicrobial-resistant microorganisms caused by the irrational use of antibiotics is a continuous threat to human and animal health (Robinson et al. 2016). In addition, previous studies have shown that the abuse of antibiotics can disrupt the homeostasis of the intestinal flora, leading to antibiotic-associated diarrhea (AAD) and allergic reactions (Slama et al. 2005). Therefore, the European Union and the United States Food and Drug Administration (FDA) have introduced several policies about the rational use of antibiotics in the animal husbandry industry over the past decade (Phillips 2007; Brüssow 2017). Nevertheless, since antibiotic use in animal husbandry is restricted, high-density farming has contributed to the development of zoonotic infections and increased the incidence of infectious diseases since the advent of intensive animal husbandry (Graham et al. 2008;

†Yuanyuan He and Feiran Li contributed equally to this work.

Handling editor: Yifei Lang.

*Correspondence:
Jiakui Li
lijk210@mail.hzau.edu.cn
[1] College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, PR China
[2] College of Animals Husbandry and Veterinary Medicine, Tibet Agricultural and Animal Husbandry University, Linzhi, Tibet 860000, PR China
[3] Xuanen Animal Husbandry and Veterinary Technical Service Center, Xuanen 445500, PR China

Leibler et al. 2017). Hence, there is an urgent need for a safe and effective alternative to antibiotics on the global market.

Recently, probiotics have been identified as an ideal alternative to antibiotics owing to their anti-infective and growth-promoting effects. Probiotics are defined as live microorganisms that benefit the host when taken at the recommended dosages (Lim et al. 2020). Accumulating evidence has shown that these probiotics have various beneficial effects, such as regulating the intestinal flora, strengthening the intestinal barrier, and increasing antimicrobial activity (Wehkamp et al. 2004; Pridmore et al. 2008; Zhong et al. 2020). Supplementing livestock feed with probiotics benefits animal gastrointestinal health, improves feed utilization, and reduces the incidence of diarrhea (Yadav and Jha 2019). The probiotic *Lactobacillus* has recently gained popularity due to its capacity to ferment carbohydrates into lactic acid. Xin et al. reported that *Lactobacillus johnsonii* BS15 could significantly promote growth performance, enhance intestinal immunity, and maintain the gut microbiota in piglets (Xin et al. 2020).

Additionally, previous studies have demonstrated that *Lactobacillus acidophilus* enhances the growth performance, intestinal morphology, barrier function, and immune response of broilers induced with *Escherichia coli* O157 (Wu et al. 2021). Lactobacillus species are potential alternatives to antibiotics due to their ability to maintain the balance of the intestinal flora and promote the growth of the body. Nevertheless, there is still a need for further screening and research on *Lactobacillus*, with the above excellent potential arising from probiotics being host- and strain specific.

Considering its host and strain specificity, *Lactobacillus* strains isolated from yaks are highly resistant to harsh environments and have antimicrobial properties. Yaks are representative species living on the Qinghai–Tibet Plateau and are characterized by adapting to low temperatures and hypoxic environments. However, due to grazing practices and harsh climatic conditions, there is a high incidence of gastrointestinal diseases in yaks, causing severe economic losses (Gomez et al. 2017). At the same time, the selection of antibiotic usage poses a severe challenge to the treatment of intestinal diseases in yaks. Numerous studies have suggested that *Lactobacillus* strains isolated from yaks are alternatives to antibiotics due to their strong tolerance and antimicrobial properties. Furthermore, *Lactobacillus* strains isolated from yaks were proven to promote growth and prevent intestinal diseases. Consequently, this study aimed to isolate *Lactobacillus* strains with excellent biological viability from the gastrointestinal tract and gastrointestinal tract (GIT) contents of yaks to facilitate the prevention of intestinal diseases in yaks.

Results

Isolation and identification of isolates

The four strains (named A4, A3.4, A1.2 and B1.9) with small colonies, a milky white color, and apparent calcium soluble circles on MRS agar plates were found to be gram-positive and catalase-negative (Fig. 1). A phylogenetic tree of the four isolated strains was constructed with MEGA V. 7.0 software. A4 was 55% homologous to *Lactobacillus fermentum*, and B1.9 was 98% homologous to *Pediococcus acidilactici*. In addition, A3.4 and A1.2

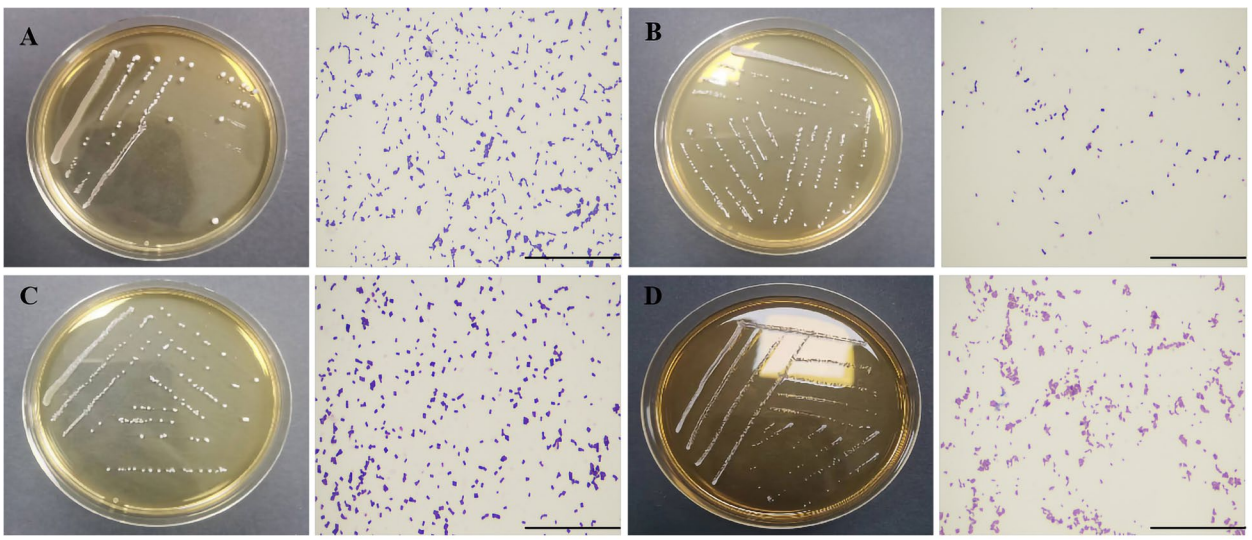

Fig. 1 Colony morphology and Gram staining findings of the isolates: **A** A4; **B** A3.4; **C** A1.2; **D** B1.9. Bar means 100 μm

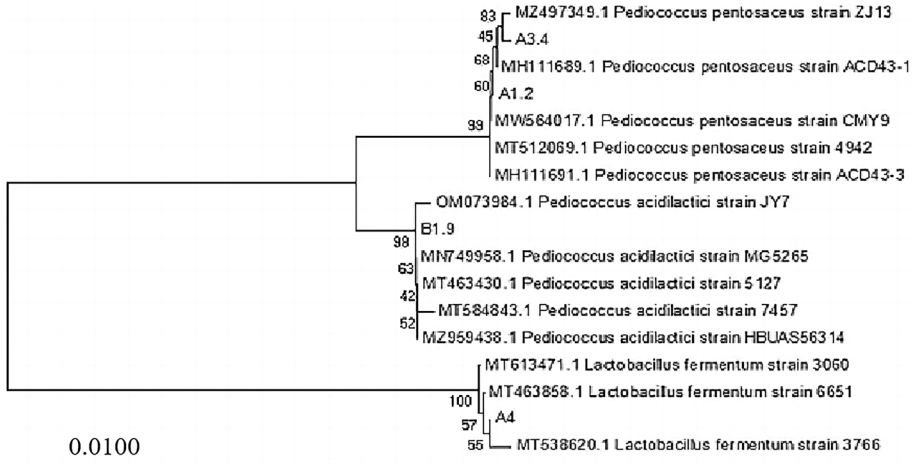

Fig. 2 Phylogenetic tree of isolated strains

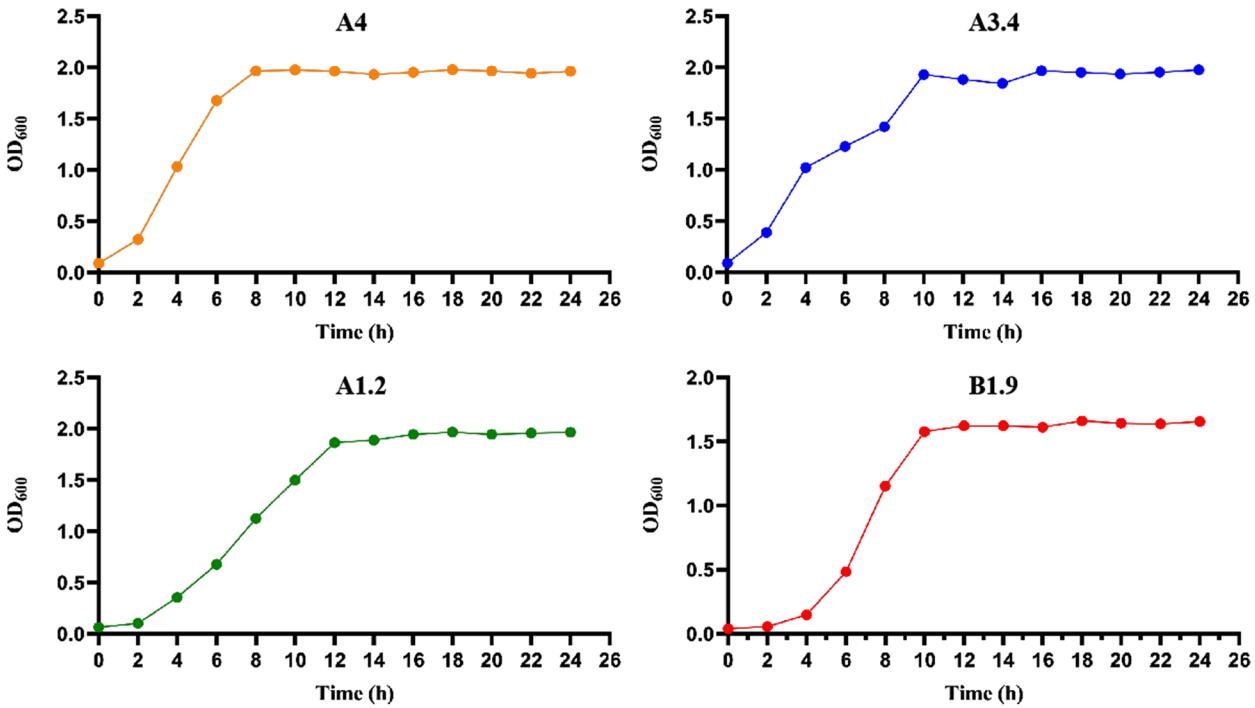

Fig. 3 Growth curves of the isolates over 24 h

were 83% and 60% homologous to *Pediococcus pentosaceus*, respectively (Fig. 2).

Growth curve

A4, A3.4 and A1.2 entered the logarithmic growth period at 2 h, while B1.9 entered at 4 h. Moreover, A4 and A1.2 reached plateau phases at 8 h and 12 h, respectively, while A3.4 and B1.9 reached the same state at 10 h (Fig. 3).

Acid and bile salt resistance test

The survival rate in bile salts and acidic environments represented the resistance of the strain to harsh climates. The different strains had different tolerances to both environmental conditions. The maximum survival rate of A4 was 51.21 ± 3.44% when the concentration of bile salt was 0.3%. Moreover, A3.4 showed greater tolerance, with a 50.61 ± 0.30% survival rate, than A1.2 and B1.9 did when the pH of the broth was maintained at 3.0

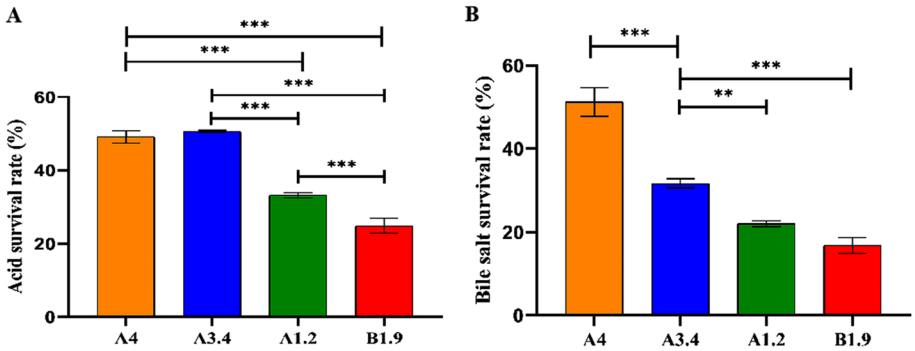

Fig. 4 Resistance of the isolated strains to acid and bile salt: **A** Resistance to acid (pH = 3.0); **B** Resistance to bile salt (0.3%). $^{**}p < 0.01$, $^{***}p < 0.001$

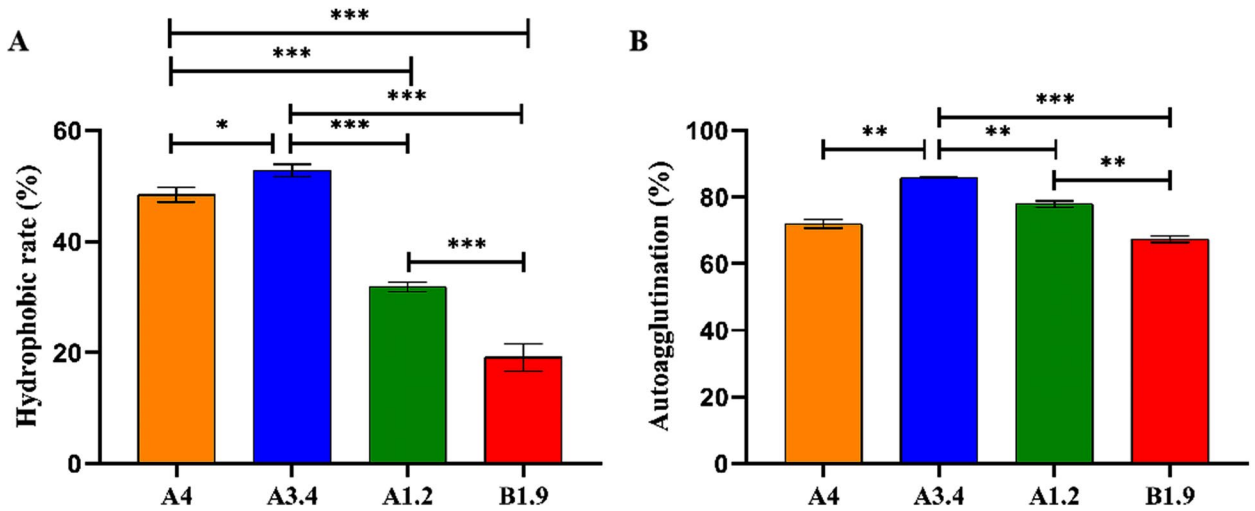

Fig. 5 Hydrophobicity (**A**) and autoagglutination (**B**) of the isolates $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

($^{***}p < 0.001$). However, there were no significant differences between the survival rates of strain A4 and strain A3.4 at pH 3.0 (Fig. 4A-B).

Hydrophobicity and autoagglutination ability

The strain's capacity to adhere is a good indicator of its capacity to colonize, and to some extent, hydrophobicity and autoagglutination are indicators of the strain's adherence. The hydrophobicity of the isolates ranged from 19.17% to 52.83% (Fig. 5A), and the autoagglutination ability of the different strains was high, between 67.43% and 85.88% (Fig. 5B). The highest hydrophobicity and autoagglutination ability of A3.4 were found among all strains, at 52.83 ± 1.10% and 85.88 ± 0.18%, respectively, which were distinctly different from those of the other strains ($^{*}p < 0.05$).

Antioxidant activity

The antioxidant capacity of strains is an essential element in assessing the beneficial potential of probiotics (Zeng et al. 2022). According to our findings, the DPPH radical scavenging ability of the four strains was generally high, ranging from 85.36–67.32% (Fig. 6A), while the hydroxyl radical scavenging ability was low, ranging from 29.77% to 18.90% (Fig. 6B); the reduction power varied considerably, ranging from 22.22–42.71% (Fig. 6C). Comparatively, the antioxidant capacity assay indicated that A3.4 had the greatest reduction power, which was significantly different from that of A4, A1.2 and B1.9 ($^{*}p < 0.001$). Moreover, A3.4 possessed a greater hydroxyl radical scavenging ability than did A1.2 and B1.9 ($^{*}p < 0.001$).

Antibiotic susceptibility test

All the isolates were sensitive to oxacillin, florfenicol and cefuroxime and exhibited high or moderate sensitivity to amoxicillin, carbenicillin, piperacillin, doxycycline, ceftazidime and cefoperazone (Table 1). However, A4 was resistant to gentamicin, and A3.4 was resistant to neomycin. At the same time, A1.2 was

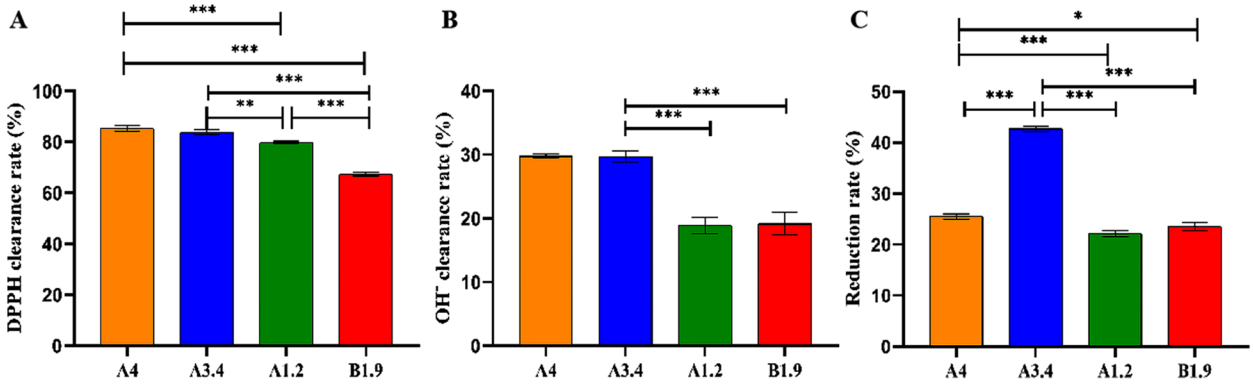

Fig. 6 Antioxidant activity of the strains: **A** DPPH clearance rate results of the isolates; **B** OH^- clearance rate of the isolates; **C** Reduction rate of the isolates, ***$p < 0.001$

resistant to gentamicin, ceftazidime and ofloxacin, and B1.9 was resistant to neomycin and cefadroxil. Generally, the isolates exhibited sensitivity to most of the standard drugs.

In vitro antibacterial tests

The inhibition effect of these isolates was evaluated based on the diameter of the zone of inhibition from the isolated strains against the indicator pathogens. All the isolates showed antibacterial activity against five pathogens, in which A3.4 showed antibacterial activity against *Pasteurella multocida* with an inhibition diameter of 22.71 ± 0.75 mm. In addition, B1.9 showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes,* with inhibition diameters of 12.533 ± 0.361 mm, 14.30 ± 0.106 mm, and 14.833 ± 0.064 mm, respectively (Fig. 7).

Table 1 Antibiotic drug sensitivity test of the isolates

Antibiotics	A4	A3.4	A1.2	B1.9
Amoxicillin	S	S	I	S
Oxacillin	S	S	S	S
Carbenicillin	S	I	I	S
Piperacillin	S	I	S	S
Neomycin	I	R	S	R
Gentamicin	R	S	R	S
Florfenicol	S	S	S	S
Doxycycline	S	S	I	S
Cephalexin	I	I	S	R
Cefradine	S	S	R	I
Ceftazidime	I	I	S	I
Cefuroxime	S	S	S	S
Cefoperazone	S	S	I	I
Ofloxacin	I	I	R	I

Notes: *S* sensitive, *R* resistant, *I* moderately sensitive. Results refer to the latest CLSI standeres

Animal safety study

During the experimental period, no mortality, disease, or diarrhea was observed in either group of mice. There was no significant difference in weight gain between the probiotic group and the control group (Fig. 8 left). Additionally, the organ indices in the probiotic group showed an increase of spleen index (Fig. 8 right), but no significant difference in liver index (Fig. 8 middle), compared to the control group.

Histopathological analysis

After H&E staining of the jejunum from both groups, the microphotographs were observed under a microscope. The probiotic and control groups showed no signs of inflammation or pathological damage, and the structures were clear and undamaged (Fig. 9).

Discussion

The use of antibiotics in agriculture has risen sharply due to the increased global demand for meat (Manyi-Loh et al. 2018). However, the irrational surge in antibiotic use has led to the emergence of multiple antibiotic-resistant pathogens that can penetrate humans and cause disease (Baran et al. 2023). Previous studies have shown that antibiotics can protect against pathogenic bacteria and suppress intestinal inflammation during an animal's growth period, improving nutrient absorption and an animal's overall health (Khan et al. 2020). However, the misuse of antibiotics can lead to intestinal diseases (Silverman et al. 2017).

Probiotics are known to be living microorganisms and are commonly used as feed additives. Probiotics not only promote the growth of host animals but also inhibit the multiplication of harmful pathogens and are favorable for host animal health by regulating the composition of the intestinal microflora and promoting nutrient absorption (Deng et al.

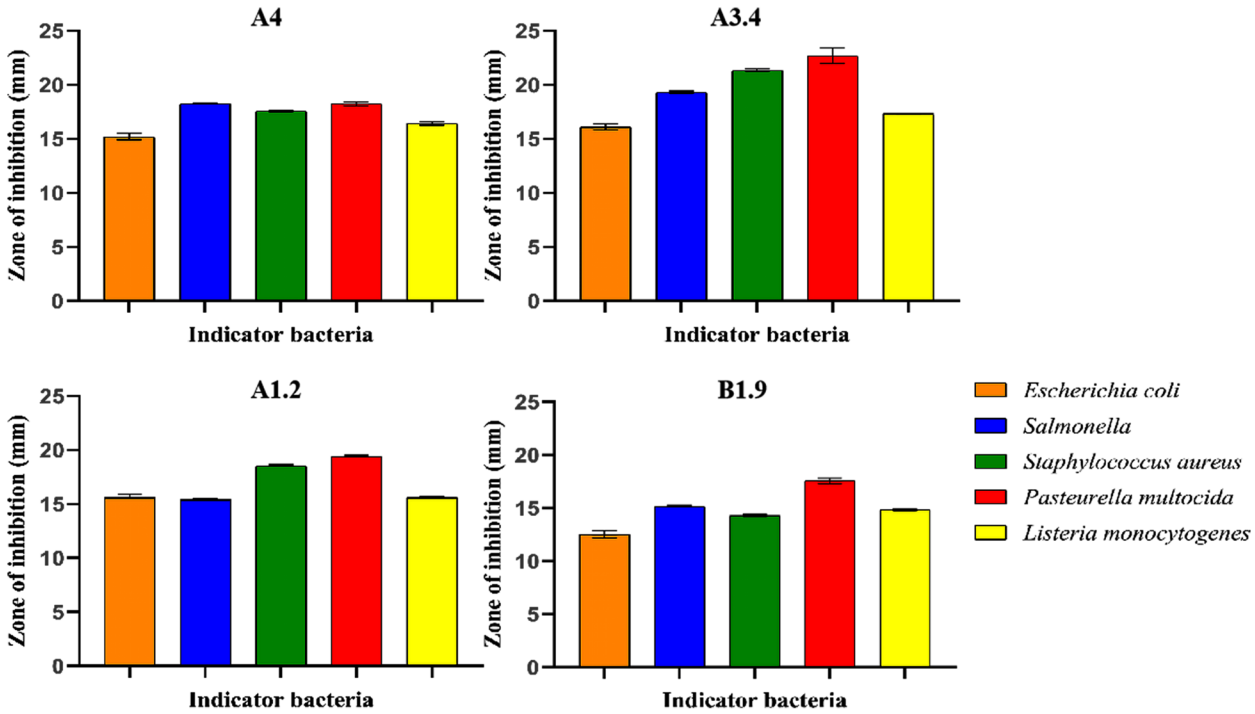

Fig. 7 Antibacterial test results

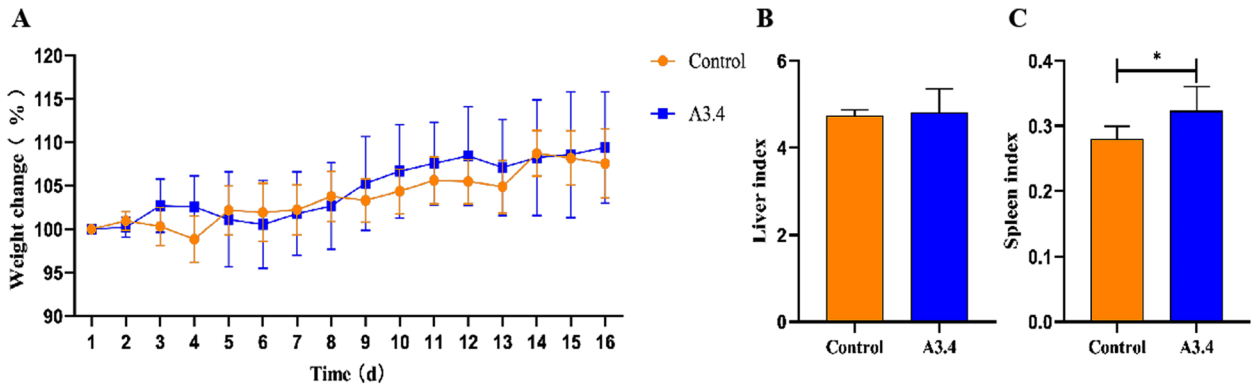

Fig. 8 Weight change and organ index results. The values represented as mean ± SD ($n = 10$). *$p < 0.05$

2021). Therefore, probiotics are treated as an alternative to antibiotics due to their unique characteristics (Goldenberg et al. 2017). In this study, we isolated A4 (*L. fermentum*), B1.9 (*P. acidilactici*), A3.4 and A1.2 (*P. pentosaceus*) from the gastrointestinal tissues and contents of yaks. We evaluated the probiotic potential of these isolated LAB strains by in vitro testing.

Probiotics can tolerate the adverse conditions of low pH and bile salts to survive and develop physiological activity in the GI tract (Ren et al. 2014). Therefore, assessing the bile tolerance and acid tolerance of LAB strains in screening for potential probiotics is essential. For successful influence and colonization of the host's small intestine, potential probiotic strains must be able to tolerate the low pH environment of the stomach (approximately 2.0–3.0). After the ingestion of food, the pH of gastric juice is usually 3.0, while the density of bile in the small intestine is typically between 0.1 and 0.3% (Liu et al. 2013; Zeng et al. 2022). In the present study, we assessed the tolerance of LAB strains isolated from yak intestines at pH 3.0 and 0.3% bile salt conditions. The results indicated that A4 exhibited greater tolerance to the bile salt

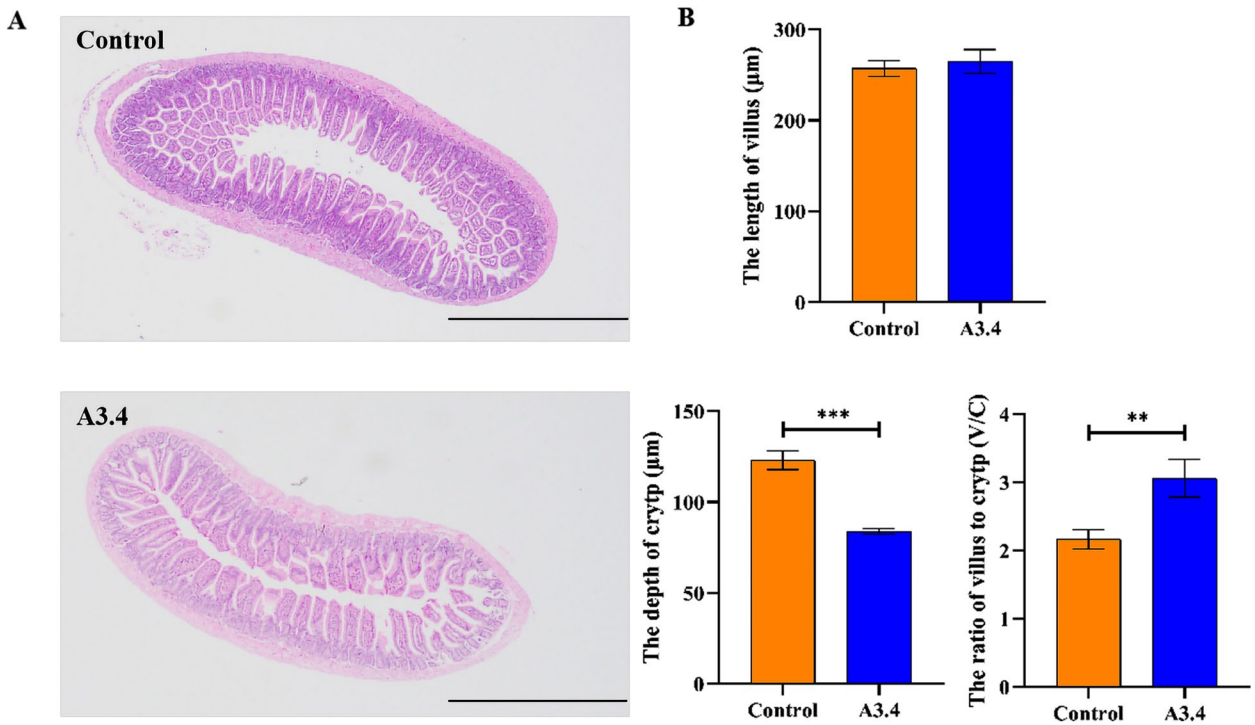

Fig. 9 Histological sections of the jejunum: **A** Histological sections of jejunum; **B** The length of villus and depth of crypt. The values are represented as mean ± SD ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.001$. Bar means 1 mm

environment, A3.4 exhibited greater tolerance to the acidic environment, and B1.9 exhibited the least tolerance under both conditions. The isolates survived in bile salts and acidic environments, which was consistent with the results of Ren et al. (2014).

The ability to hydrophobically and autoagglutinate are crucial markers for determining whether probiotics have the potential to colonize the digestive tract (He et al. 2022). Self-agglutination, which aids probiotics in adhering to and colonizing host enterocytes, is the most critical step in biofilm production. Conversely, autoaggregation allows probiotics to form a barrier that effectively prevents intestinal pathogens from adhering to enterocytes (Grilli et al. 2019; Danchik and Casadevall 2020). Among all the strains, A3.4 had the greatest hydrophobicity and autoagglutination ability (52.83 ± 1.10% and 85.89 ± 0.18%, respectively), which were distinct from those of the other strains. B1.9 had the lowest hydrophobicity and autoagglutination ability. The evolutionary tree results indicated that A3.4 and A1.2 were most likely *P. pentosaceus*, while B1.9 and A4 were more likely *P. acidilactici* and *L. fermentum*, respectively. Previous research has shown that *P. pentosaceus* ameliorates nonalcoholic fatty liver disease (NAFLD) by modulating the intestinal macrogenome and metabolic environment of mice (Yu et al. 2021). In addition, prior studies have shown that various strains of *P. pentosaceus* have anti-inflammatory, anticancer, antioxidant, detoxification, and lipid-lowering effects (Chung et al. 2021; Jiang et al. 2021; Li et al. 2021; Dong et al. 2022). Notably, in line with our findings, there were indications of encouraging adherence to *P. pentosaceus* (Kim et al. 2021).

Previous studies have suggested that strain-specific probiotics can exhibit antioxidant activity and reduce damage caused by oxidation (Wang et al. 2017). Specifically, *L. fermentum* has been speculated to contain antioxidants in vitro, inhibiting the oxidative effects of fermentation (Chen et al. 2015a). Additionally, *P. pentosaceus* has been proven to be a promising probiotic because of its antioxidant capacity through the activation of the Nrf2-Keap1 antioxidant signaling pathway (Wang et al. 2022). In our study, *P. pentosaceus* (A3.4) had the greatest DPPH scavenging ability, hydroxyl scavenging activity, and reduction power, which were significantly different from those of A1.2 and B1, which was consistent with the results of previous studies (Son et al. 2018; Diguță et al. 2020). Although A3.4 and A1.2 were both *P. pentosaceus*, the antioxidant capacity of A3.4 was much greater than that of A1.2, which may be due to the strain specificity of probiotics (Jankiewicz et al. 2023). In addition, *P. acidilactici* and *L. fermentum* (B1.9 and A4)

showed varying antioxidant activities, consistent with the findings of Mohammadi et al. (2022).

The yak (Bos grunniens) is an emblematic symbol of the Tibetan Plateau at high altitudes, yet diarrhea is a common disease in yaks that causes significant economic losses in the Tibetan Plateau region (Gao et al. 2013; Chen et al. 2015b). It has been demonstrated that supplementation with *Lactobacillus* not only accurately promotes intestinal microflora proliferation in mice but also relieves diarrhea, which might be regulated by the richness and composition of the intestinal flora (Dong et al. 2020). Previous studies have confirmed that *Lactobacillus*-produced antibacterial substances inhibit the growth of pathogenic bacteria, indicating its potential as a feed additive (Wang et al. 2018; Lin and Pan 2019; Monteiro et al. 2019). In line with the aforementioned results, the investigation showed that the isolates exhibited distinct antibacterial activity against five pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella*, and *Pasteurell*), with A3.4 (*P. pentosaceus*) demonstrating the strongest antibacterial activity (Reuben et al. 2019; Saboori et al. 2022).

The safety and probiotic properties of probiotics are equally important (Lara-Villoslada et al. 2007). Two approaches, an in vitro hemolysis test and an in vivo mouse experiment, were used to assess the safety of probiotics in this study. The results suggested that the isolates showed γ-hemolysis with no zone effect, which was consistent with the results of Sathiyaseelan et al. (2022). We also investigated the safety of the isolates via a mouse experiment. In the current study, the experimental group had no abnormal physical condition during the infusion of probiotics, and there were no significant lesions in the organs, which was in line with the findings of Sandera's study (2010).

Furthermore, regarding body weight, body weight in the probiotic group was distinctly greater than that in the control group. However, there was no significant difference in the liver index between the probiotic group and the control group, while the spleen index increased. Moreover, the depth of the villus and jejunum was also measured, which is frequently utilized as an indicator of intestinal mucosal barrier function or to reflect intestinal health status (Wang et al. 2019). The ratio of villus length to crypt depth (V/C) was greater in the probiotic group than in the control group, which was consistent with the findings of Zeng et al. (2022). Therefore, the aforementioned findings show that probiotics may strengthen the structure of the jejunal mucosa and improve the body's capacity to digest and absorb nutrients.

Conclusion

In summary, the probiotic potential of *Pediococcus pentosaceus* A3.4 was better than that of the other three isolates in many aspects according to a series of in vitro experiments. Additionally, it was shown that *Pediococcus pentosaceus* A3.4 was extremely safe, that it enhanced the growth of mice and that it had a favorable impact on intestinal development; as a result, it may be investigated further as a LAB with exceptional probiotic potential.

Methods

Isolation and identification of isolates

Samples of intestinal tissues and contents from healthy yaks were collected randomly in Tibet, China, transported in ice boxes to Huazhong Agricultural University in Wuhan and stored at -80°C for subsequent experiments.

The thawed samples (0.5 g) were weighed, and approximately 7 mL of sterile phosphate-buffered saline (PBS) was added, followed by 3 h in a 37°C shaker. The supernatant (100 μL) was aspirated in triplicate on Deman, Rogosa and Sharp (MRS) agar (Qingdao Reagent Company, China) and incubated at 37°C for 24 h. Milky white suspicious colonies were purified by selection and incubated on MRS agar plates three times until individual colonies were homogeneous in morphology. All suspicious strains were identified by morphological characterization and Gram staining.

16S rRNA sequencing technology was used to identify the isolates. First, the DNA of the isolates was extracted by bacterial DNA extraction kits (Aidlab Biotech DN11, China), and then 16S rRNA sequencing technology was employed to identify the isolates (Qin et al. 2022). Finally, MEGA V. 7.0 software was used to analyze the 16S rRNA results and construct a phylogenetic tree.

Growth curves of the isolates

The culture solutions of the isolates were added to MRS broth at 2% (*v*/*v*) and incubated at 37°C in a shaker at 150 r/min. The OD was measured every 2 h at 600 nm with a spectrophotometer for 24 h.

Acid and bile salt tolerance of the isolates

Acid tolerance measurement: The strain cultures were inoculated with 2% (*v*/*v*) inoculum in MRS broth at pH = 3.0 and in regular MRS broth. After 3 h, the absorbance was determined at 600 nm by using a spectrophotometer. Survival rates were calculated as follows, and the test was repeated three times:

$$Survival\ rates\ (\%) = \frac{\text{OD600 of the Experimental group}}{\text{OD600oftheControlgroup}} \times 100\%$$

Bile salt resistance test: The strain culture was inoculated at 2% (*v*/*v*) inoculum in MRS broth containing 0.3% bile salt and in regular MRS broth. After 3 h, the absorbance was measured at 600 nm by using a spectrophotometer. The calculation method above and the test were repeated three times.

Hydrophobicity and autoagglutination capabilities of the isolates

For the hydrophobicity assay, the isolates cultured to the stable growth phase were centrifuged at 8000 r/min for 10 min, after which the bacteria were collected. After washing twice with PBS solution, the bacteria were resuspended in PBS solution. The absorbance of the strain suspension was adjusted to 1.0 ± 0.05 at 600 nm and recorded as A0. Three milliliters of the strain suspension was mixed with 1 mL of xylene, shaken in a vortex mixer for 2 min, and then kept at 37°C for 30 min. The aqueous phase was slowly aspirated, and its absorbance (A1) was measured at 600 nm. The whole process was repeated three times. The hydrophobicity (%) of the strain was calculated as follows:

$$Hydrophobicity\ (\%) = (1 - \frac{\mathrm{A1}}{\mathrm{A0}}) \times 100\%$$

In the autoaggregation assay, the isolates were pretreated as described above, and the absorbance of the suspension was measured at 600 nm (B0). After 2 mL of each isolate was shaken for 10 s on a vortex and left for 4 h, the absorbance of the supernatant was calculated (B1). The assay was repeated three times. The autoaggregation (%) of the strains was calculated based on the following equation:

$$Auto-aggregation\ (\%) = (1 - \frac{\mathrm{B1}}{\mathrm{B0}}) \times 100\%$$

Antioxidant activity of the isolates

The isolates were pretreated as described above (hydrophobicity and autoagglutination capabilities of the isolates).

DPPH radical scavenging ability assay

The experimental course was based on the approach of Qin et al. (2022). The calculation was performed using the following formula: $DPPH\ radical\ scavenging\ rate\ (\%) = \frac{A0-A1}{A0} \times 100\%$. A1 and A0 are the absorbance values of each post-processed sample at 517 nm and a control group consisting of ultrapure water and an ethanolic solution of DPPH at 517 nm, respectively.

Hydroxyl radical scavenging activity assay

The experimental course was based on the approach of Qin (Qin et al. 2022). The calculation was executed using the following formula: $hydroxyl\ radical\ scavenging\ rate\ (\%) = \frac{B0-B1}{B0} \times 100\%$. B1 and B0 are the absorbance values of each processed sample at OD_{510} nm and the control group (ddH_2O instead of the bacterial suspension) at OD_{510} nm, respectively.

Reducing power assay

The procedure was based on the method of Tailb with minor modifications (Talib et al. 2019). Calculations were performed using the following formula: $Reducing\ rate\ (\%) = \frac{A0-A1}{A0} \times 100\%$. A1 was the absorbance value of each processed sample at OD_{700} nm, while A0 was the absorbance value of the control group (deionized water instead of the bacterial suspension) at OD_{700} nm.

Antibiotic susceptibility of the isolates

The test was performed using the Kirby-Bauer disc agar diffusion method (K-B method) recommended by the Clinical and Laboratory Standards Institute (CLSI) (Baran et al. 2023). The isolates (1×10^8 CFU/mL) were streaked onto MRS agar plates with sterile cotton swabs. The plates were incubated at 37°C for 24 h, after which the inhibition circle diameter was measured. The types of antibiotics used were amoxicillin (20 μg), carbenicillin (100 μg), piperacillin (100 μg), oxacillin (1 μg), neomycin (30 μg), gentamicin (10 μg), florfenicol (30 μg), doxycycline (30 μg), cephalexin (30 μg), cefradine (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), cefoperazone (75 μg) and ofloxacin (5 μg).

In vitro antibacterial tests

The indicator pathogens were *Escherichia coli* (*E. coli* ATCC 25922), *Salmonella enteritidis* (*S. enteritidis* NCTC 13349), *Staphylococcus aureus* (*S. aureus* ATCC 26112), and *Listeria monocytogenes* (clinical isolates) provided by the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, Hubei. *P. multocida* (*P. multocida* CUCC1764) was obtained from the College of Animals Husbandry and Veterinary Medicine, Tibet Agricultural and Animal Husbandry University, Linzhi, Tibet.

The freshly cultured indicator bacteria were applied onto LB agar plates with sterile cotton swabs. Then, the hole (diameter of 5 mm) was punctured with an aseptic perforator. Then, 100 μL of freshly cultured supernatant from the isolates was added to the wells and kept in an incubator at 37°C for 12 h. Finally, the diameters of the zones of inhibition were measured.

Hemolytic evaluation of the isolates

The freshly cultured bacterial solutions were streaked onto blood agar plates containing 5% sheep blood. Then, the plates were incubated at 37℃ for 24 h, and *S. aureus* was used as a positive control.

Animal safety assessment for A3.4

The mouse model was used for the animal safety assessment of A3.4. Briefly, 20 male C57BL/6 mice (18 ± 2 g) were housed under standard hygienic conditions with constant temperature (22 ± 2 °C), constant humidity (55 ± 2%), and a 12/12 h light–dark cycle. The animals were allowed a standard diet and free access to drinking water for the experimental period. After three days of acclimatization, the mice were randomly divided into control and probiotic groups ($n = 10$). Then, mice in the probiotic group were supplemented with 0.4 mL (1×10^9 CFU/mL) of A3.4 for 19 consecutive days, while the mice in the control group were provided with the same volume of normal saline instead. During the feeding process, the mental status, health condition, food intake, and diarrhea status of the mice were observed daily, and the weight of each mouse was recorded. After 19 d of probiotic gavage, all the mice were culled by cervical dislocation, and liver, spleen, jejunum, and blood samples were collected under sterile conditions.

All the experiments were approved and reviewed by the Institutional Animal Welfare and Research Ethics Committee guidelines of Huazhong Agricultural University, Wuhan, China (HZAUMO-2023-0276).

Histopathological analysis of the jejunum

The jejunal samples were washed in saline and stored in 4% paraformaldehyde. Then, the samples were dehydrated in various ethanol concentrations, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin-eosin (H&E) (He et al. 2022). Finally, the sections were observed under a microscope.

Statistical analysis

All data in the current study were statistically analyzed by MEGA V. 7.0 software and plotted using GraphPad Prism V. 8 software, and the data are shown as the mean ± standard deviation (SD) and were analyzed via t tests. Compared to the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Abbreviations

LAB	Lactic acid bacteria
AAD	Antibiotic-associated diarrhea
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
NAFLD	Nonalcoholic fatty liver disease

Acknowledgements

We would like to thank Pro. Li for laboratory assistance and Tibet Agricultural and Animal Husbandry University for sample availability.

Authors' contributions

YH and FL conceived and designed the experiments. YH, FL, MX, CJ, YZ, and ZQ contributed to sample collection and reagent preparation. SN, MFK, and MI revised the manuscript. YH wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Key Project of Tibet Autonomous Region (XZ202001ZY0044N; XZ202101ZD0002N-05).

Availability of data and materials

Not applicable.

Declarations

Ethics approval

All animal experiment protocols conformed to the rules of the National Guidelines for Housing and Care of Laboratory Animals (China) and were performed after obtaining approval from the Institutional Animal Care and Ethics Committee of Huazhong Agricultural University (HZAUMO-2023-0276).

Competing interests

The authors declare no competing interests.

Received: 24 October 2023 Accepted: 29 April 2024
Published online: 22 May 2024

References

Baran, A., A. Kwiatkowska, and L. Potocki. 2023. Antibiotics and bacterial resistance-A short story of an endless arms race. *International Journal of Molecular Sciences* 24: 5777. https://doi.org/10.3390/ijms24065777.

Brüssow, H. 2017. Adjuncts and alternatives in the time of antibiotic resistance and in-feed antibiotic bans. *Microbial Biotechnology* 10: 674–677. https://doi.org/10.1111/1751-7915.12730.

Cabello, F.C., and H.P. Godfrey. 2016. Even therapeutic antimicrobial use in animal husbandry may generate environmental hazards to human health. *Environmental Microbiology* 18: 311–313. https://doi.org/10.1111/1462-2920.13247.

Chen, Q., B. Kong, Q. Sun, F. Dong, and Q. Liu. 2015a. Antioxidant potential of a unique LAB culture isolated from Harbin dry sausage: In vitro and in a sausage model. *Meat Science* 110: 180–188. https://doi.org/10.1016/j.meatsci.2015.07.021.

Chen, X., B. Zhang, H. Yue, Y. Wang, F. Zhou, Q. Zhang, and C. Tang. 2015b. A novel astrovirus species in the gut of yaks with diarrhea in the Qinghai-Tibetan Plateau, 2013. *The Journal of General Virology* 96: 3672–3680. https://doi.org/10.1099/jgv.0.000303.

Chung, Y., Y. Ryu, B.C. An, Y.-S. Yoon, O. Choi, T.Y. Kim, J. Yoon, B.C. Ahn, J.H. Park, S.K. Kwon, J.F. Kim, and M.J. Chung. 2021. A synthetic probiotic engineered for colorectal cancer therapy modulates gut microbiota. *Microbiome* 9: 122. https://doi.org/10.1186/s40168-021-01071-4.

Danchik, C., and A. Casadevall. 2020. Role of cell surface hydrophobicity in the pathogenesis of medically significant fungi. *Frontiers in Cellular and Infection Microbiology* 10: 594973. https://doi.org/10.3389/fcimb.2020.594973.

Deng, Z., K. Hou, J. Zhao, and H. Wang. 2021. The probiotic properties of lactic acid bacteria and their applications in animal husbandry. *Current Microbiology* 79: 22. https://doi.org/10.1007/s00284-021-02722-3.

Diguță, C.F., G.D. Nițoi, F. Matei, G. Luță, and C.P. Cornea. 2020. The biotechnological potential of *Pediococcus* spp. isolated from Kombucha Microbial Consortium. *Foods* 9: 1780. https://doi.org/10.3390/foods9121780.

Dong, H., B. Liu, A. Li, M. Iqbal, K. Mehmood, T. Jamil, Y.F. Chang, H. Zhang, and Q. Wu. 2020. Microbiome analysis reveals the attenuation effect of *Lactobacillus* from yaks on diarrhea via modulation of gut microbiota.

Frontiers Cellular and Infection Microbiology 10: 610781. https://doi.org/10.3389/fcimb.2020.610781.

Dong, F., F. Xiao, X. Li, Y. Li, X. Wang, G. Yu, T. Zhang, and Y. Wang. 2022. Pediococcus pentosaceus CECT 8330 protects DSS-induced colitis and regulates the intestinal microbiota and immune responses in mice. *Journal of Translational Medicine* 20: 33. https://doi.org/10.1186/s12967-022-03235-8.

Gao, J., M. Liu, X. Meng, Z. Han, D. Zhang, B. Hou, K. Zhang, S. Sizhu, and J. Li. 2013. Seroprevalence of bovine viral diarrhea infection in Yaks (Bos grunniens) on the Qinghai-Tibetan Plateau of China. *Tropical Animal Health and Production* 45: 791–793. https://doi.org/10.1007/s11250-012-0290-2.

Goldenberg, J.Z., C. Yap, L. Lytvyn, C.K.-F. Lo, J. Beardsley, D. Mertz, and B. Johnston. 2017. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. *The Cochrane Database of Systematic Reviews* 12: CD006095. https://doi.org/10.1002/14651858.CD006095.pub4.

Gomez, D.E., L.G. Arroyo, M.C. Costa, L. Viel, and J.S. Weese. 2017. Characterization of the fecal bacterial microbiota of healthy and diarrheic dairy calves. *Journal of Veterinary Internal Medicine* 31: 928–939. https://doi.org/10.1111/jvim.14695.

Graham, J.P., J.H. Leibler, L.B. Price, J.M. Otte, D.U. Pfeiffer, T. Tiensin, and E.K. Silbergeld. 2008. The animal-human interface and infectious disease in industrial food animal production: Rethinking biosecurity and biocontainment. *Public Health Reports* 123: 282–299. https://doi.org/10.1177/003335490812300309.

Grilli, D.J., M.E. Mansilla, M.C. Giménez, N. Sohaefer, M.S. Ruiz, M.R. Terebiznik, M. Sosa, and G.N. Arenas. 2019. Pseudobutyrivibrio xylanivorans adhesion to epithelial cells. *Anaerobe* 56: 1–7. https://doi.org/10.1016/j.anaerobe.2019.01.001.

He, Y., F. Li, W. Zhang, M. An, A. Li, Y. Wang, Y. Zhang, M.F. Kulyar, M. Iqbal, and J. Li. 2022. Probiotic potential of Bacillus amyloliquefaciens isolated from Tibetan yaks. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-022-10027-5.

Jankiewicz, M., J. Łukasik, M. Kotowska, M. Kołodziej, and H. Szajewska. 2023. Strain-specificity of probiotics in pediatrics: A rapid review of the clinical evidence. *Journal of Pediatric Gastroenterology and Nutrition* 76: 227–231. https://doi.org/10.1097/MPG.0000000000003675.

Jiang, S., L. Cai, L. Lv, and L. Li. 2021. Pediococcus pentosaceus, a future additive or probiotic candidate. *Microbial Cell Factories* 20: 45. https://doi.org/10.1186/s12934-021-01537-y.

Khan, F., D.T.N. Pham, and Y.M. Kim. 2020. Alternative strategies for the application of aminoglycoside antibiotics against the biofilm-forming human pathogenic bacteria. *Applied Microbiology and Biotechnology* 104: 1955–1976. https://doi.org/10.1007/s00253-020-10360-1.

Kim, S., S.-P. Hong, and S.D. Lim. 2021. Physiological characteristics and antidiabetic effect of *Pediococcus pentosaceus* KI62. *Food Science of Animal Resources* 41: 274–287. https://doi.org/10.5851/kosfa.2020.e99.

Lara-Villoslada, F., S. Sierra, R. Martín, S. Delgado, J.M. Rodríguez, M. Olivares, and J. Xaus. 2007. Safety assessment of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714. *Journal of Applied Microbiology* 103: 175–184. https://doi.org/10.1111/j.1365-2672.2006.03225.x.

Leibler, J.H., K. Dalton, A. Pekosz, G.C. Gray, and E.K. Silbergeld. 2017. Epizootics in industrial livestock production: Preventable gaps in biosecurity and biocontainment. *Zoonoses and Public Health* 64: 137–145. https://doi.org/10.1111/zph.12292.

Li, H., X. Xie, Y. Li, M. Chen, L. Xue, J. Wang, J.M. Zhang, S. Wu, Q.H. Ye, S.T. Zhang, R.S. Yang, H. Zhao, L. Wu, T.T. Liang, Y. Ding, and Q.P. Wu. 2021. *Pediococcus pentosaceus* IM96 exerts protective effects against enterohemorrhagic *Escherichia coli* O157:H7 infection in vivo. *Foods* 10: 2945. https://doi.org/10.3390/foods10122945.

Lim, S.M., N.K. Lee, K.T. Kim, and H.D. Paik. 2020. Probiotic *Lactobacillus fermentum* KU200060 isolated from watery kimchi and its application in probiotic yogurt for oral health. *Microbial Pathogenesis* 147: 104430. https://doi.org/10.1016/j.micpath.2020.104430.

Lin, T.H., and T.M. Pan. 2019. Characterization of an antimicrobial substance produced by *Lactobacillus plantarum* NTU 102. *Journal of Microbiology Immunology Infection* 52: 409–417. https://doi.org/10.1016/j.jmii.2017.08.003.

Liu, W.J., Y.F. Chen, L.Y. Kwok, M.H. Li, T. Sun, C.L. Sun, X.N. Wang, T. Dan, H.P. Zhang, and T.S. Sun. 2013. Preliminary selection for potential probiotic *Bifidobacterium* isolated from subjects of different Chinese ethnic groups and evaluation of their fermentation and storage characteristics in bovine milk. *Journal of Dairy Science* 96: 6807–6817. https://doi.org/10.3168/jds.2013-6582.

Low, C.X., L.T.H. Tan, N.S. Ab Mutalib, P. Pusparajah, B.H. Goh, K.G. Chan, V. Letchumanan, and L.H. Lee. 2021. Unveiling the impact of antibiotics and alternative methods for animal husbandry: A review. *Antibiotics (Basel)* 10: 578. https://doi.org/10.3390/antibiotics10050578.

Manyi-Loh, C., S. Mamphweli, E. Meyer, and A. Okoh. 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: Potential public health implications. *Molecules* 23: 795. https://doi.org/10.3390/molecules23040795.

Mohammadi, G., M. Hafezieh, A.A. Karimi, M.N. Azra, H. Van Doan, W. Tapingkae, H. Abdelrahman, and M. Dawood. 2022. The synergistic effects of plant polysaccharide and *Pediococcus acidilactici* as a synbiotic additive on growth, antioxidant status, immune response, and resistance of Nile tilapia (Oreochromis niloticus) against Aeromonas hydrophila. *Fish Shellfish Immunology* 120: 304–313. https://doi.org/10.1016/j.fsi.2021.11.028.

Monteiro, C.R.A.V., M.S. do Carmo, B.O. Melo, M.S. Alves, C.I. Dos Santos, S.G. Monteiro, M.R.Q. Bomfim, E.S. Fernandes, and V.M. Neto. 2019. In vitro antimicrobial activity and probiotic potential of *Bifidobacterium* and *Lactobacillus* against species of Clostridium. *Nutrients* 11: 448. https://doi.org/10.3390/nu11020448.

Oliver, S.P., S.E. Murinda, and B.M. Jayarao. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. *Foodborne Pathogens and Disease* 8: 337–355. https://doi.org/10.1089/fpd.2010.0730.

Phillips, I. 2007. Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *International Journal of Antimicrobial Agents* 30: 101–107. https://doi.org/10.1016/j.ijantimicag.2007.02.018.

Pridmore, R.D., A.C. Pittet, F. Praplan, and C. Cavadini. 2008. Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-Salmonella activity. *FEMS Microbiology Letters* 283: 210–215. https://doi.org/10.1111/j.1574-6968.2008.01176.x.

Qin, S., Z. Huang, Y. Wang, L. Pei, and Y. Shen. 2022. Probiotic potential of *Lactobacillus* isolated from horses and its therapeutic effect on DSS-induced colitis in mice. *Microbial Pathogensis* 165: 105216. https://doi.org/10.1016/j.micpath.2021.105216.

Ren, D., C. Li, Y. Qin, R. Yin, S. Du, F. Ye, C.X. Liu, H.F. Liu, M.P. Wang, Y. Li, Y. Sun, X. Li, M.Y. Tian, and N.Y. Jin. 2014. In vitro evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. *Anaerobe* 30: 1–10. https://doi.org/10.1016/j.anaerobe.2014.07.004.

Reuben, R.C., P.C. Roy, S.L. Sarkar, R.-U. Alam, and I.K. Jahid. 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiology* 19: 253. https://doi.org/10.1186/s12866-019-1626-0.

Robinson, T.P., D.P. Bu, J. Carrique-Mas, E.M. Fèvre, M. Gilbert, D. Grace, S.I. Hay, J. Jiwakanon, M. Kakkar, S. Kariuki, et al. 2016. Antibiotic resistance is the quintessential One Health issue. *Transaction of the Royal Society of Tropical Medicine and Hygiene* 110: 377–380. https://doi.org/10.1093/trstmh/trw048.

Saboori, B., F. Shahidi, S. Hedayati, and A. Javadmanesh. 2022. Investigating the probiotic properties and antimicrobial activity of lactic acid bacteria isolated from an Iranian fermented dairy product, kashk. *Foods* 11: 3904. https://doi.org/10.3390/foods11233904.

Sanders, M.E., L.M.A. Akkermans, D. Haller, C. Hammerman, J. Heimbach, G. Hörmannsperger, G. Huys, D.D. Levy, F. Lutgendorff, D. Mack, et al. 2010. Safety assessment of probiotics for human use. *Gut Microbes* 1: 164–185. https://doi.org/10.4161/gmic.1.3.12127.

Sathiyaseelan, A., K. Saravanakumar, K. Han, K.V. Naveen, and M.H. Wang. 2022. Antioxidant and antibacterial effects of potential probiotics isolated from Korean fermented foods. *International Journal of Molecular Sciences* 23: 10062. https://doi.org/10.3390/ijms231710062.

Silverman, M.A., L. Konnikova, and J.S. Gerber. 2017. Impact of antibiotics on necrotizing enterocolitis and antibiotic-associated diarrhea. *Gastroenterology Clinics of North America* 46: 61–76. https://doi.org/10.1016/j.gtc.2016.09.010.

Slama, T.G., A. Amin, S.A. Brunton, T.M. File, G. Milkovich, K.A. Rodvold, D.F. Sahm, and J.D.W. Varon Jr. 2005. A clinician's guide to the appropriate and accurate use of antibiotics: The council for appropriate and rational

antibiotic therapy (CARAT) criteria. *The America Journal of Medicine* 118 (Suppl 7A): 1S-6S. https://doi.org/10.1016/j.amjmed.2005.05.007.
Son, S.H., S.J. Yang, H.L. Jeon, H.S. Yu, N.K. Lee, Y.S. Park, and H.D. Paik. 2018. Antioxidant and immunostimulatory effect of potential probiotic *Lactobacillus paraplantarum* SC61 isolated from Korean traditional fermented food, jangajji. *Microbial Pathogensis* 125: 486–492. https://doi.org/10.1016/j.micpath.2018.10.018.
Talib, N., N.E. Mohamad, S.K. Yeap, Y. Hussin, M.N.M. Aziz, M.J. Masarudin, S.A. Sharifuddin, Y.W. Hui, C.L. Ho, and N.B. Alitheen. 2019. Isolation and characterization of *Lactobacillus* spp. from kefir samples in Malaysia. *Molecules* 24: 2606. https://doi.org/10.3390/molecules24142606.
Wang, Y., Y. Wu, Y. Wang, H. Xu, X. Mei, D. Yu, Y. Wang, and W. Li. 2017. Antioxidant properties of probiotic bacteria. *Nutrients* 9: 521. https://doi.org/10.3390/nu9050521.
Wang, L., H. Zhang, M.U. Rehman, K. Mehmood, X. Jiang, M. Iqbal, X.L. Tong, X. Gao, and J.K. Li. 2018. Antibacterial activity of *Lactobacillus plantarum* isolated from Tibetan yaks. *Microbial Pathogensis* 115: 293–298. https://doi.org/10.1016/j.micpath.2017.12.077.
Wang, K., G. Cao, H. Zhang, Q. Li, and C. Yang. 2019. Effects of Clostridium butyricum and *Enterococcus faecalis* on growth performance, immune function, intestinal morphology, volatile fatty acids, and intestinal flora in a piglet model. *Food Function* 10: 7844–7854. https://doi.org/10.1039/c9fo01650c.
Wang, L., Q. Liu, Y. Chen, X. Zheng, C. Wang, Y. Qi, Y.C. Dong, Y. Xiao, C. Chen, T.H. Chen, et al. 2022. Antioxidant potential of *Pediococcus pentosaceus* strains from the sow milk bacterial collection in weaned piglets. *Microbiome* 10: 83. https://doi.org/10.1186/s40168-022-01278-z.
Wehkamp, J., J. Harder, K. Wehkamp, B. Wehkamp-von Meissner, M. Schlee, C. Enders, C. Sonnenborn, S. Nuding, S. Bengmark, K. Fellermann, J.M. Schroder, and E. Stange. 2004. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: A novel effect of a probiotic bacterium. *Infection and Immunity* 72: 5750–5758. https://doi.org/10.1128/IAI.72.10.5750-5758.2004.
Wu, Z., K. Yang, A. Zhang, W. Chang, A. Zheng, Z. Chen, Z. Chen, H. Cai, and G.H. Liu. 2021. Effects of *Lactobacillus acidophilus* on the growth performance, immune response, and intestinal barrier function of broiler chickens challenged with *Escherichia coli* O157. *Poultry Science* 100: 101323. https://doi.org/10.1016/j.psj.2021.101323.
Xin, J., D. Zeng, H. Wang, N. Sun, Y. Zhao, Y. Dan, K.C. Pan, B. Jing, and X.Q. Ni. 2020. Probiotic *Lactobacillus johnsonii* BS15 promotes growth performance, intestinal immunity, and gut microbiota in piglets. *Probiotics and Antimicrobial Proteins* 12: 184–193. https://doi.org/10.1007/s12602-018-9511-y.
Yadav, S., and R. Jha. 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. *Journal of Animal Science and Biotechnology* 10: 2. https://doi.org/10.1186/s40104-018-0310-9.
Yu, J.S., G.S. Youn, J. Choi, C.H. Kim, B.Y. Kim, S.J. Yang, J.H. Lee, T.S. Park, B.K. Kim, S.W. Roh, et al. 2021. *Lactobacillus lactis* and *Pediococcus pentosaceus*-driven reprogramming of gut microbiome and metabolome ameliorates the progression of nonalcoholic fatty liver disease. *Clinical Translational Medicine* 11: e634. https://doi.org/10.1002/ctm2.634.
Zeng, Z., J. Zhang, Y. Li, K. Li, S. Gong, F. Li, P.P. Wang, M. Iqbal, M.F. Kulyar, and J.K. Li. 2022. Probiotic potential of *Bacillus licheniformis* and *Bacillus pumilus* isolated from Tibetan yaks, China. *Probiotics and Antimicrobial Proteins* 14: 579–594. https://doi.org/10.1007/s12602-022-09939-z.
Zhong, Y., C. Zheng, J.H. Zheng, and S.C. Xu. 2020. The relationship between intestinal flora changes and osteoporosis in rats with inflammatory bowel disease and the improvement effect of probiotics. *European Review for Medical and Pharmacological Sciences* 24: 5697–5702. https://doi.org/10.26355/eurrev_202005_21361.

Publisher's Note