Article

Screening of *Lactobacillus plantarum* subsp. *plantarum* with potential probiotic activities for inhibiting ETEC K88

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Abstract: For screening excellent lactic acid bacteria (LAB) strains to inhibit *Escherichia* (*E.*) *coli* (ETEC) K88, inhibitory activities of more than 1100 LAB strains isolated from different materials and kept in the lab were evaluated in this study. Nine strains with inhibition zone at least 22.00 mm (including that of hole puncher 10.00 mm) and good physiological and biochemical characteristics identified by 16S DNA gene sequencing and *rec*A gene multiple detection, were assigned to *Lactobacillus* (*L.*) *plantarum* subsp. *plantarum* (5), *L. fermentum* (1), *L. reuteri* (1), *W. cibaria* (1) and *E. faecalis* (1), respectively. As investigated for their tolerance abilities and safety, only strain ZA3 possessed high hydrophobicity and auto-aggregation abilities, had high survival rate in low pH, bile salt environment and GI fluids, sensitive to ampicillin, resistant to norfloxacin and amikacin, without hemolytic activity and didn't carry antibiotic resistance genes, exhibited broad spectrum activity against a wide range of microorganisms, and antibacterial substance may attribute to organic acids, especially lactic acid and acetic acid. The results indicated that the selected strain *L. plantarum* subsp. *plantarum* ZA3 could be considered a potential probiotic to inhibit ETEC K88 for further research.

Keywords: *L. plantarum* subsp. *plantarum*; ETEC K88; antimicrobial; probiotics

1. Introduction

ETEC K88 is a pathogenic variant of *Escherichia (E.)* coli that colonizes the surface of gastrointestinal cells, when K88 infects the host and produces enterotoxins, which disrupt intestinal cellular electrolyte homeostasis, leading to fluid loss and ultimately to secretory diarrheal disease in newborns and piglets [1-3]. Post-weaning diarrhea (PWD) caused by ETEC K88 has a high mortality rate, while in China and the United States, the average annual mortality of PWD cases is as high as 15% and 15.5%, respectively, and the direct economic losses amount to more than 145 million US dollars [4]. Antibiotics have been long used to reduce diarrhea and enhance growth performance in weaned piglets, while long-term use of antibiotics caused the emergence of resistant strains of animal and food-borne pathogens, further intestinal microbial imbalance, according to announcement No. 194 of the Ministry of

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Agriculture and Rural Affairs, China officially stopped the production, import, operation and use of some pharmaceutical feed additives as of this 1st of July. Thus, it is necessary to find a new effective alternative to antibiotics for the treatment and prevention of bacterial infection diseases including diarrhea caused by ETEC K88.

It has been confirmed that probiotics are microorganisms that can have beneficial effects on host health, mainly through the production of antibacterial substances, competition with pathogenic bacteria for nutrients and adhesion sites and enhancing intestinal mucosal barrier integrity to play an immunomodulatory role against diarrhea in weaned piglets [5]. Lactic acid bacteria (LAB) are the most frequently used probiotics, and several studies conducted on newly weaned piglets have noted that LAB can increase the abundance of lactobacilli and bifidobacterial, inhibit colonization of ETEC K88/F4 and improve production of short-chain fatty acid [6]. Patil et al. [7] found *Lactobacillus (L.) sobrius* may be effective in the reduction of the *E. coli* F4 colonization and may improve the weight gain of infected piglets; Probiotic bacterium *L. rhamnosus* GG was effective in ameliorating PWD induced by *E. coli* K88, modulation of intestinal microflora and enhancement of intestinal antibody defense [8]. In conclusion, above research indicate that LAB have a good inhibitory effect on pathogenic bacteria in piglets, especially on intestinal imbalance caused by ETEC K88.

Excellent LAB strains should have characteristics of fast propagation, easy cultivation, strains to maintain a certain activity during production and storage, and a certain tolerance to processing technology in application; moreover, the unstable performance and high cost of LAB used in production are still a constraint to their application, and the excellent probiotic LAB are still urged to be discovered. This study evaluate the probiotic properties of the LAB strains isolated from different sources by determining their inhibitory activities to ETEC K88, and after physiological and biochemical characteristics, cell surface properties and tolerance to simulated human gastrointestinal (GI) tract and bile test, selected excellent strains were identified by 16S rRNA gene sequencing and *recA* gene multiple detection, strains that performed well in the probiotic test were then evaluated for safety, and the antimicrobial substances and organic acids produced by the fermentation of the strains were determined.

2. Results

2.1. Inhibitory Activities to ETEC K88 of LAB Strains Isolated from Different Sources

Table 1 showed inhibitory activities to ETEC K88 of LAB strains isolated from different sources. As can be seen, 40 strains (ZA1 to ZA40, Zhengzhou University Agricultural College. Strains were uniformly rebranded as the ZA series for better documentation and preservation.) with inhibition zone at least 18.00 mm (including that of hole puncher 10.00 mm, next same) were selected for further study. Among these strains, ZA2, ZA3, ZA7, ZA8, ZA10, ZA15, ZA18, ZA19, ZA24, ZA28, ZA30, ZA33 and ZA34 had more than 22.00 mm inhibition zone.

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Table 1. Antimicrobial activities to ETEC K88 of representative LAB isolates.

Isolates	Antimicrobial activity	Separation source	Isolates	Antimicrobial activity	Separation source
ZA1	+++	weaned piglet feces	ZA21	+++	rice silage
ZA2	++++		ZA22	+++	
ZA3	++++		ZA23	+++	
ZA4	+++		ZA24	++++	
ZA5	+++		ZA25	+++	
ZA6	+++		ZA26	+++	
ZA7	++++	feed grass	ZA27	+++	
ZA8	++++		ZA28	++++	
ZA9	+++		ZA29	+++	mixture silage
ZA10	++++		ZA30	++++	
ZA11	+++		ZA31	+++	
ZA12	+++		ZA32	+++	
ZA13	+++		ZA33	++++	
ZA14	+++		ZA34	++++	
ZA15	++++	Qula	ZA35	+++	
ZA16	+++		ZA36	+++	
ZA17	+++		ZA37	+++	corn silage
ZA18	++++	cassave silage	ZA38	+++	
ZA19	++++		ZA39	+++	
ZA20	+++		ZA40	+++	

Note: +, diameter of inhibition zone: 10.00-14.00 mm; ++, 14.00-18.00 mm; +++, 18.00-22.00 mm; ++++, more than 22.00 mm; -, no inhibition zone was detected; the diameter of inhibition zone including that of hole puncher (10.00 mm).

2.2. Physiological and Biochemical Characteristics of Selected LAB Isolates

Physiological and biochemical characteristics of selected LAB isolates are shown in table 2. All strains were able to grow in 3.0 and 6.5 (w/v, %) NaCl, and at pH 4.0, 4.5, 5.0, 5.5, 6.0, 8.0 and 9.0. Except ZA1, ZA2, ZA3, ZA8, ZA9, ZA10, ZA11, ZA18, ZA28, ZA29, ZA33, ZA34 and ZA40 could grow at 5°C, 50°C and pH 3.0, other strains couldn't. In addition, ZA6, ZA15, ZA18, ZA26, ZA27, ZA31, ZA32 and ZA33 could produce gas from glucose, while other isolates could not.

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Table 2. Physiological and biochemical characteristics of selected LAB isolates.

Isolates	Growth at temperature (°C)				Growth in NaCl (w/v, %)		Growth at pH									Fermentation type
	5	10	45	50	3.0	6.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	8.0	9.0	
ZA1	w	+	+	+	+	+	w	+	+	+	+	+	+	+	+	Homo
ZA2	w	+	+	+	+	+	w	w	+	+	+	+	+	+	w	Homo
ZA3	+	+	+	w	+	+	+	+	+	+	+	+	+	+	+	Homo
ZA4	+	+	+	w	+	+	-	-	+	+	+	+	+	+	+	Homo
ZA5	w	w	+	w	+	+	-	w	+	+	+	+	+	+	+	Homo
ZA6	w	+	+	w	+	+	w	w	+	+	+	+	+	+	+	Hetero
ZA7	-	+	w	-	+	+	-	-	+	+	+	+	+	+	+	Homo
ZA8	w	+	+	w	+	w	+	+	+	+	+	+	+	+	+	Homo
ZA9	+	+	+	w	+	+	w	w	+	+	+	+	+	+	+	Homo
ZA10	w	+	w	-	+	+	+	+	+	+	+	+	+	+	+	Homo
ZA11	w	+	+	+	+	+	w	+	+	+	+	+	+	+	+	Homo
ZA12	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	Homo
ZA13	w	+	+	+	+	+	w	+	+	+	+	+	+	+	+	Homo
ZA14	w	+	+	+	+	w	w	+	+	+	+	+	+	+	+	Homo
ZA15	-	w	w	-	+	+	w	+	+	+	+	+	+	+	w	Hetero
ZA16	w	+	+	+	+	+	w	+	+	+	+	+	+	+	+	Homo
ZA17	-	+	w	+	+	w	-	w	w	+	+	+	+	+	+	Homo
ZA18	w	+	w	w	+	+	+	w	+	+	+	+	+	+	+	Hetero
ZA19	w	+	w	-	+	w	w	w	+	+	+	+	+	+	+	Homo
ZA20	w	+	+	+	+	w	w	w	+	+	+	+	+	+	+	Homo
ZA21	w	+	+	w	+	+	-	w	+	+	+	+	+	+	+	Homo
ZA22	-	+	+	+	+	w	-	-	w	+	+	+	+	+	+	Homo
ZA23	+	+	w	-	+	w	-	w	+	+	+	+	+	+	+	Homo
ZA24	+	+	w	w	+	w	-	w	w	+	+	+	+	+	+	Homo
ZA25	-	+	+	+	+	+	-	w	+	+	+	+	+	+	+	Homo
ZA26	-	+	w	w	+	+	w	+	+	+	+	+	+	+	+	Hetero
ZA27	w	+	+	+	+	w	-	-	w	+	+	+	+	+	+	Hetero
ZA28	+	+	+	w	+	+	w	+	+	+	+	+	+	+	+	Homo
ZA29	w	+	+	+	+	w	w	w	+	+	+	+	+	+	+	Homo
ZA30	-	+	+	w	+	+	-	-	w	+	+	+	+	+	+	Homo
ZA31	w	+	+	-	+	+	-	+	+	+	+	+	+	+	+	Hetero
ZA32	-	+	+	-	+	+	-	w	+	+	+	+	+	+	+	Hetero
ZA33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Hetero
ZA34	w	+	+	w	+	+	+	+	+	+	+	+	+	+	+	Homo
ZA35	w	+	w	-	+	w	-	w	+	+	+	+	+	+	+	Homo
ZA36	-	+	+	w	+	+	w	w	+	+	+	+	+	+	w	Homo
ZA37	w	+	+	-	+	+	-	-	+	+	+	+	+	+	+	Homo
ZA38	w	+	+	+	+	w	-	w	+	+	+	+	+	+	+	Homo
ZA39	-	+	+	+	+	+	-	w	+	+	+	+	+	+	+	Homo
ZA40	w	+	+	+	+	w	w	+	+	+	+	+	+	+	+	Homo

Note: +, positive; -, negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative.

2.3 16S DNA Gene Sequence Analysis and recA Gene Multiple Detection

Comprehensive inhibitory activity, physiological and biochemical results, ZA2, ZA3, ZA8, ZA10, ZA18, ZA19, ZA28, ZA33 and ZA34 were selected for 16S DNA gene analysis. 16S DNA sequence analyzing was used for determining the molecular classification of representative LAB strains, and phylogenetic trees that were constructed using 9 strains, based on evolutionary distances determined by a neighbor-joining method were shown in figure 1 and 2. These 9 strains were placed in the cluster comprised of the genera *Lactobacillus*, *Weissella* (*W.*) and *Enterococcus* (*E.*).

As shown in figure 1, strains ZA2, ZA3, ZA8, ZA10 and ZA28 placed in *L. plantarum* cluster, were couldn't distinguished by 16S DNA sequencing. *rec*A gene PCR amplification products of these 5 strains, and type strains in *L. plantarum* cluster including *L. casei*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* were shown in figure 3. As can be seen, all these 5 strains and *L. plantarum* subsp. *plantarum* JCM 1149^T had 318 bp products, while the negative control *L. casei* did not produce any amplicons, so, strains ZA2, ZA3, ZA8, ZA10 and ZA28 could be assigned to *L. plantarum* subsp. *plantarum* for the reason

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that. ZA19 and ZA33 were also placed in the cluster of the genus *Lactobacillus*, and they could be identified as *L. fermentum* and *L. reuteri* both supported by 100% bootstrap values, respectively, and both between 99% shared similarity in their 16S DNA.

In figure 2, strain ZA18 was placed in the *Weissella* cluster, with the species *W. cibaria* LMG 17699^T being the most closely related species, which was supported by 99% bootstrap analysis in the phylogenetic tree and more than 99% similarity in the 16S rRNA gene sequence, therefore, strain ZA18 belonged to *W. cibaria*. Strain ZA34 placed in the cluster of the genus *Enterococcus* in the phylogenetic tree was clearly identified as *E. faecali*, since it formed a very well-defined cluster (100% bootstrap) with *E. faecalis.*

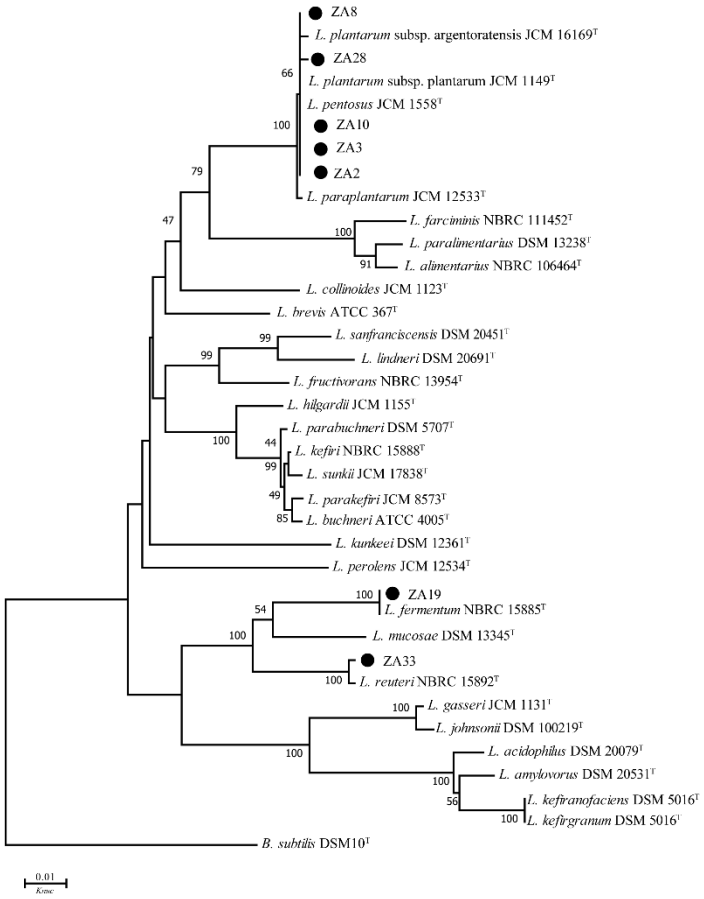

Figure 1. Phylogenetic tree of selected *Lactobacillus* strains.

Note: Bootstrap values for 1000 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *L.* = *Lactobacillus*, *B.* = *Bacillus*. *Knuc* = nucleotide substitution rate.

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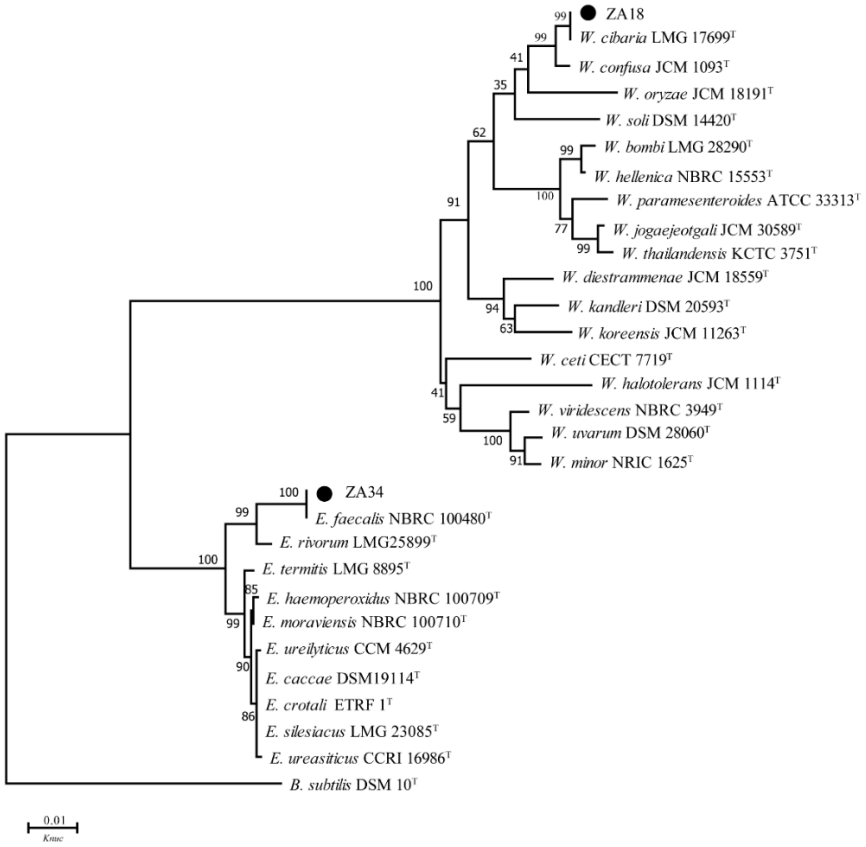

Figure 2. Phylogenetic tree of ZA18 and ZA34.

Note: Bootstrap values for 1000 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *W.* = *Weissella*, *E.* = *Enterococcus*, *B.* = *Bacillus*. *Knuc* = nucleotide substitution rate.

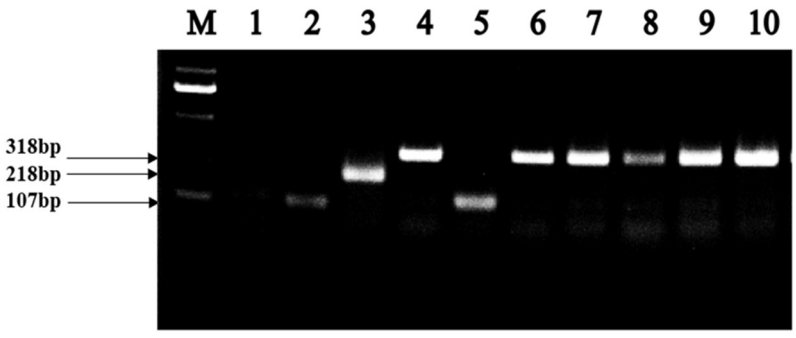

Figure 3. Amplification products obtained from the *recA* multiplex assay.

Note: Lane M contained a 2000 bp PLUS DNA ladder. Lanes 1, 2, 3, 4 and 5, PCR amplication products from *L. casei* JCM 16167T (negative control), *L. paraplantarum* JCM 12533T, *L. pentosus* JCM 1558T, *L. plantarum* subsp. *plantarum* JCM 1149T and *L. plantarum* subsp. *argentoratensis* JCM 16169T, respectively; Lane 6, 7, 8, 9, and 10, PCR amplification product from ZA3, ZA2, ZA28, ZA8 and ZA10.

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2.4 Cell Surface Properties of Selected LAB Isolates

The hydrophobicity and auto-aggregation ability of 9 selected LAB isolates are shown in figure 4. Figure 4 (a) exhibited strains had significant differences in hydrophobicity, among these 9 strains, ZA3 had the highest hydrophobicity at 59.7%, and the lowest was ZA31, only 9.9%. The auto-aggregation ability of isolates was presented on figure 4 (b), all tested isolates showed lower auto-aggregation ability at 2 h, while at 8 h increased significantly, and ZA3 also showed the highest auto-aggregation at 78.95% in comparison to other LAB isolates tested. Thus, ZA2, ZA3, ZA8, ZA10, ZA21 and ZA28 were used for further testing.

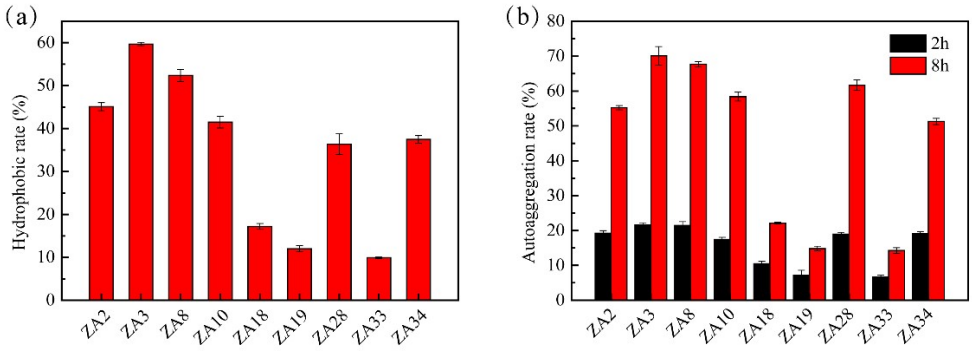

Figure 4. Cell surface hydrophobicity and auto-aggregation ability of lactic acid bacterial isolates.

2.5 Acid Production Capacity and Growth Curve of Selected LAB Isolates

Acid production capacity and growth curve of 6 selected LAB isolates are shown in figure 5.The following conclusions was no significant difference in the acid-producing ability of ZA2, ZA3, ZA8, ZA10, ZA21 and ZA28 within 48 h, but ZA3 had the lowest pH at 3.0 after 48 h fermentation (figure 5 (a)). Figure 5 (b) showed the growth curve of these 6 isolates in 24 h, and from which could see the growth adaptation period of all 6 strains was from 0 to 2 h, and logarithmic growth period was from 2 to 16 h.

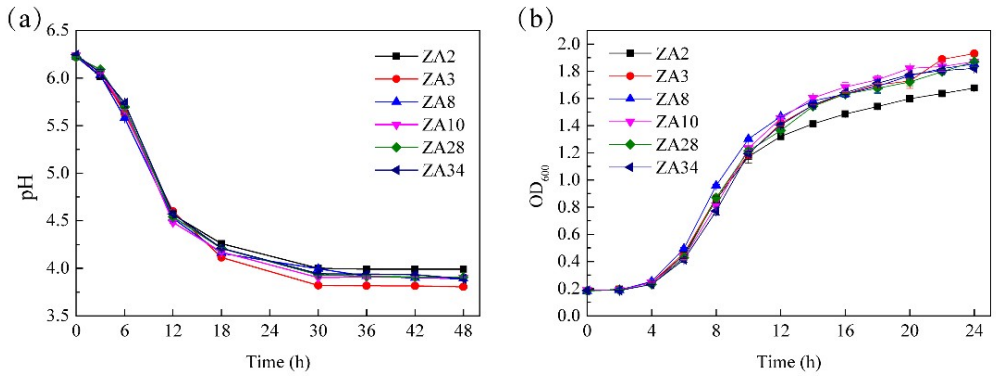

Figure 5. Acid production capacity and growth curve of 6 lactic acid bacterial isolates.

2.6 pH 2.5 and Bile Salt Resistance of Selected LAB isolates

Table 3 showed pH 2.5 resistance of 6 selected LAB isolates. OD values of all 6 test LAB

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isolates were significantly different after treated at pH 2.5 for 2, 4 and 6 h. All isolates had certain vigor after 2 h incubation, of which ZA3, ZA8, ZA10 and ZA21 with the OD value 1.228, 1.456, 1.386 and 1.174, respectively, among which the highest was ZA8 with 1.456. After 4 h incubation, only ZA3 and ZA8 still had certain vigor, while ZA3 with 1.200 OD value still. As for 6 h incubation, no isolate had viability.

From table 4, all 6 LAB isolates tested exhibited different viability after exposure to 0.5% bile salt for 2, 4 and 6 h. Only ZA2 and ZA3 had vitality with OD 0.896 and 1.481 after 2 h incubation, respectively, while other strains without any activity. After 4 h incubation, all strains were almost inactive (OD value of ZA2 was 0.474) except ZA3, which still had 1.341 OD value. As after 6 h, the OD value of ZA3 was also 1.115.

Based on the excellent pH 2.5 and bile salt tolerance of ZA3 in selected isolates, only ZA3 was selected for subsequent experiments.

Table 3. Biomass of 6 lactic acid bacteria isolates grown for 20 h after treatment in different acidic environments with different times.

Isolates	OD_{600}						
	Initial (pH 6.8)	pH 2.5			pH 6.2		
		2 h	4 h	6 h	2 h	4 h	6 h
ZA2	0.149±0.003	0.767±0.209	0.259±0.050	0.192±0.027	1.403±0.017	1.462±0.038	1.446±0.018
ZA3	0.144±0.004	1.228±0.097	1.200±0.019	0.260±0.051	1.730±0.056	1.808±0.026	1.813±0.028
ZA8	0.153±0.003	1.456±0.063	0.912±0.143	0.242±0.017	1.610±0.151	1.776±0.028	1.801±0.011
ZA10	0.152±0.005	1.386±0.114	0.387±0.080	0.151±0.009	1.719±0.044	1.716±0.035	1.772±0.017
ZA28	0.146±0.002	0.305±0.076	0.202±0.045	0.184±0.034	1.720±0.037	1.755±0.091	1.765±0.003
ZA34	0.146±0.005	1.174±0.109	0.317±0.112	0.178±0.016	1.616±0.083	1.681±0.058	1.749±0.003

Table 4. Biomass of 6 lactic acid bacteria isolates regenerating for 20 h after treatment in bile salt at different times.

Isolates	OD_{600}						
	Initial	Control			0.5% bile salt (w/v)		
		2 h	4 h	6 h	2 h	4 h	6 h
ZA2	0.152±0.003	1.444±0.033	1.472±0.022	1.503±0.026	0.896±0.148	0.474±0.242	0.351±0.102
ZA3	0.148±0.001	1.756±0.070	1.794±0.038	1.813±0.018	1.481±0.118	1.341±0.110	1.115±0.178
ZA8	0.151±0.009	1.675±0.098	1.770±0.039	1.797±0.053	0.199±0.024	0.178±0.005	0.171±0.004
ZA10	0.179±0.004	1.730±0.164	1.727±0.021	1.736±0.022	0.237±0.077	0.199±0.028	0.178±0.008
ZA28	0.151±0.007	1.736±0.078	1.775±0.030	1.774±0.013	0.171±0.006	0.170±0.003	0.162±0.007
ZA34	0.151±0.005	1.631±0.024	1.695±0.048	1.718±0.036	0.189±0.019	0.172±0.005	0.168±0.002

2.7 Survival of ZA3 After Simulation GI Exposure

Figure 6 illustrated the viable count (log CFU/mL) of the strain ZA3 during GI exposure. The population of ZA3 before in SGF was 8.72 log CFU/mL, while after 3 h incubation in SGF, the population of ZA3 was 8.20 log CFU/mL, and the survival ratio was 94.03%. As for in SIF, initial population of ZA3 was 8.01 log CFU/mL, the population reduced to 7.92 log CFU/mL after 4 h treatment, that was to say, the survival ratio of ZA3 after SIF phase was 98.88%.

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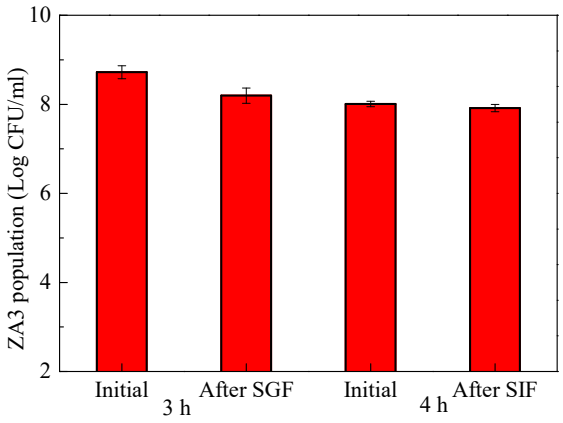

Figure 6. Surviving of ZA3 during a simulated gastrointestinal (GI) exposure.

2.8 Safety evaluation of ZA3

2.8.1 Safety Properties of ZA3

The results of virulence factors genes, biogenic amines genes and antibiotic resistances genes amplification of ZA3 are shown in table 5. PCR results revealed that ZA3 did not harbour any virulence genes such as collagen gene associated with adhesin (ace), gelatinase gene (gelE) and cytolysin gene (cylA), biogenic amines genes as histidine decarboxylase (hdc), tyrosine decarboxylase (tdc) and ornithine decarboxylase (odc), and antibiotic resistances gene as viz vancomycin resistance gene (vanA) and tetracycline resistance gene (tetM).

Table 5. Detection of virulence factor gene, biogenic amine production gene and antibiotic resistance gene in ZA3.

Isolates	Virulence factors genes			Biogenic amines genes			Antibiotic resistance genes	
	ace	gelE	cylA	hdc	tdc	odc	vanA	tetM
ZA3	-	-	-	-	-	-	-	-

2.8.2 Assessment of Antibiotic Susceptibility

Table 6 showed antibiotic susceptibility of isolate ZA3. ZA3 demonstrated sensitive to namely, carbenicillin, carbenicillin, ampicillin, clindamycin, erythromycin and chloramphenicol, resistant to norfloxacin and amikacin, and intermediate resistant to gentamicin and penicillin.

Table 6. Antibiotic susceptibility test of isolate ZA3.

Isolate	CB	CZ	AM	GM	NOR	CC	P	E	C	AK
ZA3	S	S	S	I	R	S	I	S	S	R

Note: 1. S: Susceptible; I: Intermediate resistant; R: Resistant.

2. Refer to the latest CLSI standard. CB: carbenicillin; CZ: carbenicillin; AM: ampicillin; GM: gentamicin; NOR: norfloxacin; CC: clindamycin; P: penicillin; E: erythromycin; C: chloramphenicol; AK: amikacin.

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2.8.3 Hemolytic Activity of ZA3

As shown in figure 7, compared with positive control *Staphylococcus aureus* ATCC 6538^T having blood hemolysis activity in figure 7 (b), ZA3 in figure 7 (a) showed no hemolytic activity.

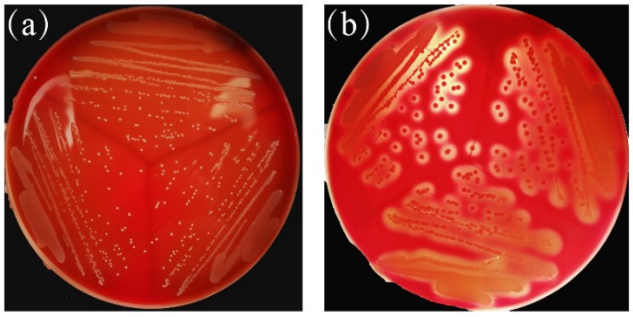

Figure 7. Hemolytic activity of ZA3.

(a) ZA3, (b) positive control: *Staphylococcus aureus* ATCC 6538^T.

2.9 Antimicrobial Spectrum Test of ZA3

Results of the agar well diffusion in table 7 displayed that ZA3 demonstrated powerful antimicrobial activity against all selected microorganisms tested in this work. As can be seen that inhibition zone diameter more than 22.00 mm were *Pseudomonas aeruginosa* ATCC 15692^T and *Listeria monocytogenes* ATCC 51719^T, as for *E. coli* ATCC 11775^T, *Staphylococcus aureus* ATCC 6538^T, *Bacillus subtilis* ATCC 19217^T, *Micrococcus luteus* ATCC 4698^T and *Salmonella enterica* ATCC 43971^T, inhibition zone diameter of them were all above 18.00 mm.

Table 7. Antibacterial spectrum of ZA3.

Isolate	Indicator bacterias						
	E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*L. monocytogenes*	*M. luteus*	*S. enterica*
ZA3	+++	++++	+++	+++	++++	+++	+++

Notes: 1. +, diameter of inhibition zone: 10.00-14.00 mm; ++, 14.00-18.00 mm; +++, 18.00-22.00 mm; ++++, more than 22.00 mm; -, no inhibition zone was detected; the diameter of inhibition zone including that of hole puncher (10.00 mm).

2. *E. coli*: *Escherichia coli* ATCC 11775^T; *P. aeruginosa*: *Pseudomonas aeruginosa* ATCC 15692^T; *S. aureus*: *Staphylococcus aureus* ATCC 6538^T; *B. subtilis*: *Bacillus subtilis* ATCC 19217^T; *L. monocytogenes*: *Listeria monocytogenes* ATCC 51719^T; *M. luteus*: *Micrococcus luteus* ATCC 4698^T; *S. enterica*: *Salmonella enterica* ATCC 43971^T.

2.10 Carbohydrate Utilization Patterns of ZA3

Carbohydrate utilization patterns of ZA3 are shown in table 8, and results indicated that ZA3 could use galactose, D-glucose, D-fructose, mannose, L-sorbose, rhamnose, mannitol, aorbitol, maltose, lactose, melibiose, saccharose, trehalose, gluconate, N-acetyl glucosamine and amygdalin as carbon sources, and ribose could be weakly used, other than that, the remaining carbon sources as glycerol, D-arabinose, L-arabinose, D-xylose, dulcitol, inositol, salicin, melezitose, D-raffinose, starch, xylitol and L-arabitol were completely unavailable.

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Table 8. Carbohydrate utilization patterns of ZA3.

Substrate	ZA3	Substrate	ZA3
Glycerol	-	Salicin	-
D-Arabinose	-	Cellobiose	-
L-Arabinose	-	Maltose	+
Ribose	w	Lactose	+
D-Xylose	-	Melibiose	+
Galactose	+	Saccharose	+
D-Glucose	+	Trehalose	+
D-Fructose	+	Melezitose	-
Mannose	+	D-Raffinose	-
L-Sorbose	+	Starch	-
Rhamnose	+	Xylitol	-
Dulcitol	-	L-Arabitol	-
Inositol	-	Gluconate	+
Mannitol	+	*N*-acetyl glucosamine	+
Sorbitol	+	Amygdalin	+

Note: +, positive; -, negative; w, weakly positive.

2.11 Identification of the Antimicrobial Substance Produced by ZA3

Effects of pH, enzymes and hydrogen peroxide on the antimicrobial activity of ZA3 against ETEC K88 are shown in table 9. The inhibition zone diameter of fermentation liquid and supernatant of ZA3 were both above 18.00 mm. The results summarized that the hydrogen peroxide did not affect antimicrobial activity of ZA3 based on the inhibition zone diameter were still above 18.00 mm after treated by hydrogen peroxide. At the same time, after treated by pepsinum and trypsase, the inhibition zone diameter was still above 18.00 mm, as for proteinase *K*, the diameter was also between 14.00-18.00 mm. However, when tested effects of different pH, the antimicrobial activity of ZA3 decreased with increasing pH, while which was not affected by pH 3.0 and 4.0 obviously, slightly decreased at pH 4.5, and a complete loss of activity was observed at pH values ranging from 5.5 to 10.0. Therefore, antibacterial substances produced by ZA3 may be acid.

Table 9. Antimicrobial activity of ZA3 to ETEC K88 after different treatment.

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Treatment	Antimicrobial activity
ZA3	
fermentation liquid	+++
supernatant	+++
hydrogen peroxide	+++
proteinase *K*	++
pepsinum	+++
trypsase	+++
pH	
3.0	+++
4.0	+++
4.5	++
5.0	+
5.5	-
6.0	-
6.5	-
7.0	-
10.0	-

Note: +, diameter of inhibition zone: 10.00-14.00 mm; ++, 14.00-18.00 mm; +++, 18.00-22.00 mm; ++++, more than 22.00 mm; -, no inhibition zone was detected; the diameter of inhibition zone including that of hole puncher (10.00 mm).

2.12 Organic Acid Produced by Fermentation of ZA3

Liquid chromatography was used to analyze the acid production of ZA3 after 24 h fermentation. As shown in figure 8, 4 kinds of organic acid were mainly detected in the fermentation broth, which were citric acid, succinic acid, lactic acid and acetic acid, and the content was 0.822, 0.576, 2.545 and 1.729 mg/ml, respectively.

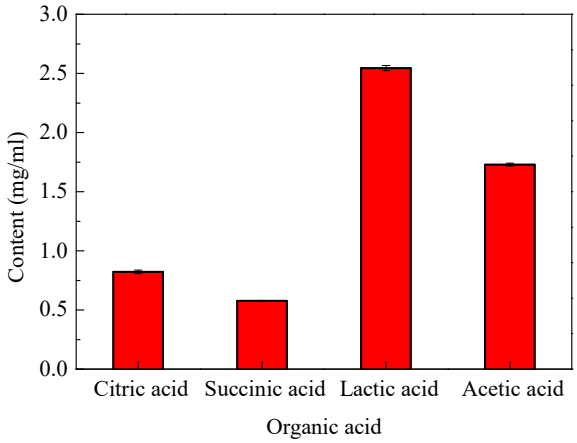

Figure 8. The organic acid produced by fermentation of ZA3.

3. Discussion

In this study, to obtain functional probiotic bacteria that inhibit ETEC K88, more than 1100 LAB from different sources were tested and 40 isolates with an inhibition zone diameter of 18.00 cm or more were selected. ETEC is the main pathogenic bacterium that causes diarrhea in humans and young animals. As one of the indispensable floras with important physiological functions in human and animal body, LAB has the incomparable host bacteria advantages of other receptor strains. Ziadi et al. [9] reported the strain *L. plantarum* F3 showed antagonistic activity against *E. coli* with an inhibition zone diameter of 21.00 mm by the agar well diffusion method, while in our study, 13 isolates had more than 22.00 mm inhibition zone by using the same test method.

One of the most important criteria for selecting LAB with probiotic properties is the ability to tolerate the environment of the gastrointestinal tract, with the pH of pig gastric juices being as low as 2.0 and bile with a pH of about 8.0. Dushku et al. [10] isolated and identified 40 LAB strains from the gastrointestinal tract of local snails, which were artificially simulated to survive the gastrointestinal tract only 2 strains; Rao et al. [11] screened *L. plantarum* AT4 for low pH and up to 0.5% bile salts. This study found 40 selected strains with excellent inhibition abilities, 13 homo-fermentative isolates could grow at 5-50°C and pH 3.0-10.0, and ferment glucose without producing gas. Relative to hetero-fermentative LAB producing a mix of lactic acid and acetic acid, homo-fermentative LAB promotes rapid fermentation, primarily producing lactic acid and rapidly reducing the pH, preventing the growth of other undesirable spoilage organisms, which indicated that these strains satisfying the demands for growth in relatively extreme environments. In a word, the excellent physiological and biochemical characteristics provide tremendous potential for selected strains in practical applications [12].

Species identification is the basis for conducting scientific experiments, and the identification of the genus provides an indirect understanding of the bacteria's habits, metabolism and pathogenic patterns. In this study, phylogenetic trees were constructed based on the evolutionary distances of their 16S DNA sequences using the neighbour-joining method to identify selected LAB isolates at the species level. Selected strains were placed in cluster comprised of genera *Lactobacillus*, *Weissella* and *Enterococcus*. Since strains ZA2, ZA3, ZA8, ZA10 and ZA28 were clearly assigned to the genus *Lactobacillus* and grouped in the *L. plantarum* cluster on phylogenetic tree with type strains *L. casei* JCM 16167^T, *L. paraplantarum* JCM 12533^T, *L. pentosus* JCM 1558^T, *L. plantarum* subsp. *plantarum* JCM 1149^T and *L. plantarum* subsp. *argentoratensis* JCM 16169^T, which couldn't be separated by 16S rDNA [13]. By means of *rec*A gene PCR amplification products, all these 5 strains and *L. plantarum* subsp. *plantarum* JCM 1149^T had 318 bp products, while the negative control *L. casei* and other 3 type strains did not produce any amplicons, thus, ZA2, ZA3, ZA8, ZA10 and ZA28 strains could also be accurately identified as *L. plantarum* subsp. *plantarum*.

Adherence is a prerequisite for strains to colonize the gut and increase their viability, and only if the strain is able to adhere and colonize the intestinal tract can it promote immune regulation and stimulate the intestinal barrier and metabolism function [14]. Adherence mainly includes cell surface hydrophobicity, self-aggregation and co-aggregation to indicator bacteria and its effect on intestinal cells. It has been shown that high hydrophobicity and auto-aggregation ability could promote the colonization of beneficial microorganisms in the gastrointestinal tracts of human hosts [15]. In this study, ZA3 from weaned pig feces showed the highest hydrophobic activity (59.70%) and auto-aggregation ability (78.95%) in comparison

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to other LAB isolates tested, and the results are consistent with previous studies that the higher the surface hydrophobicity of these isolates, the stronger the self-aggregation ability [16].

As a functional LAB, it should overcome several challenging environmental conditions, such as extremely high or low pH, salt and bile, all are the most common factors [17]. In the present study, ZA3 was found still had certain vigor after 4 h incubation in different acidic or bile salt environments and exhibited 94.03 % survival rate after 3 h in simulated gastric juice. The results obtained by Joghataei et al. [18] displayed that *L. fermentum* FH19 exhibited the highest survival rate (96%) after 3 h in SGF, comparatively speaking, ZA3 with 98.88% survival ratio after SIF phase in this study indicating that it has a significant effect on SGF and SIF tolerance. Lee et al. [19] reported *L. plantarum* C182 possesses significant level of resistance against 0.3% bile salts, while ZA 3 had vitality with OD value 1.115 after exposure to 0.5% bile salt for 6 h.

Safety is also one of the basic criteria for screening strains, LAB, especially *Lactobacillus*, are usually considered safe. However, it has recently been discovered that *Lactobacillus* and *Bifidobacterium* are frequently isolated from diseased tissues such as endocarditis and sepsis [20,21]. Therefore, it is necessary to re-evaluate their safety. In addition, the safety evaluation of probiotics has become more important with the isolation and application of new probiotics and the emergence of genetically modified probiotics. As isolated strains *Lactobacillus* MMP4 wanted to be used in the dairy industry, Choudhary et al. [22] used PCR to investigate whether strain MMP4 was antibiotic resistant genes, and had safety parameters such as gelatinase and hemolytic activity. In this study, all tested strains were PCR-negative for all the virulence factors including virulence factors genes, biogenic amines genes and antibiotic resistances genes, and antibiotic susceptibility and hemolytic activity, which are at risk for genetic transfer. Moreover, ZA3 was sensitive to ampicillin, resistant to norfloxacin and amikacin and absence of hemolysis on blood agar, indicating that it could have potential as a safety probiotic candidate.

The ultimate goal of screening LAB is application, and the ability to utilize a wider variety of carbon sources indicating that the strain ZA3 was easy to culture and has greater viability, which is of greater value in research and production.

There is a wide variety of antibiotics, and antibiotic inhibition spectrum varies by antibiotic type, when using probiotics instead of antibiotics, which probiotics should be used instead of which antibiotics? Is it a complete replacement or a partial replacement? It is difficult for feed and breeding companies to make these decisions. Moreover, probiotics typically exhibit narrow killing spectrum, inhibiting only those bacteria that are closely related to them. Therefore, it is very important to screen strains with a wide antimicrobial spectrum and define the range of antimicrobial spectrum of the strains. In this study, results of screening for the potential antagonistic activity against important pathogens showed ZA3 exhibit broad spectrum activity against a wide range of microorganisms, including gram-positive and gram-negative bacteria. Additionally, because the intestinal flora is a complex system and it is difficult to introduce new microorganisms into this competitive environment, it produces substances as bacteriocins, organic acids, short-chain fatty acids and hydrogen peroxide that inhibit pathogens in the gut of growth and reduce the occurrence of diarrhea [23]. ZA3 had strong antimicrobial activity after excluding the effects of hydrogen peroxide by proteinase *K* and eliminating hydrogen peroxide from the cell-free culture supernatants, however, all

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activities disappeared completely when the cell-free supernatant was treated with neutralizing pH, which indicated that the antimicrobial activity may attributed to the production of organic acids. Similarly, as one of the antimicrobial compounds, Tirloni et al. [24] and Bah et al. [25] proposed organic acids can significantly inhibit the growth of pathogenic bacteria; Silva et al. [26] reported *L. lactis* subsp. *lactis* presented antimicrobial activity against pathogens which may be related to their lactic acid production, low pH values and antimicrobial compounds. Furthermore, some research results showed that the main organic acids metabolites of LAB fermentation were pyruvate, lactic acid, acetic acid, citric acid, oxalic acid and malic acid etc. [24,27,28]. ZA3 mainly produced 4 kinds of organic acids as citric acid, succinic acid, lactic acid and acetic acid after 24 h fermentation, respectively, and lactic acid and acetic acid were significantly higher than other two acids. Such a conclusion is the same as that of Mun et al. [29], who researched lactic acid and acetic acid in *L. plantarum* EM fermentation broth was the main antibacterial active substances. In summary, it is worthwhile to investigate ZA3 effect on diarrhea in animals induced by ETEC K88.

4. Materials and Methods

4.1 Screening of LAB Restrain ETEC K88 Activity

More than 1100 LAB strains isolated from weaned piglet feces, feed grass, Qula, rice silage, mixture silage and corn silage were used in this study. All LAB isolates were incubated in MRS broth (De Man, Rogosa, Sharpe) at 30 °C.

The antagonistic effect of LAB isolates against ETEC K88 were first determined by the agar well diffusion technique [9]. The target bacteria ETEC K88 (Purchased from the China Veterinary Culture Collection Center, CVCC) was grown on LB liquid culture medium and incubated at 37 °C with 180 rpm for 12 h. Subsequently, 100 uL of the overnight culture of K88 was added to LB agar medium cooled to 50 °C and shaken for mixing well before pouring onto the surface of the already coagulated LB agar plates. After solidification, a hole was punched in the center of the plate with a hole punch (diameter 10 mm) and 200 μL of cultures of different strains (16 h) were placed in the hole and diffused at 4 °C for 2 h before measuring inhibition zone.

4.2 Molecular Identification of Representative Strains

4.2.1 16S rRNA Analysis

The 16S rRNA gene of selected strains were amplified by PCR using the 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACGACTT-3′) universal primer sets. Amplifications by PCR were performed in a total volume of 50 μL DNA thermal cycler, which containing 25 μL 2 × Taq Plus Mastermix, 27 F and 1492 R primer 1 μL each, and finally add sterile distilled water to 50 μL. Single colonies cultured for 24 h were added to each reaction system, separately, and the PCR condition was: initial activation at 94 °C for 5 min; 33 cycles at 94 °C for 50 s, 52 °C for 50 s and 72 °C for 50 s; and a final cycle at 72 °C for 17 min. The PCR products were placed on a 1% agarose gel and electrophoresed with EB (Ethidium Bromide Solution) staining. The successful amplification was analyzed by sequencing service (MGI Tech Co., Ltd, Beijing, China), and resulting sequences were compared with sequences in the GenBank database using the BLAST program available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

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4.2.2 *RecA* Multiple Sequence

Means of partial amplification product comparison of the *rec*A gene was used to distinguish strains of *L. plantarum* cluster, including *L. casei*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* [13]. A multiplex PCR assay (20 μL) was performed with *rec*A gene-based primers para F (5′-GTCACAGGCATTACGAA AAC-3′), pent F (5′-CAGTGGCGCGGTTGATATC-3′), plan F (5′-CCGTTTATGCGGAACACC TA-3′) and pREV (5′-TCGGGATTACCAAACATCAC-3′). The PCR mixture was composed of primers para F, pent F and pRE V (0.25 μM each), 0.12 μM primer plan F, 25 μL 2 × Taq Plus Mastermix, add sterile distilled water to 40 μL. Single colonies including type strains *L. casei* JCM 16167^T (negative control), *L. paraplantarum* JCM 12533^T, *L. pentosus* JCM 1558^T, *L. plantarum* subsp. *plantarum* JCM 1149^T and *L. plantarum* subsp. *argentoratensis* JCM 16169^T and strains of this cluster indistinguishable after 16S rRNA analysis were added to each reaction system, separately. The PCR condition was initial denaturation at 94 °C for 5 min, 33 cycles of denaturation at 94 °C (30 s), annealing at 56 °C (10 s) and elongation at 72 °C (30 s), and final extension at 72 °C for 5 min. The PCR products were visualized on a 2% agarose gel in 0.5×TAE (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) buffer.

4.3 Cell Surface Hydrophobicity and Auto-aggregation of Representative Strains

The cell surface hydrophobicity and auto-aggregation assay were performed as Somashekaraiah et al. [16] and wang et al. [30], respectively, and both with some modifications. LAB strains cultivated in MRS broth at 30 °C for 16 h were washed twice with PBS (8, 000 rpm, 4 °C, 10 min) and resuspended in PBS buffer followed by absorbance measurement at 600 nm (OD 600, marked as A0).

For cell surface hydrophobicity analysis, 3 mL cell suspension was blended with 1 mL xylene, the two phases system was mixed by vortexing for 2 min and incubated at 37 °C without shaking for 30 min for separation of the aqueous and organic phases. The water phase was carefully removed and its absorbance at 600 nm was measured (OD 600, marked as A1). The percentage of cell surface hydrophobicity (H%) was calculated using the following formula:

$$H\% = (1- A1/ A0) \times 100\% \quad (1)$$

As for auto-aggregation, after made the sample stand awhile before incubating at 30 °C, and the upper suspension was checked for absorbance at 600 nm at time intervals of 0, 2, and 8 h (OD 600, the absorbance at each particular time marked as different Atime). The auto-aggregation was measured (in percentage) using the following formula:

$$\text{auto-aggregation}\% = [1- (Atime/A0) \times 100] \% \quad (2)$$

4.4 Determination of Growth Curve and Acid Production Capacity of Representative Strains

Each single LAB colony were picked into 20 mL sterile MRS, and the optical density at 600 nm (OD 600) and colony forming units (cfu) /mL were determined immediately at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h after inoculation at 30 °C, respectively. pH of each fermentation solution was determined at 0, 6, 12, 18, 24, 30, 36, 42 and 48 h after inoculation, respectively.

4.5 Low pH and Bile Salt Tolerance of Representative Strains

Fresh MRS solution containing 0.5% (wt/vol) bile salt and MRS broth at pH 2.5 were used

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to assess low pH and bile salt tolerance, and no bile salt and pH 6.8 were set as control, respectively. The viability was determined after 0, 3 and 6 h incubation, and the biomass of each LAB isolates grown for 20 h after different treatment times in different acidic and bile salt environments was determined by the optical density at 600 nm.

4.6 Survival of Representative Strains in GI Fluids

The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were performed as described by Massounga et al. [31] with modifications. Briefly, for SGF, 3.5 g/L pepsin was suspended in 0.2% w/v sterile NaCl solution and adjusted pH to 2.0, made the total volume of the solution up to 100 mL and filtered through a 0.22 μm filter membrane. For SIF, 1 g/L trypsin, 18 g/L bile salt from ox and 11 g/L $NaHCO_3$ were suspended in 0.2% w/v sterile NaCl solution, adjusted the pH of the solution 6.8, again, brought the total solution volume to 100 mL with 0.22 μm membrane filtration. 2% 10^8 cfu/mL LAB solution was added to 20 ml SGF and incubated 3 h, as for SIF was 4 h. Viable colonies were determined with plate counts on MRS agar after 0, 3 and 7 h incubation.

4.7 Pathogenicity Evaluation of Strain ZA3

4.7.1 Safety Evaluation

The strain ZA3 was screened for the presence of genetic traits related to virulence factors, biogenic amines and antibiotic resistance using the PCR protocols [32]. The target genes including gelE (gelatinase) (F: 5′-TATGACAATGCTTTTTGGGAT-3′, R: 5′-AGATGCACCCG AAATAATATA-3′), cylA (cytolisin) (F: 5′-GAATTGAGCAA AAGTTCAATCG-3′, R: 5′-GTCTG TCTTTTCACTTGTTTC-3′), ace (adhesion of collagen) (F: 5′-ACTCGGGGATTGATAGGC-3′, R: 5′-GCTGCTAAAGCTGCGCTT-3′), vanA (vancomyc- in resistance) (F: 5′-TCTGCAATA GAGATAGCCGC-3′, R: 5′-GGAGTAGCTATCCCAGCATT-3′), tetM (tetracycline resistance) (F: 5′-ATTACACTTCCGATTTCGG-3′, R: 5′-GTTAAATAGTGTTCTTGGAG-3′), hdc (histidine decarboxylase) (F: 5′-AGATGGTATTGTTTCTTATG-3′, R: 5′-AGACCATACACCATAACCTT-3′), tdc (tyrosine decarboxy- lase) (F: 5′-GAYATNATNGGNATNGGNYTNGAYCARG-3′, R: 5′-CCRTARTCNGGNATAGCRAARTCNGTRTG-3′) and odc (ornithine decarboxylase) (F: 5′-GTNTTYAAYG CNGAYAARCANTAYTTYGT-3′, R: 5′-ATNGARTTNAGTTCRCAYTTYTCNG GG-3′).

4.7.2 Antibiotic Susceptibility

Antibiotic susceptibility was determined by disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute [33]. Susceptibility tests of the isolates against 10 antibiotics, carbenicillin, cefamezin, ampicillin, gentamicin, norfloxacin, clindamycin, penicillin, erythromycin, chloramphenicol and amikacin, respectively, were determined by the disc diffusion method in a nutrient broth medium. Susceptibility to antimicrobials were measured after 24 h incubation of the mean inhibition zone diameter.

4.7.3 Hemolytic Activity

Hemolytic activity was analyzed on blood agar as the manufacturer's instructions. Fresh bacterial ZA3 was streaked on Columbia blood agar plates, and *Staphylococcus aureus* ATCC6538^T was used as positive control.

4.8 Antimicrobial Activity

The antimicrobial activity of ZA3 against pathogenic bacteria was assessed by the method of agar well diffusion, *E. coli* ATCC 11775^T, *Pseudomonas aeruginosa* ATCC 15692^T, *Staphylococcus aureus* ATCC6538^T, *Bacillus subtilis* ATCC 19217^T, *Listeria monocytogenes* ATCC 51719^T, *Micrococcus luteus* ATCC 4698^T and *Salmonella enterica* ATCC 43971^T were used as indicator bacterium.

4.9 Carbohydrate Utilization Patterns of ZA3

Thirty common carbon sources including glycerol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, melezitose, D-raffinose, starch, xylitol, L-arabitol, gluconate, N-acetyl glucosamine and amygdalin were used to detect carbohydrate utilization patterns of ZA3 by replacing the carbon source each in turn in the MRS medium.

4.10 Identification of the Antimicrobial Substance of ZA3

The antimicrobial substance of ZA3 was assessed according to Ni et al. [34] and exclusion experiments for acid and catalase inhibition, and protease (proteinase *K*, trypsin and pepsin) degradation were performed, respectively.

4.11 Determination of Organic Acids Produced by ZA3

The organic acid produced by ZA3 was determined with high performance liquid chromatography (HPLC) (column: Carbomix H-NP10: 8%, 7.8 × 300 mm, Sepax Technologies, Inc, Delaware, USA; detector: DAD, 214 nm, Agilent 1200 Series, Agilent Technologies Co., MNC, CA, USA; eluent: 2.5 mmol/L H_2SO_4, 0.6 mL/min; temperature: 55°C). Overnight ZA3 bacteria cultures of LAB grown in MRS broth were centrifuged at 8,000 × g for 10 min and filtered through a 0.22 μm filter membrane. The organic acids lactic acid, acetic acid, propionic acid, butyric acid, succinic acid, citric acid and malic acid were detected.

4.12 Statistical Analyses

Each test was performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) or Paired t-test (SPSS 22.0). All data were shown as mean ± standard error of mean (SEM), and $P < 0.05$ indicated significant difference in statistics.

5. Conclusions

In this study, the inhibitory activities of 1100 LAB strains from different sources were tested to inhibit ETEC K88, of which ZA3 isolated from weaned pig feces and identified as *L. plantarum* subsp. *plantarum* had good inhibition ability and growth performance, excellent safety features as well as good hydrophobicity and auto-aggregation, high survival rate in SGF and SIF, broad spectrum activity against a wide range of microorganisms, and antibacterial substance may attributed to organic acids. Therefore, ZA3 might be a suitable candidate for further study for its protective effects against ETEC K88 infections in weaned piglets.

Author Contributions: H.-L.P. and G.-Y.Q. designed experiments; W.-W.W. carried out experiments; H.M., H.-J.Y., Z.-F.T. and Y.-P.W. analyzed experimental results. W.-W.W. and H.-L.P. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The 16Sr RNA gene sequence of strains ZA3, ZA2, ZA28, ZA8, ZA10, ZA19, ZA33, ZA18 and ZA34 used to support the findings of this study have been deposited in the GenBank repository with accession number MT597900, MT597901, MT597902, MT597903, MT597904, MT597905, MT597906, MF597907 and MF597910, respectively.

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