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Original article

Antibacterial spectrum of four compounds from yeasts in koumiss

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Abstract

Koumiss has beneficial therapeutic effects on bacterial diseases. Four antibacterial compounds from yeasts (*Kluyveromyces marxianus* and *Saccharomyces cerevisiae*) in koumiss were evaluated for their antibacterial effects against three Gram-negative bacteria, three Gram-positive bacteria and five pathogenic *Escherichia coli* strains. The antibacterial compounds from yeasts in koumiss were extracted, and their main components were determined. The inhibition zones were analyzed, and their minimum inhibition concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined. Aqueous phases of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* at pH 2.0 and 8.0 produced larger inhibition zones than those in other phases, and then antibacterial compounds from *K. marxianus* (K2, pH=2.0; K8, pH=8.0) and *S. cerevisiae* (S2, pH=2.0; S8, pH=8.0) were obtained. Their main components were organic acids and killer toxins. K2 had more propanoic acid and S2 had more oxalic acid than others. The inhibition zones of K2, K8, S2 and S8 against three Gram-negative bacteria and three Gram-positive bacteria were 12.03-23.30 mm, their MICs were 0.01-0.13 g/mL, and MBCs were 0.03-0.50 g/mL. Meantime, the inhibition zones of K2, K8, S2 and S8 against five pathogenic *E. coli* were 16.10-25.26 mm, their MICs were 0.03-0.13 g/mL, and MBCs were 0.13-1.00 g/mL.

These four antibacterial compounds from yeasts in koumiss had broad antibacterial spectrum. In addition, K2 and S2 were better than K8 and S8.

Key words: *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Escherichia coli*, minimum inhibition concentration, minimum bactericidal concentration

Introduction

Escherichia coli are normal intestinal flora in animals, most of which are not pathogenic. However, some serotypes are pathogenic, such as *E. coli* O_8 and *E. coli* $O_{78,}$ which are sources of bacterial diseases in animal husbandry together with common pathogenic bacteria. Different pathogenic bacteria cause various clinical symptoms and pathological changes. Moreover, the morbidity and mortality are higher when pathogenic bacteria combine with other pathogens, consequently resulting in large economical losses. Their carriers are

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the sources of infection when the excreta contact feed, water, and grassland, resulting in enormous economic losses in the breeding industry (Boerlin et al. 1999, Sun et al. 2014). For example, *E. coli* O_8 has high incidence within animal husbandry in Inner Mongolia. Calves suffer from diarrhea after infection and die in severe cases (Gow et al. 2008, Mainda et al. 2015).

Antibiotics are used in treating diseases caused by pathogenic bacteria and *E. coli*. Although antibiotics have broad antibacterial spectrum and have obvious therapeutic effects, their abuse results in drug resistance, and the antibiotic residues in animals could endanger human health and safety. In spite of their beneficial effects against pathogenic bacteria, they also harm normal flora to a certain degree, leading to micro-ecological imbalance, physiological disorder, and increased sensitivity to exogenous infection (Cizman 2003, Levy and Marshall 2004, Tadesse et al. 2012, Dwivedi et al. 2015). Therefore, more attention is being paid to natural medicines, probiotics, and some other green products to replace these antibiotics.

Koumiss is a common fermented mare's milk with beneficial therapeutic effects on cardiovascular disease, tuberculosis, and diarrhea as it can nourish vessels, relieve ill moods, and improve digestion (Wu et al. 2009). Yeasts are the main microorganisms in koumiss, playing an important role in koumiss fermentation and endowing them with its therapeutic effects. Some yeasts have been shown to have antibacterial effects on *E. coli* possibly by producing antibacterial compounds, such as killer toxins and organic acids in metabolism (Etienne-Mesmin et al. 2011). We have isolated *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* from koumiss, and demonstrated that their antibacterial compounds had obvious antibacterial effects on *E. coli* O_8 (Chen et al. 2017, Chen et al. 2019). However, the antibacterial spectrum of these compounds from yeasts in koumiss against usual pathogenic bacteria and *E. coli* remain obscure. The present study aimed at investigating the main components of antibacterial compounds from *K. marxianus* and *S. cerevisiae* in koumiss, analyzing their antibacterial spectrum, and selecting the best antibacterial compound. The study will provide a scientific basis for utilizing antibacterial compounds from yeasts in koumiss to treat diseases caused by pathogenic bacteria.

Materials and Methods

Yeasts and pathogenic bacteria

K. marxianus and *S. cerevisiae* were isolated and identified in koumiss samples collected from the Hulunbeier area of Inner Mongolia, China. *E. coli* O_1, *E. coli* O_2, *E. coli* O_8, *E. coli* O_{78}, *E. coli* O_{86} were dominant pathogenic *E. coli* strains previously isolated from 101 cow rectum feces (Huasai et al. 2012) and stored in our laboratory of the Inner Mongolia Agricultural University (Hohhot, China) at -70°C. Three Gram-negative bacteria: *Salmonella Typhimurium* ATCC 14028, enterohemorrhagic *E. coli* CICC 21530, *Pseudomonas aeruginosa* ATCC 27853; three Gram-positive bacteria: *Bacillus cereus* CMCC 63301, *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* CVCC 1886 were obtained from Bianzhen, Nanjing, China.

For each experiment, bacteria were thawed and subcultured in nutrient broth medium (BD, New York, USA) (Dou et al. 2016). Yeasts were thawed and subcultured in potato dextrose agar (PDA) medium (BD, New York, USA).

Preparation of antibacterial compounds from yeasts in koumiss

K. marxianus and *S. cerevisiae* were cultivated in PDA liquid medium with potato 300 g/L, dextrose 20 g/L, chloramphenicol 0.1 g/L, and were harvested following 72 h incubation at 25°C. The suspensions were centrifuged (10 000 r/min, 15 min), the supernatants were filtered through a sterile 0.22 μm syringe filter, and divided into two parts. One half of the supernatant was adjusted to pH 2.0 and the second half was adjusted to pH 8.0 by 1 M NaOH and 1 M HCl (Yongsheng, Tianjin, China). Ethyl acetate (99.5%; Yongsheng, Tianjin, China) of twice the volume of supernatant was added and shaken for 4 h. Organic and aqueous phases were separated using a separating funnel, to which 50 mL sterile water were added to the organic phase and ethyl acetate removed by rotary evaporators. The residual antibacterial activity of the organic phase on *E. coli* O_8 (10^8 colony-forming unit (CFU)/mL) was determined. The aqueous phase was centrifuged (6 000 r/min, 15 min), and the residual antibacterial activity of the aqueous phase supernatant on *E. coli* O_8 was determined (Skovgaard et al. 2013). In two control groups, sterile water was adjusted to pH 2.0 or pH 8.0. The phases which had larger inhibition zones were freeze-dried for 48 h, then antibacterial compounds from yeasts in koumiss were obtained (Chen et al. 2017). Their killer toxins were determined using an enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China), following the manufacturer's instructions. Their organic acids were determined by High Performance Liquid Chromatography (HPLC) (GB/T5009.157-2003).

Table 1. The inhibition zones of each phase solution against *E. coli* O_8.

Solution	Inhibition zone (mm)	
	K. marxianus	*S. cerevisiae*
pH 2 aqueous phase	24.31±0.81[a]	23.36±0.17[a]
pH 2 organic phase	15.22±0.80[e]	15.79±0.64[c]
Mixture of pH 2	20.76±0.46[c]	20.91±0.45[b]
pH 2 control	13.74±0.47[f]	13.74±0.47[d]
pH 8 aqueous phase	22.67±0.38[b]	22.29±1.06[ab]
pH 8 organic phase	8.17±0.08[g]	12.22±0.21[d]
Mixture of pH 8	16.86±0.34[d]	13.76±1.31[d]
pH 8 control	-	-

In the same column, values with different small letter superscripts signify significant difference (P<0.05).

Antibacterial tests

The concentrations of each bacteria were adjusted to 1×10^8 CFU/mL by sterile saline. Aliquots of 100 µL of each bacterium suspension were spread on nutrient agar medium (peptone 10 g/L, beef extract 3 g/L, sodium chloride 5 g/L, agar 15 g/L, final pH 7.3±0.2) plates. Three Oxford cups (8 mm diameter) were placed equidistantly on each plate. Aliquots of 200 µL of each antibacterial compound from yeast in koumiss were added to one Oxford cup and incubated for 24 h at 37°C. Inhibition zones were measured using a vernier caliper (Zhang et al. 2013). The tests were carried out in triplicate.

MICs and MBCs

Minimum inhibition concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using a broth microdilution method (Skovgaard et al. 2013). Briefly, aliquots of 100 µL of each bacterial suspension were adjusted to 10^6 CFU/mL, and mixed with 100 µL of each antibacterial compound from yeast in koumiss in each well. Antibacterial compounds were prepared by a double serial dilution method, and the concentrations of each solution ranged from 0.01-1.00 g/mL. MIC was determined as the lowest concentration that inhibited visible bacterial growth after 24 h. One control well was included in each measurement. After incubation for 24 h, aliquots of 10 µL from each well were spotted onto nutrient agar medium plates. MBC was determined as the lowest concentration with no bacterial growth after 48 h. The experiments were carried out in triplicate.

Statistical analyses

All data were expressed by mean±standard deviation (SD). The data of each group were subjected to analysis of variance (ANOVA) test. Statistical analyses were performed using SPSS version 13.0 software. $p<0.05$ was considered statistically significant.

Results

Preparation of antibacterial compounds from yeasts in koumiss

Aqueous phases of *K. marxianus* at both pH 2.0 and pH 8.0 produced significantly larger inhibition zones than those in other phases ($p<0.05$, Table 1), which were then freeze-dried for 48 h, and K2 and K8 were obtained. Aqueous phases of *S. cerevisiae* at both pH 2.0 and pH 8.0 produced significantly larger inhibition zones than those in other phases ($p<0.05$), which were then freeze-dried for 48 h, and S2 and S8 were obtained. The main components in K2, K8, S2 and S8 were organic acids and killer toxins. Among these components, K2 had more propanoic acid than others ($p<0.05$, Table 2). S2 had more oxalic, tartaric, formic, ascorbic, lactic, citric and malic acids than others ($p<0.05$), but it had no propanoic acid. The contents of killer toxins did not differ significantly among the four compounds ($p>0.05$).

Effects of compounds from yeasts in koumiss against Gram-negative and Gram-positive bacteria

Effects of antibacterial compounds from yeasts in koumiss against Gram-negative bacteria were better than those against Gram-positive bacteria (Table 3). The inhibition zone of S8 on *S. typhimurium* was larger than that in other groups ($p<0.05$). There were no significant differences among the inhibition zones of four antibacterial compounds from yeasts in koumiss against enterohemorrhagic *E. coli* ($p>0.05$). The inhibition zone of K8 against *P. aeruginosa* was the largest, but there were no significant differences among the

Table 2. Main components of antibacterial compounds from yeasts in koumiss (mg/100 g).

Antibacterial compound	*K. marxianus*		*S. cerevisiae*	
	K2	K8	S2	S8
Oxalic acid	83.43±7.59^{b}	50.63±3.01^{c}	159.21±2.22^{a}	73.91±6.55^{b}
Tartaric acid	259.82±11.89^{c}	212.98±10.80^{d}	403.87±14.68^{a}	324.77±7.73^{b}
Formic acid	484.83±15.55^{b}	248.09±11.85^{d}	768.27±10.09^{a}	375.34±15.86^{c}
Ascorbic acid	743.90±14.96^{b}	405.32±7.15^{d}	1157.53±34.75^{a}	615.68±11.32^{c}
Acetic acid	49.67±2.77^{c}	62.96±5.76^{b}	82.61±6.63^{a}	81.56±6.80^{a}
Lactic acid	680.79±26.55^{c}	672.96±20.12^{c}	971.03±28.85^{a}	838.57±27.17^{b}
Propionic acid	2711.30±56.34^{a}	1874.70±39.23^{c}	0.00±0.00^{d}	2340.91±86.85^{b}
Citric acid	281.08±15.34^{b}	190.02±10.91^{b}	6595.90±86.06^{a}	244.11±4.82^{b}
Malic acid	673.12±12.30^{b}	13.15±1.90^{d}	778.12±15.45^{a}	93.52±4.12^{c}
Protein concentrations	74.99±4.40^{a}	74.13±5.00^{a}	70.99±4.59^{a}	68.31±3.40^{a}

In the same row, values with different small letter superscripts signify significant difference ($p<0.05$).

Table 3. Antibacterial spectrum of compounds from yeasts in koumiss.

	Pathogenic bacterium	Inhibition zone (mm)			
		K2	K8	S2	S8
Gram- negative bacteria	*Salmonella Typhimurium*	19.04±0.74^{b}	20.31±0.90^{b}	19.45±0.77^{b}	23.30±0.43^{a}
	Enterohemorrhagic E. coli	18.37±0.75^{a}	20.03±0.97^{a}	20.19±0.83^{a}	18.47±0.31^{a}
	Pseudomonas aeruginosa	21.08±1.41^{ab}	22.19±1.17^{a}	19.01±1.06^{b}	21.03±1.11^{ab}
Gram- positive bacteria	*Bacillus cereus*	17.80±0.52^{a}	15.35±1.33^{ab}	15.01±1.45^{b}	9.00±0.88^{c}
	Staphylococcus aureus	17.01±1.03^{b}	20.50±1.02^{a}	15.15±1.04^{b}	15.40±0.79^{b}
	Streptococcus agalactiae	12.03±0.57^{c}	16.17±0.58^{a}	14.67±0.39^{b}	16.00±0.82^{ab}

In the same row, values with different small letter superscripts signify significant difference ($p<0.05$).

inhibition zones of K8, K2, and S8 against *P. aeruginosa* ($p>0.05$). The inhibition zones of K2 and K8 against *B. cereus* were larger than those in other groups ($p<0.05$), but there were no significant differences between these two groups ($p>0.05$). The inhibition zone of K8 against *S. aureus* was larger than those in other groups ($p<0.05$). The inhibition zones of K8 and S8 against *S. agalactiae* were larger than those in other groups ($p<0.05$), but there were no significant differences between these two groups ($p>0.05$).

MICs and MBCs of antibacterial compounds from yeasts in koumiss against Gram-negative and Gram-positive bacteria

In the MICs and MBCs tests against Gram-negative bacteria, the following antibacterial effects were the best (Table 4): K8 and S8 against *S. Typhimurium*; S8 against enterohemorrhagic *E. coli*; K2 and S2 against *P. aeruginosa*; In the MICs and MBCs tests against Gram-positive bacteria, the following antibacterial effects were the best (Table 4): K2 and S2 against *B. cereus*; K8 against *S. aureus*; S2 on *S. agalactiae*.

Effects of compounds from yeasts in koumiss against five pathogenic *E. coli* strains

There were no significant differences among the inhibition zones of four antibacterial compounds from yeasts in koumiss against *E. coli* O_1 and *E. coli* O_2 ($p>0.05$, Table 5). The inhibition zone of K2 against *E. coli* O_8 was larger than those in other groups ($p<0.05$). The inhibition zones of K2 and S2 against *E. coli* O_{78} were larger than those in other groups ($p<0.05$), but there were no significant differences between these two groups ($p>0.05$). The inhibition zone of K2 against *E. coli* O_{86} was larger than those in other groups ($p<0.05$).

MICs and MBCs of antibacterial compounds from yeasts in koumiss against five pathogenic *E. coli* strains

The best antibacterial effects were as follows (Table 6): K2 and S2 against *E. coli* O_1, *E. coli* O_8 and *E. coli* O_{78}; K2 and S2 against *E. coli* O_2; K2 against *E. coli* O_{86}.

Table 4. MICs and MBCs of antibacterial compounds from yeasts in koumiss against Gram-negative and Gram-positive bacteria (g/mL).

	Pathogenic bacterium	K2		K8		S2		S8	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram- negative bacteria	*Salmonella Typhimurium*	0.06	0.25	0.02	0.25	0.06	0.13	0.02	0.13
	Enterohemorrhagic *E. coli*	0.06	0.25	0.13	0.25	0.06	0.13	0.02	0.13
	Pseudomonas aeruginosa	0.02	0.25	0.06	0.50	0.02	0.03	0.06	0.50
Gram- positive bacteria	*Bacillus cereus*	0.01	0.13	0.13	0.25	0.01	0.06	0.06	0.13
	Staphylococcus aureus	0.06	0.25	0.02	0.25	0.06	0.13	0.13	0.50
	Streptococcus agalactiae	0.02	0.25	0.02	0.25	0.01	0.13	0.02	0.13

Table 5. Effects of antibacterial compounds from yeasts in koumiss against five pathogenic *E. coli* strains.

Pathogenic *E. coli*	Inhibition zone (mm)			
	K2	K8	S2	S8
E. coli O_1	24.38±1.14^a	22.56±0.87^a	23.12±0.94^a	21.64±0.97^a
E. coli O_2	20.50±1.63^a	20.08±1.55^a	20.26±1.03^a	18.32±0.58^a
E. coli O_8	23.22±0.49^a	20.16±0.71^b	21.32±0.86^b	19.58±0.33^b
E. coli O_{78}	25.26±0.79^a	16.10±0.80^c	23.62±1.01^a	21.04±0.39^b
E. coli O_{86}	23.66±0.85^a	20.02±0.90^b	19.28±0.47^b	17.02±0.39^c

In the same row, values with different small letter superscripts signify significant difference ($p<0.05$).

Table 6. MICs and MBCs of antibacterial compounds from yeasts in koumiss against five pathogenic *E. coli* strains (g/mL).

Pathogenic *E. coli*	K2		K8		S2		S8	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli O_1	0.03	0.13	0.06	0.25	0.03	0.13	0.06	0.50
E. coli O_2	0.03	0.25	0.13	0.50	0.03	0.50	0.13	1.00
E. coli O_8	0.03	0.13	0.13	0.25	0.03	0.13	0.13	0.25
E. coli O_{78}	0.03	0.13	0.13	-	0.03	0.13	0.13	0.50
E. coli O_{86}	0.03	0.13	0.06	0.25	0.06	1.00	0.13	1.00

Discussion

Aqueous phases of *K. marxianus* pH 2.0, *K. marxianus* pH 8.0, *S. cerevisiae* pH 2.0, and *S. cerevisiae* pH 8.0, were freeze-dried to obtain K2, K8, S2 and S8. The inhibition zone of the control at pH 2.0 was smaller than the inhibition zones of aqueous phases at pH 2.0. So, low pH had an effect to inhibit *E. coli* O_8, but antibacterial compounds from yeasts in koumiss played the main roles to inhibit *E. coli* O_8. Their organic acids and killer toxins were different, so the antibacterial effects might be diverse.

Effects of four antibacterial compounds from yeasts in koumiss on three Gram-negative bacteria, three Gram-positive bacteria, five *E. coli* and their antibacterial spectrum were determined by the Oxford cup method and the broth microdilution method. Combining these two methods allowed the determination of the effects of four antibacterial compounds from yeasts in koumiss on the growth of pathogenic bacteria and *E. coli* (Wookey et al. 2004, Elayaraja et al. 2014). Fakruddin et al. (2017) reported that a *Saccharomyces cerevisiae* strain isolated from fruit showed better antibacterial effect against *B. cereus*, *S. Typhimurium*, *P. aeruginosa*, and *E. coli*, which is consistent with our results.

Gram-positive bacteria are generally more sensitive to essential oils than Gram-negative bacteria due to their outer membrane and a unique periplasmic space (Nikaido 1996). The essential oil degrades the cell wall of the organism by interacting with the essential oil component which causes disruption of the cytoplasmic membrane and damages the membrane protein (Sikkema et al. 1994). However, some substances containing essential oil also show antibacterial effects against Gram-negative bacteria, which may be due to the synergistic effect of essential oil and other constituents. For instance, the essential oil of *Anethum sowa* L had appreciable antimicrobial activity (Saleh-E-In et al. 2017). The four antibacterial compounds from

yeasts in koumiss had better antibacterial effects on Gram-positive bacteria and Gram-negative bacteria, possibly because of the antibacterial compounds from yeasts in koumiss had essential oil, thereby, their antibacterial effects being similar to Chinese herbal medicines.

The effects of four antibacterial compounds from yeasts in koumiss against *S. Typhimurium*, *B. cereus*, and *S. aureus* using the Oxford cup method were consistent with the results obtained by the broth microdilution method, but the results of the other three pathogenic bacteria were not consistent with the results obtained by the broth microdilution method. This may due to their different pH values. Moreover, S2 had better antibacterial effects against *B. cereus*, *P. aeruginosa*, *S. agalactiae* at low concentration.

The four antibacterial compounds from yeasts in koumiss had better antibacterial effects against five pathogenic *E. coli*. Their effects as determined by the Oxford cup method were consistent with the results obtained by the broth microdilution method, suggesting that combination of these two methods could correctly reflect their antibacterial spectrum and antibacterial effects. The MICs of *Glycyrrhiza*, *Rheum officinale*, and *Sophora flavescens* of Chinese herbal medicine against *E. coli* were between 0.004-0.375 g/mL (Liu et al. 2003), approaching the MICs of the four antibacterial compounds from yeasts in koumiss against *E. coli*. It seems that the four antibacterial compounds from yeasts in koumiss may have the same antibacterial activities as Chinese herbal medicines.

In summary, four antibacterial compounds from yeasts in koumiss had better antibacterial effects on three Gram-negative, three Gram-positive bacteria, and five strains of *E. coli*, demonstrating that they had a broad antibacterial spectrum, with potential to be used as broad-spectrum antibacterial agents. Compared with the four antibacterial compounds from yeasts in koumiss, the antibacterial effects of K2 and S2 were better than those of K8 and S8. However, the antibacterial effects of K8 and S8 on some bacteria were better, showing their potential to be utilized as targeted antibacterial agents.

We utilized antibacterial compounds from yeasts in koumiss which are natural antibacterial agents, and found that they had broad antibacterial spectrum and better antibacterial effects. Previous reports revealed that the physiological properties, metabolic performance, and growth rates of different sources of yeasts vary (Csoma et al. 2010, Lane et al. 2011). The research on antibacterial compounds from yeasts in koumiss is in its early stages. It will provide theoretical foundation for exploiting and utilizing them.

Conclusions

Four antibacterial compounds from yeasts in koumiss had broad antibacterial spectrum. In addition, K2 and S2 were better than K8 and S8.

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