



J. Dairy Sci. 101:1–8
<https://doi.org/10.3168/jds.2017-13349>
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Baicalin inhibits *Escherichia coli* isolates in bovine mastitic milk and reduces antimicrobial resistance

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ABSTRACT

In this study, we aimed to evaluate the inhibitory effect of baicalin on *Escherichia coli* in vitro and the effects of baicalin treatment on antimicrobial resistance of the *E. coli* isolates. Through isolation, purification, and identification, a total of 56 *E. coli* strains were isolated from 341 mastitic milk samples. The study of inhibition effect of baicalin on the *E. coli* strains in vitro was focused on permeability and morphology of the isolates using an alkaline phosphatase kit and scanning electron microscopy. Furthermore, the resistance spectrum of the isolates to the common antimicrobial agents was tested at sub-minimum inhibitory concentrations of baicalin by the agar dilution method. Extended-spectrum β -lactamase and plasmid-mediated quinolone resistance genes were amplified by PCR before and after incubation with baicalin. The results revealed that baicalin has certain inhibitory effects on the isolates in vitro. The alkaline phosphatase enzyme activity was significantly increased from 1.246 to 2.377 U/100 mL, and the surface of *E. coli* was concave and shriveled. Analysis of the resistance spectrum and PCR amplification showed that, after administration with baicalin, the sensitivity of most strains to the selected antimicrobial agents was enhanced. Strikingly, the drug-resistant genes from 71.43% (40/56) of these isolates were found to have drug-resistant genes to different extents. Altogether, the current study confirmed both the inhibitory effect on *Escherichia coli* in vitro and the reduction of antimicrobial resistance by baicalin. This is the first comprehensive study to report on baicalin, a traditional Chinese medicine that acts on *E. coli* isolated from the mastitic milk samples.

Key words: baicalin, *Escherichia coli*, multi-drug resistance, bovine mastitis

INTRODUCTION

Bovine mastitis, inflammation of the mammary gland, is a damaging and costly disease facing dairy farmers throughout the world (Halasa et al., 2007; Heikkilä et al., 2012). Due to the large-scale adoption of the 5-point plan, mastitis has been successfully controlled in many countries for decades (Neave et al., 1969; Hillerton et al., 1995; Zadoks et al., 2002), although the incidence of pathogens, such as *Escherichia coli* (Olde Riekerink et al., 2010), *Staphylococcus aureus* (Olde Riekerink et al., 2008), and *Streptococcus uberis* (Levison et al., 2016), is still prevalent on many farms. As a major pathogen causing environmental mastitis, *E. coli* is difficult to be cured because of its ubiquitous existence, easy transmission, and rapidly emerging drug resistance properties (Olde Riekerink et al., 2010; Steeneveld et al., 2011; Saini et al., 2012; Dahmen et al., 2013).

For successful implementation of a clinical *E. coli* mastitis control program, it is important to identify the pathogen. This microbiological analysis can be done through isolation and identification of pathogenic bacteria from infected cows. Antimicrobial agents are used for therapeutic as well as preventive measures against bacterial infections including bovine mastitis in farm animals (Lindeman et al., 2013). Unfortunately, several recent studies have reported the increasing occurrence of highly multi-drug-resistant *E. coli* isolated from food-producing animals from various countries including China (Rao et al., 2014; Xu et al., 2015; Seni et al., 2016). Therefore, the need for a safe, novel, cost-effective, alternative therapeutic strategy, especially of herbal origin, is extremely urgent. Susceptibility of bacteria to antimicrobial agents decreases with increasing resistance. Because mechanisms of bacterial resistance are numerous and intractable, reducing or eliminating antimicrobial resistance is challenging.

Traditional Chinese medicines have long been used to treat a wide range of pathologies, and research about their mechanisms of pharmacological activity is very extensive. Some reports have shown that *Scutellaria baicalensis*, one of the most popular and multi-purpose herbs, has strong antimicrobial effects in vitro. Related

Received June 19, 2017.

Accepted October 11, 2017.

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research has reported that *S. baicalensis* contains numerous flavonoids, such as baicalin, baicalein, wogonin, and chrysin (Li and Chen, 2005). Some of these flavonoids such as baicalin have been demonstrated to have antioxidant (Chan et al., 2008), antibacterial (Huang, 1999), antiviral (Huang et al., 2000), and antiinflammatory activities (Huang et al., 2006). In addition to its multi-purpose pharmacological actions, baicalin's bacteriostatic mechanisms include destruction of bacterial cell membranes; inhibition of bacterial DNA, RNA, and protein biosynthesis; and degradation of endotoxins (Wu, 2011). However, no research has evaluated the effects of baicalin on *E. coli* isolated from mastitic milks. Therefore, in the present study, we isolated *E. coli* from mastitic milk samples and investigated the antibacterial activity of baicalin against *E. coli* in vitro and its effect on drug resistance.

MATERIALS AND METHODS

Materials

Baicalin (purity >95%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Antimicrobial pretreated paper discs and biochemistry reaction tubes were purchased from Hangzhou Tianhe Micro-organism Reagent Co. Ltd. (China). Mueller-Hinton, MacConkey (MAC), eosin methylene blue agar, Luria-Bertani (LB), and Mueller-Hinton broth medium were purchased from Beijing Aoboxing Bio-Tech Co. Ltd. (China). Taq DNA polymerase was purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd. (China). An alkaline phosphatase (AKP) kit was purchased from Nanjing Jiancheng Bioengineering Institute (China). All other chemicals were of reagent grade.

Bacteriological Examination

Milk samples of mastitic cows ($n = 341$) were collected from 26 dairy herds located in 4 cities (Baoji, Weinan, Xi'an, and Yangling) of Shaanxi province of China. Milk samples were collected using procedures outlined by Rajala-Schultz et al. (2004). Briefly, dirt, bedding, and hair were brushed from teats using a dry paper towel. Teats were then dipped in 4% sodium hypochlorite. Germicide was allowed to contact teats for at least 30 s before being thoroughly dried with single-service paper towels. Teat ends were scrubbed with cotton balls moistened with 70% ethyl alcohol. The 10-mL milk samples collected from each mammary quarter at the front end were discarded. Five to 10 mL of milk from each mammary quarter was collected

into a separate sterile polypropylene test tube. Samples were frozen and transported immediately to the laboratory for microbiological culture.

The milk samples were inoculated in LB broth and incubated for 12 to 18 h at 37°C. Then, the incubated samples were streaked on a MAC plate for 18 to 24 h at 37°C. With a sterile loop, 1 or 2 pink colonies from the MAC plate were picked up and streaked on the other MAC plate for 18 to 24 h at 37°C. Further identification of *E. coli* was performed according to the methods described by the National Mastitis Council (Barnes Pallesen et al., 1987). Routine bacterial examination, such as colony morphology, Gram stain test, typical growth on eosin methylene blue agar (Hogan et al., 1999), and micro-biochemical reaction were performed. The specific 16S rRNA sequence of *E. coli* was also analyzed by PCR (Zhu et al., 2007). The primer (forward: 5'-AAGAAGCTTGCTTCTTTGCTG-3'; reverse: 5'-GAGCCCGGGGATTTCACAT-3') was finally used to amplify the 16S rRNA gene. The cycling conditions were an initial step of 5 min at 94°C, 35 cycles of 30 s at 94°C, 40 s at 54°C, and 1 min at 72°C, followed by 10 min at 72°C.

Antibacterial Activity

The minimal inhibitory concentration (MIC) of baicalin against the test bacteria was determined by broth dilution according to a modified method of Bandyopadhyay et al. (2008). With a sterile loop, 4 to 5 colonies of *E. coli* were picked up and suspended in 3 mL of LB broth and adjusted to 1×10^4 cfu/mL by dilution with LB broth. Fifty microliters of the bacteria suspension was added into each well of a sterile cell culture 96-well plate. Baicalin were diluted with LB broth to prepare a range of concentrations (1, 3, 6, 9, and 12 mg/mL); then, 100-μL samples were added into each test well containing the bacteria. For the positive control, LB broth was added at 100 μL in wells containing 50 μL of bacteria. For the negative control, LB broth was added at 150 μL in wells containing no bacteria. Finally, 15 μL of resazurin (1 mg/mL) was added into each well. Three parallels of the well were made in each group. The MIC was defined as the lowest concentration of the samples at which the microorganism did not demonstrate visible growth after 24 h of incubation at 37°C. Microorganism growth was indicated by color change of the resazurin from blue to pink.

Bacteria Cell Wall Permeability

To examine the effect of baicalin on permeating of the *E. coli* cell wall, the methods of Zhou et al. (2016)

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