

Raw cow's milk relatively inhibits quorum sensing activity of *Cromobacterium violaceum* in comparison to raw she-camel's milk

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Abstract

Milk from different animal species has variable levels of antimicrobial factors against some of spoilage bacteria. For example, they are significantly present in higher concentration in she-camel's milk than in cattle or buffalo and they are more heat-resistant than their counterparts in cattle and buffalo. Spoilage bacteria are known to communicate with each other by release of signaling molecules, a phenomenon described as quorum sensing (QS). Some food matrices inhibit these signaling compounds. In this study we screened QS inhibitory activities in raw milk of cattle and camel. Ten samples each of fresh raw cow's milk and she-camel's milk from apparently healthy animals were screened using the bacterial model *Cromobacterium violaceum*. The tested cow's raw milk samples were able to inhibit the production of QS signalling molecules acyl-homoserine lactones (AHLs) produced by *C. violaceum*. However, she-camel's milk samples were less effective in inhibiting such AHLs. Thus, one of the factors which influence the inhibitory activity could be derived from variation in milk chemical composition, especially in the percentage of fat which is significantly higher in tested cow's milk samples (2.22±0.12) than in tested she-camel's milk samples (1.44±0.35). Natural inhibition of QS signaling by cow's milk may offer a unique means to control foodborne pathogens and reduce microbial spoilage.

Keywords: Quorum, Sensing, Inhibition, *Cromobacterium violaceum*, Milk.

Introduction

Milk is an established and healthy food source of energy, proteins, vitamins, and minerals. In addition to its value as a nutrient source, interest has arisen in the ability of milk to kill bacteria and in how this knowledge can be applied to mastitis control, human health, and functional foods for people.

Milks from different species contain different amounts of various antimicrobial factors. Cow's milk has high lactoperoxidase, but low lactoferrin and lysozyme, while human breast milk has high lactoferrin and lysozyme, but low lactoperoxidase.

She-camel's milk contains all essential nutrients as cow's milk (Elagamy *et al.*, 1998), and also has a high biological value due to the higher content of antimicrobial factors such as lysozyme, lactoferrin and immunoglobulins (Elagamy *et al.*, 1992). The ability to alter the activity of these anti-microbial factors in milk could have an impact on shelf-life of raw milk and development of additional health and functional foods based upon these factors.

On the other hand, some pathogenic and spoilage bacteria are able to regulate the phenotypic characteristics as a function of cell density under the control of chemical signal molecules (Gram *et al.*,

2002). These auto-inducer molecules have been identified as oligopeptides in Gram-positive bacteria and acylated homoserine lactones (AHLs) in Gram-negative bacteria (Novick *et al.*, 1993).

The ability of the bacteria to communicate, sense and respond to population density is termed "quorum sensing" (QS). Worthily, many bacterial physiological functions such as luminescence, virulence, motility, sporulation, biofilm formation, etc., are regulated by QS systems (Gram *et al.*, 2002). It was, therefore, hypothesized that if QS systems regulate bacterial mechanisms in food spoilage, then inhibition of the communication underlying the QS systems could be a good strategy to reduce or prevent the spoilage reactions.

Investigating the importance of QS during bacterial pathogenesis and spoilage, research has focused on inhibiting QS using bacterial biosensors and indicators (Choo *et al.*, 2006). Screening for AHL production from bacterial strains has typically relied on bacteriological monitoring systems, which consist of a phenotypic response, activated through an AHL-receptor protein (Ravn *et al.*, 2001). This is the case of *C. violaceum*, a Gram-negative water and soil bacterium highly sensitive to most short-chained un-

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substituted AHLs, whose phenotypic response is the production of violacein, a water-insoluble purple pigment with antibacterial activity (McClellan *et al.*, 1997).

Except for the halogenated furanones from the red alga *Delisea pulchra*, most of the identified QS-inhibiting compounds of non-bacterial origin have come from plant origin (Teplitski *et al.*, 2000; Bauer and Teplitski, 2001; Choo *et al.*, 2006).

Although QS inhibitory activity of food of animal origin such as poultry meat-derived fatty acids has been reported (Widmer *et al.*, 2007), no information has been published regarding the QS inhibitory activity in milk from different animal species. The purpose of the current study was to investigate the QS inhibitory activity of cow's and she-camel's milk against *C. violaceum* to discover possible existence of novel and natural QS inhibitors.

Materials and Methods

Bacterial strains and culture conditions

Ten samples each of fresh raw cow's and she-camel's milk from apparently healthy animals were collected from Tripoli zoo, Libya, and submitted to the laboratory within approximately 6 hours of milking in an ice box container.

The bacterial strains used in this study are listed in Table 1. Bacterial strains were cultured in nutrient medium at optimum growth temperature of 25°C.

Table 1. Bacterial strains used in this study

ATCC12472 ^a	<i>C. violaceum</i> wild-type.
CV026 ^b	<i>C. violaceum</i> CV026 (a mini-Tn5 mutant) was used as an indicator strain for the detection of <i>N</i> -acyl-homoserine lactones (AHLs) with <i>N</i> -acyl side chains of four to eight carbons. CV026 responds to AHLs by inducing the synthesis of the purple pigment violacein (McClellan <i>et al.</i> , 1997).

^aAmerican Type Culture Collection (ATCC) reference strain.
^bCV026 strain was kindly provided by Professor Juan E. González (Department of Molecular and Cell Biology, University of Texas, Austin, TX).

The release of QS signals from *C. violaceum* was verified by CV026 QS white reporter strain (McClellan *et al.*, 1997). Briefly, the assay was modified to detect QS inhibitory activity in raw milk as follows: 100 µl of an overnight broth culture of CV026 reporter strain was spread on sterile nutrient plate agar using sterile bent glass rod. The agar plate was left to dry, then five wells each of 200 µl capacity were made in the agar using a sterile instrument.

10 ml of each milk sample was sterilized using Minisart® 0.45 µm-pore filter. 1 ml of each filtered sample was inoculated with 10 µl of an overnight broth culture of ATCC12472 wild-type *C. violaceum*.

Finally, 100 µl of each sterile sample and ATCC12472-mixed sample was introduced into each well in CV026-containing nutrient agar plate with incubation at 25°C for 48 hours.

The chemical composition of milk samples was determined by Lactostar automatic milk analyzer (Funke-Gerber, Germany). Lactostar analyzer was calibrated and adjusted for obtaining three successive readings for every sample; all readings were calculated and statistically recorded as average ± standard deviation (SD).

Results and Discussion

Milk is protected to different extents against microbial contaminations by natural inhibitory systems, including the lactoperoxidase/thiocyanate/hydrogen peroxide (LP) system, lactoferrins, lysozyme, immunoglobulins and free fatty acids (Elagamy *et al.*, 1992; Kappeler *et al.*, 1999). The concentration and the activity of each of these antimicrobial systems/substances depend on the animal species and on the stage of lactation. She-camel's milk is reported to have a stronger inhibitory system than that of cow's milk (Elagamy *et al.*, 1992). In particular; the levels of lysozyme and lactoferrins are reported to be two and three folds higher than those of cow's milk, respectively (Barbour *et al.*, 1984; De Valdez *et al.*, 1988; Kappeler *et al.*, 1999).

Antimicrobial proteins naturally present in milk have the ability to inhibit and/or kill a broad spectrum of bacteria. They are more heat-resistant than their counterparts in cattle and buffalo milk. In addition, the biological activity of protective proteins in heat-treated camel milk at 75°C/30 min is higher than that of cattle and buffalo milk proteins (Elagamy, 2000). The she-camel's milk and cow's milk have a bacteriostatic effect against *Listeria monocytogenes* and *Escherichia coli* O78:K80 (Benkerroum *et al.*, 2004). Cow's milk inhibited the metabolic activity of *E. coli* through the presence of both xanthine oxidase (XO) activity and the presence of nitrite, implying that XO-generated nitric oxide functions as an antibacterial agent (Hancock *et al.*, 2002).

Food spoilage is a consequence of the degrading enzymatic activity of some food-associated bacteria. Several proteolytic, lipolytic, chitinolytic, and pectinolytic activities associated with the deterioration of goods are regulated by QS, suggesting a potential role of such cell-to-cell communication in food spoilage (Ammor *et al.*, 2008).

QS-signaling relies on the release of a low-molecular mass signaling molecule into the extracellular milieu. Accumulation of the signal, which is often an AHL above a threshold concentration, indicative of a critical cell population density, activates the relevant gene expression (Swift *et al.*, 1996).

C. violaceum CV026 QS reporter strain is a non-pigmented mutant and production of the purple pigment can be induced by providing exogenous AHLs signal molecules (McClellan *et al.*, 1997). Thus, development of purple color around all she-camel's milk samples which were mixed with wild-type *C. violaceum* indicated production of AHLs signal molecules (Figure 1). On the other hand, absence of the purple color indicated presence of QS inhibitory activity in all cow's milk samples (Figure 1).

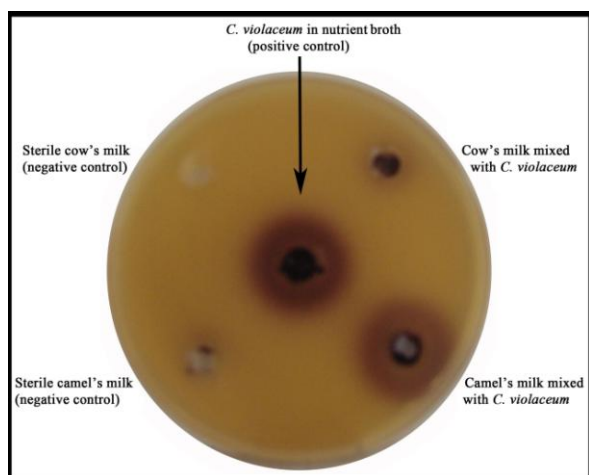


Fig. 1. QS-signaling detection assay. *C. violaceum* CV026 QS-reporter stain was evenly spread on the surface nutrient agar plate and five sample wells were made. The central well was inoculated with nutrient broth containing wild-type *C. violaceum* as a positive control. Sterile milk samples were used as negative control. Cow's milk (upper-right well) inhibited production of AHLs signaling molecules from wild-type *C. violaceum* (no purple color). She-camel's milk (lower-right well) did not inhibit production of AHLs signaling molecules from wild-type *C. violaceum* (purple color).

The identification of species-specific signals enables the competitive inhibition of QS in pathogens in or on food using natural or synthetic signal analogues. The chemical complexities of food environments offer challenges for detection, identification and control of such signaling processes with respect to food-borne bacteria (Novak, 2006).

Some components of she-camel's milk are different from those in cow's milk, and their values also differ from cow's milk and are variably estimated by different researchers. Insulin, vitamin C, niacin and some unsaturated fatty acids are higher in she-camel's milk. The absence of β -lactoglobulin and the different components of proteins in she-camel's milk may prevent allergic reactions (Wernery, 2007).

Interestingly, animal fat plays an important role in QS-inhibitory activity of food of animal origin such as in poultry, the QS-inhibitory activity is derived from several fatty acids as linoleic acid, oleic acid, palmitic

acid, and stearic acid were each tested for inhibition at 0.1, 1, and 10 mM concentrations (Widmer *et al.*, 2007). Fat percentage in she-camel's milk ($1.44 \pm 0.35\%$) is significantly less than in cow's milk ($2.22 \pm 0.12\%$) (Table 2); the increase in percentage of fat in raw cow's milk may confer QS-inhibitory activity.

Table 2. Chemical composition of tested milk samples

Constituents %	Cow's milk	She-camel's milk
Fat	2.22 ± 0.12	1.44 ± 0.35
Protein	3.81 ± 0.24	3.49 ± 0.27
Lactose	5.58 ± 0.37	5.15 ± 0.39
Solid not Fat (SnF)	10.23 ± 0.67	9.4 ± 0.72
Minerals	0.98 ± 0.1	1.04 ± 0.19

In camel, the hydration status of the animal body would determine the fat content of the milk, as well as the type of forage eaten. With the increase in water content of milk produced by thirsty camels, there was a decrease in the fat content, from 4.3 to 1.1 percent (Yagil and Etzion, 1980). Compared to cow, buffalo and ewe milk fat, camel milk fat contains less short-chained fatty acids, while the same long-chained fatty acids can be found (Dhingra, 1934).

Psychrotrophs, particularly strains of *Pseudomonas fluorescens*, dominate the microflora of refrigerated raw milk and secrete heat-stable extracellular enzymes (proteases and lipases), which survive pasteurization and even ultra-heat treatments (UHT) and degrade the casein and fat components of raw milk causing a reduction in cheese yield, gelation of UHT milk and off flavors in many dairy products (Dunstall *et al.*, 2005).

These enzymes are usually produced in the late log/early stationary growth phases when the cell density is high. This fact indicated that induction of these enzymes may be a candidate for QS. Inhibition of QS signal molecule by furanone enhanced the shelf life of fermented milk (Rani and Agrawal, 2009).

In this study we presented additional natural modulation of QS by inhibition in cow's milk, which make it suitable for use in a variety of applications, including the food shelf-life extension and for improving the health of man.

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