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Publication Date

2020-03-01

DOI

10.1016/j.idairyj.2019.104581

Peer reviewed

HHS Public Access

Author manuscript

Int Dairy J. Author manuscript; available in PMC 2021 March 01.

Published in final edited form as:

Int Dairy J. 2020 March ; 102: . doi:10.1016/j.idairyj.2019.104581.

The antimicrobial activity of bovine milk xanthine oxidase

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Abstract

Mammalian milk is a source of antimicrobial compounds such as xanthine oxidase (XO). The interplay of infant saliva, which contains the substrates for XO activity, and human milk containing XO has been recently shown to inhibit the growth of pathogenic bacteria. Based on the complex and protective mechanism observed in human milk, we hypothesized that bovine milk XO operates similarly, thus representing an opportunity to investigate its functionality in broader health implications. We demonstrated that bovine milk-hypoxanthine mixture (0 to 400 μM) inhibited several Gram-negative and -positive bacterial pathogens in a dose-dependent manner. Kinetic experiments revealed that XO catalyzed hypoxanthine reduction (K_m, 58.0 μM; V_{max}, 5.1 μmol^{-1} min^{-1} mg) resulted in the production of antimicrobial hydrogen peroxide. These results demonstrate that the antimicrobial properties of bovine milk XO are similar to those of human milk XO with significant implications for the development of novel products targeting infant health.

1. Introduction

Lactation is a biological process that has evolved to deliver a complete postnatal nutrition for newborns. Its biodynamic aspect is evidenced by compositional changes in milk throughout the lactation period to better suit the infant requirements (Hinde & German, 2012). Milk delivers all the required essential nutrients, substrates, and fuel molecules for the growth and health of the neonate. More recent research is documenting that milk has also been selected through evolution for its ability to protect the infant from bacterial pathogens. For example, milk creates a specific microenvironment for the gut microbiota of the neonate (Petherick, 2010; Smilowitz, Lebrilla, Mills, German, & Freeman, 2014; Walker, 2010). Milk contains

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antimicrobial factors and in many cases those antimicrobials are not fully functional or become functional during the transit through the gastro intestinal tract. Such a dynamic protective strategy is emerging as a common theme for milk-based antimicrobials and may reflect the unique selective pressures on milk as a product of evolution. For example, the fragments of milk proteins released during infant digestion have antimicrobial properties. These peptides are typically inactive within the sequence of the parent protein and become active when released by proteolysis (Dallas et al., 2013).

Xanthine oxidase (XO) is an example of an even more complex mechanism of protection because its substrates are not present in milk. However, with all components in place except the enzyme substrate, the secretion/addition of xanthine or hypoxanthine as substrates ostensibly controls the entire antimicrobial process. The enzyme complex works in combination with the lactoperoxidase system to produce reactive oxygen and nitrogen species that serve as antimicrobials in situ, thus protecting the mammary gland against mastitis and the offspring against bacterial infections (Al-Shehri et al., 2015; Björck & Claesson, 1979; Harrison, 2004, 2006; Millar et al., 2002; Stevens et al., 2000; Vorbach, Harrison, & Capecchi, 2003). XO has broad substrate specificity and can reduce nitrite, yielding reactive nitrogen species such as nitric oxide and peroxynitrite, a strong antibacterial agent (Hancock et al., 2002; Martin, Hancock, Salisbury, & Harrison, 2004; Millar et al., 1998). XO can also reduce oxygen to generate the reactive oxygen species superoxide (O^{2-}) and hydrogen peroxide in the presence of hypoxanthine or xanthine (Fig. 1). Non-human milk also contains XO and the biochemical properties of the XOs differ significantly among the species (Atmani, Benboubetra, & Harrison, 2004; Benboubetra, Baghiani, Atmani, & Harrison, 2004).

Until recently, milk XO was thought to perform solely a structural function in the membrane of the fat globule, a hypothesis based on the fact that milk does not contain its substrates. However, it has been proposed that XO-derived hydrogen peroxide exerts its antimicrobial effect by acting as a substrate for the lactoperoxidase system in bovine milk (Björck & Claesson, 1979) (Table 1). It has been shown that the addition of 100 μM hypoxanthine (XO substrate) to breast milk promotes the production of H_2O_2 (Stevens et al., 2000) (Table 1). Moreover, experiments carried out with animals, particularly bovine calves, suggest that animals supplemented with active XO put on weight at a faster rate than their untreated pen mates and show a 50% reduction in the incidence of infective scours (Millar et al., 2002). Hydrogen peroxide has been well described chemically and its generation has been demonstrated to inactivate various microorganisms (Baldry, 1983). Furthermore, a recent study suggested that the observed antimicrobial activity of human milk XO results from the interplay of infant saliva, which contains the required hypoxanthine and xanthine substrates for XO activity, and human milk containing XO and lactoperoxidase (Al-Shehri et al., 2015) (Table 1). During nursing, the interaction between breast milk and infant saliva enables the production of antimicrobial N and O species thus influencing the early oral microbiota and possibly the infant gut. However, the implications of XO enzyme and its biological properties towards infant health have not been well characterized.

Considering that milk is heat treated prior to human consumption to ensure its microbial safety and to improve its shelf life (Rowe & Donaghy, 2011), the choice of the heat

treatment has a significant impact on this enzyme activity. We have recently demonstrated the effects industrial heat treatments such as of batch pasteurization (BP), high temperature short time (HTST), and ultra high temperature (UHT) on the inactivation of xanthine oxidase and its antimicrobial properties. XO activity was preserved by HTST (100%), but partial (8%) and nearly complete (95%) enzyme inactivation was observed when milk was subjected to BP and UHT, respectively (Ozturk, German, & de Moura Bell, 2019).

While this complex antimicrobial mechanism has been demonstrated in human milk, there is a lack of information about the mechanism of action of bovine milk XO. We hypothesized that bovine milk XO operates similarly to human milk XO and, therefore, we investigated the functions of bovine milk XO under conditions that mimic the oral cavity (oxygen, temperature, and pH). In this study, the ability of endogenous bovine XO to generate the antimicrobial radical hydrogen peroxide under conditions mimicking the oral cavity was evaluated on the growth of *Staphylococcus aureus*. Further, we examined the effects of bovine milk-hypoxanthine mixture on the growth of a range of both Gram-negative pathogens including *Escherichia coli* O157:H7, *Klebsiella pneumoniae* and *Salmonella endocarditis* and Gram-positive pathogens including *S. aureus* MRSA, *Listeria monocytogenes* and *Enterococcus faecalis*. Antimicrobial activities were evaluated at high (10^6 cfu mL^{-1}) and low (10^3 cfu mL^{-1}) bacterial inoculum with hypoxanthine concentrations in the range of 0 to 400 μM.

2. Materials and methods

2.1. Milk samples

Fresh raw bovine milk was obtained from a UC Davis local dairy farm (Davis, California, USA) and stored immediately at −80 °C until use.

2.2. Enzyme assays and kinetic parameters

For bovine milk XO activity and kinetic parameters assays, bovine milk (stored at −80 °C) was diluted 1:100 in Tris buffer immediately before analysis. 50 μL diluted samples were mixed with 50 μL of 100 μM Ampliflu Red (Sigma-Aldrich Pty Ltd, Australia) and 0.8 U mL^{-1} horseradish peroxidase (Sigma-Aldrich Pty Ltd, Sydney, Australia) in 100 mM Tris-HCl buffer. The 100 μL mixture (sample + peroxidase reagent) was incubated at pH 7.5 for 30 min and the production of H_2O_2 resulting from the oxidation of hypoxanthine to uric acid was measured fluorometrically using a M5 plate reader (Molecular Devices, San Jose, CA, USA), for 30 min at a regular interval of 10 min. H_2O_2 in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with Ampliflu Red (10-acetyl-3,7-dihydroxyphenoxazine) reagent to generate the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 530 ± 12.5 nm and 590 ±17.5 nm, respectively. To confirm H_2O_2 was produced by XO activity only, the XO-specific inhibitor (allupurinol) was used. The fluorescence signal was measured in a temperature-controlled Molecular Devices M5 plate reader (excitation 540 nm, emission 590 nm). For each milk sample, a corresponding assay substrate blank (i.e., excluding hypoxanthine) was subtracted from each data point and the H_2O_2 standard curve was plotted. Sample ΔA (A_2−A_1) to the H_2O_2 standard curve was applied to get B nmol of H_2O_2

(H_2O_2 generated between t_1 and t_2 in the reaction). XO activity (nmol^{-1} min mL^{-1} = mU mL^{-1} = U L^{-1}) was measured according to the following equation:

$$\text{XO activity} = (B \times dilution\ factor)/[(t_2 - t_1) \times V] \quad (1)$$

where B is the amount of H_2O_2 (nmol) generated by XO from standard curve, t_1 and t_2 are the time of the first (A_1) and second readings (A_2) (in min), respectively and V is the pre-treated sample volume (mL) added into the reaction well. One unit of XO is defined as the amount of enzyme that catalyzes the oxidation of hypoxanthine, yielding 1.0 μmol of uric acid and H_2O_2 per minute at 37 °C.

2.3. Kinetic parameters

Steady state kinetic studies were carried out to determine the parameters of the Michaelis-Menten kinetic model, V_{max} and K_m, for milk XO using Graph Prism 6.0 software with non-linear plotting techniques (Michaelis, Menten, Johnson, & Goody, 2011). XO kinetic parameters were determined using final hypoxanthine concentrations of 0, 25, 50, 100, 200, and 400 μM and bovine milk XO was assayed using 100 μM hypoxanthine. Measurements were replicated at least three times. Accordingly, the reaction rate (v) of XO and the substrate concentration [S] were determined according to the Michaelis–Menten equation:

$$v = k_c[Eo][S]/(S + K_m) \quad (2)$$

where [Eo] is the concentration of enzyme, and k_c and K_m are the catalytic constant of hydrolysis and the Michaelis constant, respectively.

2.4. Antimicrobial activity assays

To demonstrate the mechanism of action of bovine milk XO and to compare our results with the existing literature, a proof-of-concept antimicrobial assay that commonly uses incubation time of 24 h and conditions that mimic the oral cavity (oxygen, temperature, and pH) (Al-Shehri et al., 2015) was employed. To determine the effects of hydrogen peroxide, *S. aureus* ATCC 29213 was used as a model microorganism for inhibition of bacterial growth. Bacterial stocks were diluted in nutrient broth to a final concentration of 100 colony-forming units (cfu) mL^{-1}. Subsequently, 50 μL aliquots were added to microtiter plate wells with serial dilutions of H_2O_2 in nutrient broth to achieve final concentrations of 0, 50, 100, 200, 300 and 400 μM. Microtiter plates were incubated at 37 °C for 24 h with shaking. Bacterial growth was determined turbidometrically using a microtiter plate reader (Biotek-Synergy-2 Microplate Reader, Biotek, Winooski, VT, USA) as absorbance at 600 nm. All assays were set up with triplicate samples unless noted otherwise.

To understand the inhibition of bacterial growth by XO, *S. aureus* ATCC 29213, was used as a model microorganism (Al-Shehri et al., 2015). Raw milk was diluted 1:20 in sterile 100 mM Tris-HCl buffer, pH 7.5. Bacteria (diluted in sterile phosphate buffer saline) were incubated with raw milk alone (control), raw milk and xanthine (milk + X), raw milk and hypoxanthine (milk + HX), and raw milk and xanthine + hypoxanthine (milk + X + HX). Each microtiter well contained 50 μL of xanthine, or hypoxanthine, or xanthine and hypoxanthine solutions with a final concentration of 0 (control), 25, 50, 100, 200, and 400

μM, 25 μL diluted milk (1:20) and 25 μL bacteria solution with a final concentration of 100 cfu mL^{-1} (in triplicate). The 96-well plates were incubated for 24 h at 37 °C with mixing at 200 rpm. Serial dilutions were made and aliquots were then spotted onto Tryptic soy agar plates, and incubated overnight at 37 °C for determining viable cfu. The assays were replicated at least three times, and error bars represent one standard deviation.

We investigated the effects of bovine milk-hypoxanthine mixture on the growth of a range of Gram-negative and Gram-positive bacterial pathogens including *E. coli* O157:H7*, K. pneumoniae, Sal. endocarditis, S. aureus* MRSA*, L. monocytogenes* and *Ent. faecalis* including high (10^6 cfu mL^{-1}) and low (10^3 cfu mL^{-1}) concentration of microorganism using final hypoxanthine concentrations (0, 50, 100, 200, 300, 400 μM). This experimental design was used to simulate a typical number of microorganisms that would be ingested and may result in an infection in the oral cavity of neonates. These assays were prepared as per the above protocol, in 96-well microtiter plates and the growth incubation conditions were used as described in the above.

2.5. Statistics

The parameters of the Michaelis-Menten kinetic model, V_{max} and K_m, for bovine milk XO were estimated using Graph Prism 6.0 software with non-linear plotting techniques (Woolf, 1931). The statistical significance of the differences compared with control values are indicated above the bars using t-test, **** $p < 0.0001$.

3. Results and discussion

3.1. Enzyme activity and kinetic parameters

Measured XO activity of bovine milk samples in this study were 170 ± 5.3 U L^{-1}. The results herein presented are in agreement with those previously presented by Ozturk et al. (2019) (200 U L^{-1}) and Demott and Praepanitchai (1978) (208 U L^{-1}), in spite of the fact that different methodologies were used: XO activities were quantified by spectrophotometric measurement of either the rate of oxidation of xanthine to uric acid (Ozturk et al., 2019) or the rate of formation of vanillic acid from vanillin (Demott & Praepanitchai, 1978). Overall, there is a wide range of variation for bovine milk XO activity reported in the literature, which might be, in part, due to different reaction conditions used by different methods to quantify XO activity (temperature, pH, and reaction substrates). For example, Zikakis and Wooters (1980) reported an average XO activity (38 °C and pH 7.5) of 61.2 μL O_2^{-1} (40 U L^{-1}), while Cerbulis and Farrell (1976) reported XO activities (25 °C and pH 7.4) for raw bovine milk from 75.7 to 151 U L^{-1} by directly using 133 μM xanthine. The observed differences could be also expected due to variations in milk associated with the sexual cycle of the cow, season, feeding regime, and breed (Cerbulis & Farrell, 1977).

The kinetics of XO in raw milk were determined using various initial concentrations of hypoxanthine over 30 min for each reaction. The reaction rate for each substrate concentration was for the first 30 min, and initial rates of reaction were therefore calculated from the equation (1). The assumption that [S]>>[E] is not valid after 30 min. Therefore, the enzyme concentration becomes limited and the reaction rate slows. The velocity of

hypoxanthine conversion by native XO measured using different substrate concentrations, is shown in Fig. 2. The direct plot was created by plotting the reaction rate (v) against the initial substrate concentration (S). K_m refers to the minimum substrate concentration that yields half of V_{max}. Based on the kinetic experiments with bovine milk XO, an apparent K_m for hypoxanthine of 60 μM and a V_{max} value of 5 μmol min^{-1} mg^{-1} were obtained. These results are in agreement with those reported by (Ozturk et al., 2019) where a V_{max} value of 7 μmol min^{-1} mg^{-1} has been reported for bovine milk XO with xanthine as substrate. These results were important for the design and interpretation of subsequent studies of bacterial inhibition by milk-hypoxanthine mixtures.

3.2. Antimicrobial properties of xanthine oxidase

There is increasing evidence that human milk XO has physiological functions associated with the production of antimicrobial, reactive oxygen and nitrogen species if the substrate is available. (Al-Shehri et al., 2015; Hancock et al., 2002; Stevens et al., 2000; Vorbach et al., 2003). XO uses O_2 as its electron acceptor and produces superoxide (O_2•) and H_2O_2 (Fig. 1). H_2O_2 has been commonly used for infection and microbial control in health care, biotechnology industry and environmental applications. We have evaluated whether the addition of micromolar concentrations of H_2O_2 would inhibit or stimulate bacterial growth. A turbidity assay was used to assess in vitro bacterial growth in standard nutrient growth medium supplemented with micromolar concentrations of H_2O_2 ranging from 0 to 400 μM, incubated at 37 °C for 24 h. The dose-dependent bactericidal effects of different concentrations of H_2O_2 are shown in Fig 3. The results demonstrated the inhibition of the Gram-positive *S. aureus* by 200 μM H_2O_2 (Fig. 3).

Having demonstrated direct inhibition of *S. aureus* growth by micromolar concentrations of H_2O_2, a more physiological assay was developed, where the H_2O_2 was generated by XO through the interaction between hypoxanthine or xanthine and bovine milk in vitro. To demonstrate inhibition of bacterial growth under physiological conditions (at pH 7.5 and 37 °C, in the presence of oxygen), the viability of *S. aureus* was examined in a medium comprising bovine milk mixed with serial dilutions of hypoxanthine. H_2O_2 generated by bovine milk and hypoxanthine mixture inhibited, in a dose-dependent manner, the viability of *S. aureus* (Fig. 4b). *S. aureus* inhibition required the addition of >100 μM hypoxanthine. It has previously been shown that addition of 100 μM hypoxanthine to breast milk promoted the production of H_2O_2 thus completely inhibiting bacterial overgrowth in milk for at least 7 days (Stevens et al., 2000).

For the use of xanthine as a substrate for the production of H_2O_2, the viability of *S. aureus* was assessed in a medium comprising bovine milk mixed with serial dilutions of xanthine from 0 to 400 μM. The bovine milk-xanthine mixture inhibited, in a dose-dependent manner, the viability of *S. aureus*. Over 200 μM of supplemented xanthine was needed to completely inhibit *S. aureus* growth (Fig. 4a). When both hypoxanthine and xanthine were added in the milk, the inhibition of *S. aureus* was achieved at levels >100 μM of each substrate ($p < 0.05$). The addition of the XO inhibitor allopurinol eliminated XO-milk generated inhibition of *S. aureus* suggesting that XO was responsible for the H_2O_2 production observed during enzymatic and microbiology assays (Fig. 4d). When allopurinol was added to prevent XO

generation of H_2O_2, no inactivation of *S. aureus* was observed (Fig. 4d) Inhibition of XO by allopurinol restored normal *S. aureus* growth, demonstrating the quantitative contribution of XO in milk to the overall killing of *S. aureus* in the milk system.

The effects of a bovine milk-hypoxanthine mixture were investigated on the growth of each of the six microorganisms at high (10^6 cfu mL^{-1}) and low (10^3 cfu mL^{-1}) (Figs. 5 and 6). We found that all species grew in bovine milk (0 μM hypoxanthine). At concentrations ≥ 50 μM hypoxanthine, *E. coli* O157:H7 *and S. aureus* MRSA were completely inhibited, independent of the initial inoculum. For *Sal. endocarditis* there was no microorganism recovery at levels ≥50 μM hypoxanthine at the low inoculum. However, *K. pneumoniae* was inhibited fully at levels >100 μM hypoxanthine with low initial inoculum. Whereas, at higher inoculum, *K. pneumoniae* was not inhibited. Additionally, 100% inhibition of *L. monocytogenes* required the addition of >200 μM hypoxanthine, whereas *Ent. faecalis* was inhibited by 60% in the presence of 400 μM hypoxanthine. In general, the bovine milk and hypoxanthine mixture inhibited, in a dose-dependent manner, the viability of these pathogens, with the initial inoculum being relevant in specific cases. Sweeney et al. (2018) investigated the effect of breastmilk XO and lactoperoxidase system and saliva (27 μM hypoxanthine and 20 μM xanthine) on the growth of ten microbial species, including high and low initial concentrations of microorganisms over time. They found that for the microorganisms tested at low (200 cfu) concentration [*Staphylococcus epidermidis, S. aureus* (MSSA), *Pseudomonas aeruginosa*] and at high (10^7 cfu) concentration [*Streptococcus pyogenes, S. aureus* (MSSA), and *Ps. aeruginosa*], after 24h incubation, the decrease in viability was significant for the groups where hypoxanthine and xanthine were added (Sweeney et al., 2018).

The abundance of XO in milk has been recognized for more than a century (Massey & Harris, 1997); however, its physiological function has remained a matter of speculation. Recent studies demonstrated an increasing evidence that XO has additional physiological functions associated with the production of antibacterial reactive oxygen species and reactive nitrogen species in the presence of its substrates (Al-Shehri et al., 2015; Björck & Claesson, 1979; Harrison, 2004; Stevens et al., 2000; Vorbach et al., 2003); however, it has remained unclear if bovine milk XO operates similarly as human milk XO in the oral cavity. Under conditions that mimic the oxygen, temperature and pH, the bovine milk xanthine oxidase and hypoxanthine mixture inhibited, the viability of a variety of human pathogens, in a dose-dependent manner. Results presented herein demonstrate that the antibacterial activity of bovine milk against microorganisms in the mouth is dependent on XO-catalyzed reduction of hypoxanthine and/or xanthine, leading to H_2O_2 production. These findings not only contribute significantly to the long-standing question of the physiological significance of XO in milk but are also relevant to a proposed biological function for this protein in mammalian newborns. The present data present opportunities for important applications in settings where the normal interaction of breast milk and saliva could be limited, particularly in the case of preterm infants. Considering the importance of XO antimicrobial activity during the breastfeeding process, which involves a short contact time between XO (in the milk) and its substrate (in the saliva) and the presence of endogenous H_2O_2 in the milk, the evaluation of the effects of shorter incubation times on the antimicrobial activity of XO is warranted. While the abundance of xanthine oxidase in human milk is a compelling

argument for its efficacy and safety, it is important to consider the pro-oxidative effects of ROS and NOS when using substrate concentrations exceeding the values naturally present in the infant saliva (50 μM) (Al-Shehri et al., 2015). A safety dossier addressing potential risks to the infant would be required when using substrate concentrations above 50 μM.

4. Conclusions

In the present study, under conditions that simulate the oxygen, temperature and pH conditions present in the oral cavity, the bovine milk xanthine oxidase and hypoxanthine mixture inhibited, in a dose-dependent manner, the viability of several human pathogens. These results suggest that this group of enzymes should be investigated beyond their immediate biological properties towards infant health.

Acknowledgements

This research was supported in part by funding from the National Institutes of Health awards R01AT007079 and R01AT008759.

References

Al-Shehri SS, Knox CL, Liley HG, Cowley DM, Wright JR, Henman MG, et al. (2015). Breastmilk-saliva interactions boost innate immunity by regulating the oral microbiome in early infancy. PLoS ONE, 10, Article e0135047.

Atmani D, Benboubetra M, & Harrison R (2004). Goats' milk xanthine oxidoreductase is grossly deficient in molybdenum. Journal of Dairy Research, 71, 7–13. [PubMed: 15068060]

Baldry MGC (1983). The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. Journal of Applied Bacteriology, 54, 417–423. [PubMed: 6409877]

Benboubetra M, Baghiani A, Atmani D, & Harrison R (2004). Physicochemical and kinetic properties of purified sheep's milk xanthine oxidoreductase. Journal of Dairy Science, 87, 1580–1584. [PubMed: 15453470]

Björck L, & Claesson O (1979). Xanthine oxidase as a source of hydrogen peroxide for the lactoperoxidase system in milk. Journal of Dairy Science, 62, 1211–1215.

Blake D, Stevens C, Eisenthal R, Harrison R, Millar TM, & Rachel E (2004). Ingestible compositions comprising antibacterial agents US Patent US20040120938A1.

Cerbulis J, & Farrell HM (1977). Xanthine oxidase activity in dairy products. Journal of Dairy Science, 60, 170–176.

Dallas DC, Guerrero A, Khaldi N, Castillo PA, Martin WF, Smilowitz JT, et al. (2013). Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective Antimicrobial peptides. Journal of Proteome Research, 12, 2295–2304. [PubMed: 23586814]

Demott BJ, & Praepanitchai OA (1978). Influence of storage, heat, and homogenization upon xanthine oxidase activity of milk. Journal of Dairy Science, 61, 164–167. [PubMed: 641238]

Hancock JT, Salisbury V, Ovejero-Boglione MC, Cherry R, Hoare C, Eisenthal R, et al. (2002). Antimicrobial properties of milk: Dependence on presence of xanthine oxidase and nitrite. Antimicrobial Agents and Chemotherapy, 46, 3308–3310. [PubMed: 12234868]

Harrison R (2004). Physiological roles of xanthine oxidoreductase. Drug Metabolism Reviews, 36, 363–375. [PubMed: 15237859]

Harrison R (2006). Milk xanthine oxidase: Properties and physiological roles. International Dairy Journal, 16, 546–554.

Hinde K, & German JB (2012). Food in an evolutionary context: Insights from mother's milk. Journal of the Science of Food and Agriculture, 92, 2219–2223. [PubMed: 22729619]

Lipmann F, & Owen CR (1943). The antibacterial effect of enzymatic xanthine oxidation. Science, 98, 246–248. [PubMed: 17752701]

Martin HM, Hancock JT, Salisbury V, & Harrison R (2004). Role of xanthine oxidoreductase as an antimicrobial agent. Infection and Immunity, 72, 4933–4939. [PubMed: 15321984]

Massey V, & Harris CM (1997). Milk xanthine oxidoreductase: The first one hundred years. Biochemical Society Transactions, 25, 750–755. [PubMed: 9388538]

Michaelis L, Menten ML, Johnson KA, & Goody RS (2011). The original Michaelis constant: Translation of the 1913 Michaelis-Menten paper. Biochemistry, 50, 8264–8269. [PubMed: 21888353]

Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, & Blake DR (1998). Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Letters, 427, 225–228. [PubMed: 9607316]

Millar TM, Kanczler JM, Bodamyali T, Blake DR, & Stevens CR (2002). Xanthine oxidase is a peroxynitrite synthase: Newly identified roles for a very old enzyme. Redox Report, 7, 65–70. [PubMed: 12189051]

Ozturk G, German JB, & de Moura Bell MLNJ (2019). Effects of industrial heat Treatments on the kinetics of inactivation of antimicrobial bovine milk xanthine oxidase. npj Science of Food, 3, Article 13.

Petherick A (2010). Development: Mother's milk: A rich opportunity. Nature, 468, S5–S7. [PubMed: 21179083]

Rowe M, & Donaghy J (2011). Microbiological aspects of dairy ingredients In Chandan RC & Kilara A (Eds.), Dairy ingredients for food processing (pp. 59–101). Oxford, UK: Wiley Blackwell.

Smilowitz JT, Lebrilla CB, Mills DA, German JB, & Freeman SL (2014). Breast Milk oligosaccharides: Structure-function relationships in the neonate. Annual Review of Nutrition, 34, 143–169.

Stevens CR, Millar TM, Clinch JG, Kanczler JM, Bodamyali T, & Blake DR (2000). Antibacterial properties of xanthine oxidase in human milk. Lancet, 356, 829–830. [PubMed: 11022933]

Sweeney EL, Al-Shehri SS, Cowley DM, Liley HG, Bansal N, Charles BG, et al. (2018). The effect of breastmilk and saliva combinations on the in vitro growth of oral pathogenic and commensal microorganisms. Scientific Reports, 8, Article 15112.

Vorbach C, Harrison R, & Capecchi MR (2003). Xanthine oxidoreductase is central to the evolution and function of the innate immune system. Trends in Immunology, 24, 512–517. [PubMed: 12967676]

Walker A (2010). Breast milk as the gold standard for protective nutrients. Journal of Pediatrics, 156, S3–S7. [PubMed: 20105662]

Woolf B (1931). The addition compound theory of enzyme action. Biochemical Journal, 25, 342–348. [PubMed: 16744585]

Zikakis JP, & Wooters SC (1980). Activity of xanthine oxidase in dairy products. Journal of Dairy Science, 63, 893–904. [PubMed: 6893206]

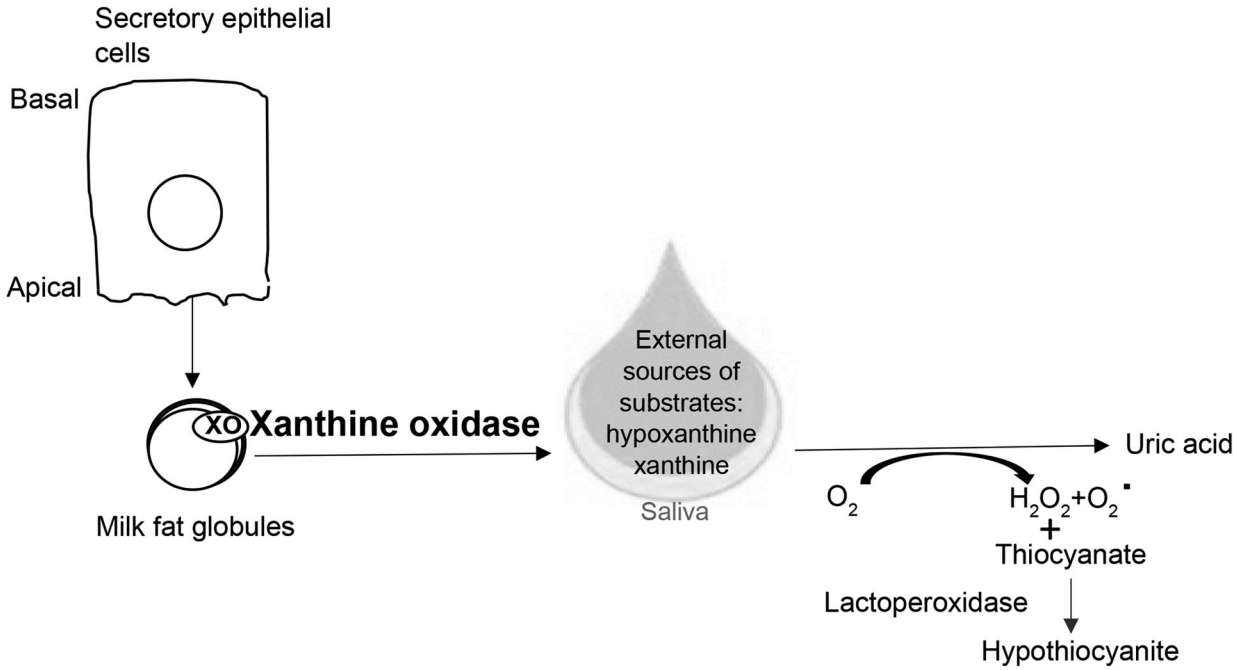

Fig. 1.

Human milk xanthine oxidase (XO) has antimicrobial properties in the presence of its substrates such as hypoxanthine and xanthine. XO is the most abundant protein component of the milk fat globule membrane. XO uses O_2 as its electron acceptor and produce superoxide (O_2•), hydrogen peroxide (H_2O_2), catalyzing the conversion of hypoxanthine into xanthine and then further into uric acid (UA). H_2O_2 subsequently acts as a substrate for the milk enzyme lactoperoxidase present in both milk and saliva to convert thiocyanate into the antibacterial hypothiocyanite (adapted from Vorbach et al., 2003).

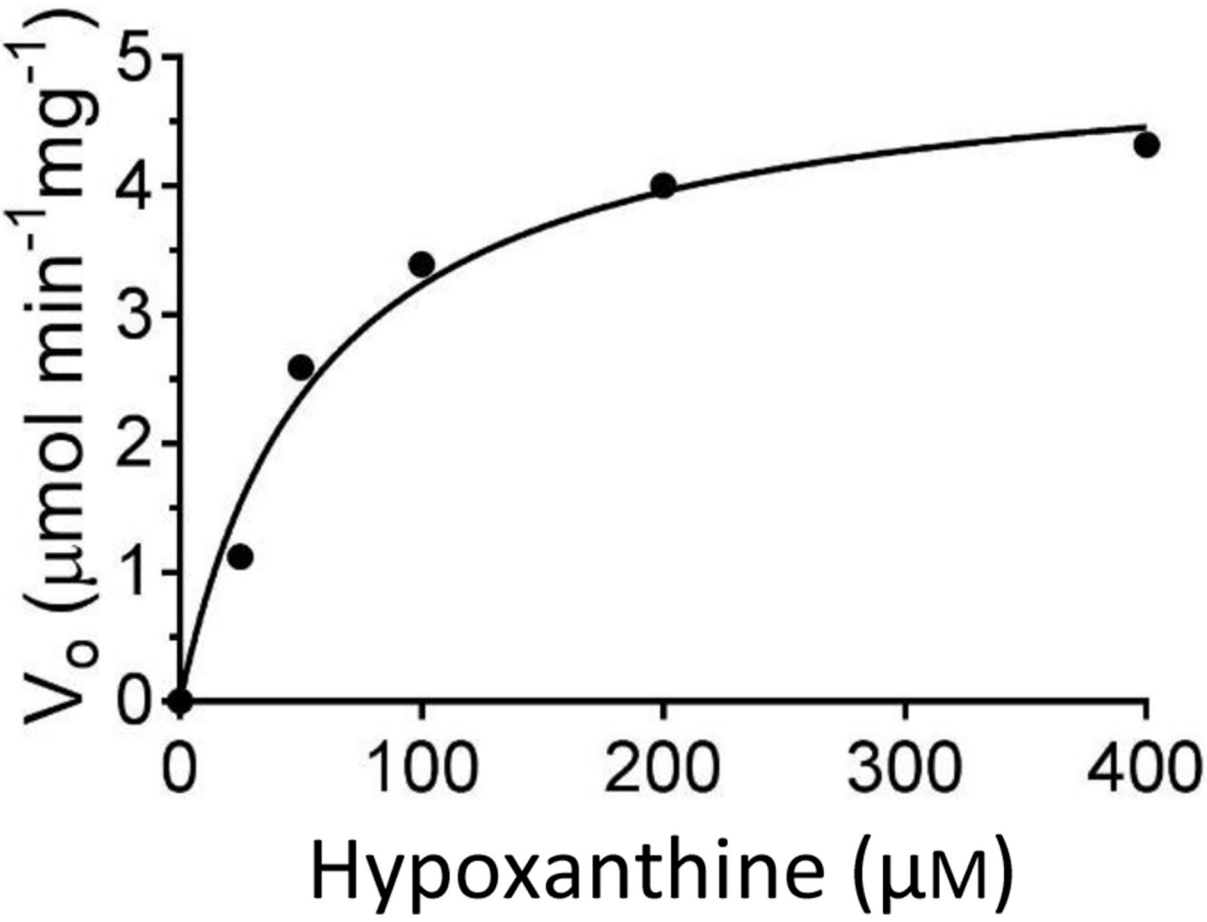

Fig. 2.

Michaelis-Menten kinetics of raw bovine milk xanthine oxidase for H_2O_2 generation. The K_m, and V_{max} data were obtained using the Michaelis-Menten equation.

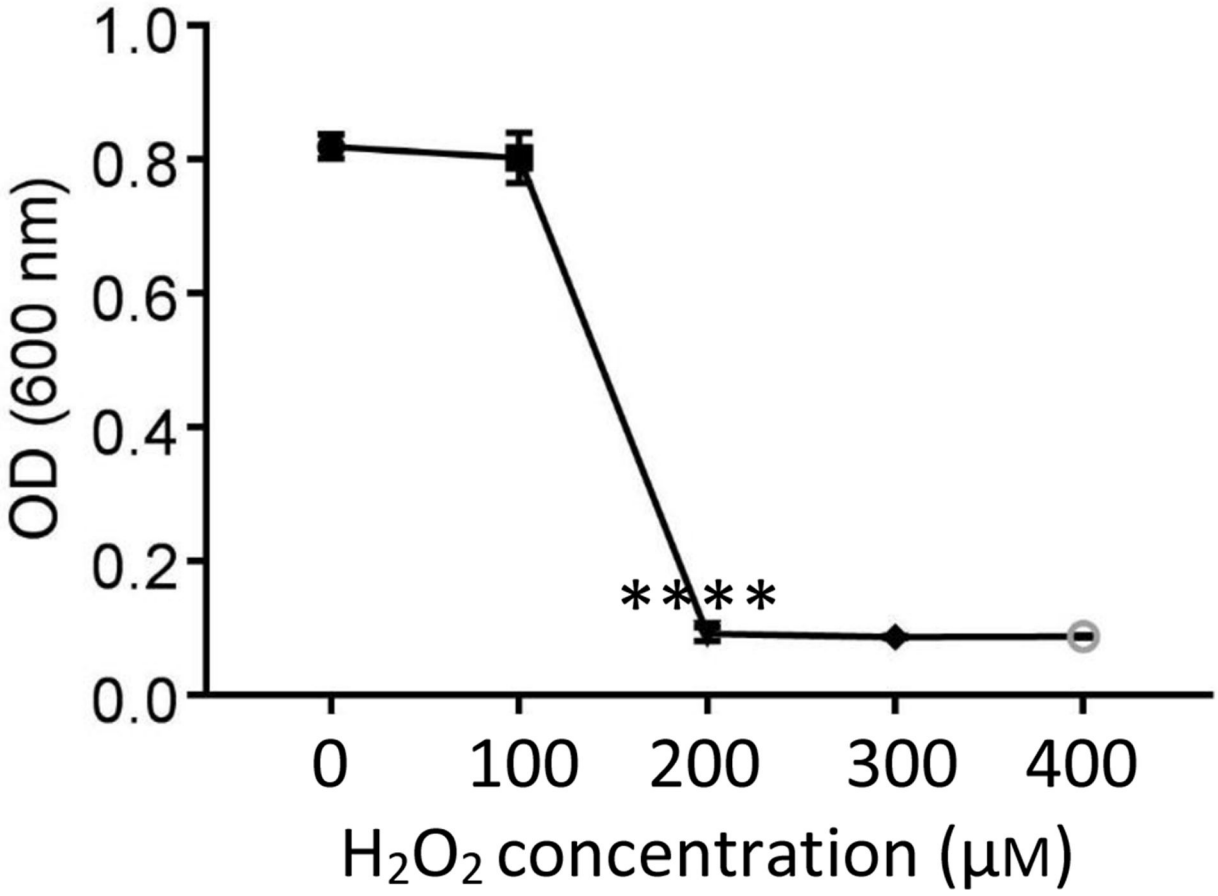

Fig. 3.

The effect of increasing H_2O_2 concentration (0–400 μM) on *S. aureus* growth. H_2O_2 was added to nutrient broth inoculated with *S. aureus* and incubated for 24 h at 37 °C. The concentration of cells was determined as turbidity by measuring absorbance at 600 nm. Values represent the mean ± SD (n = 6); **** p <0.0001 t test was used to compare means.

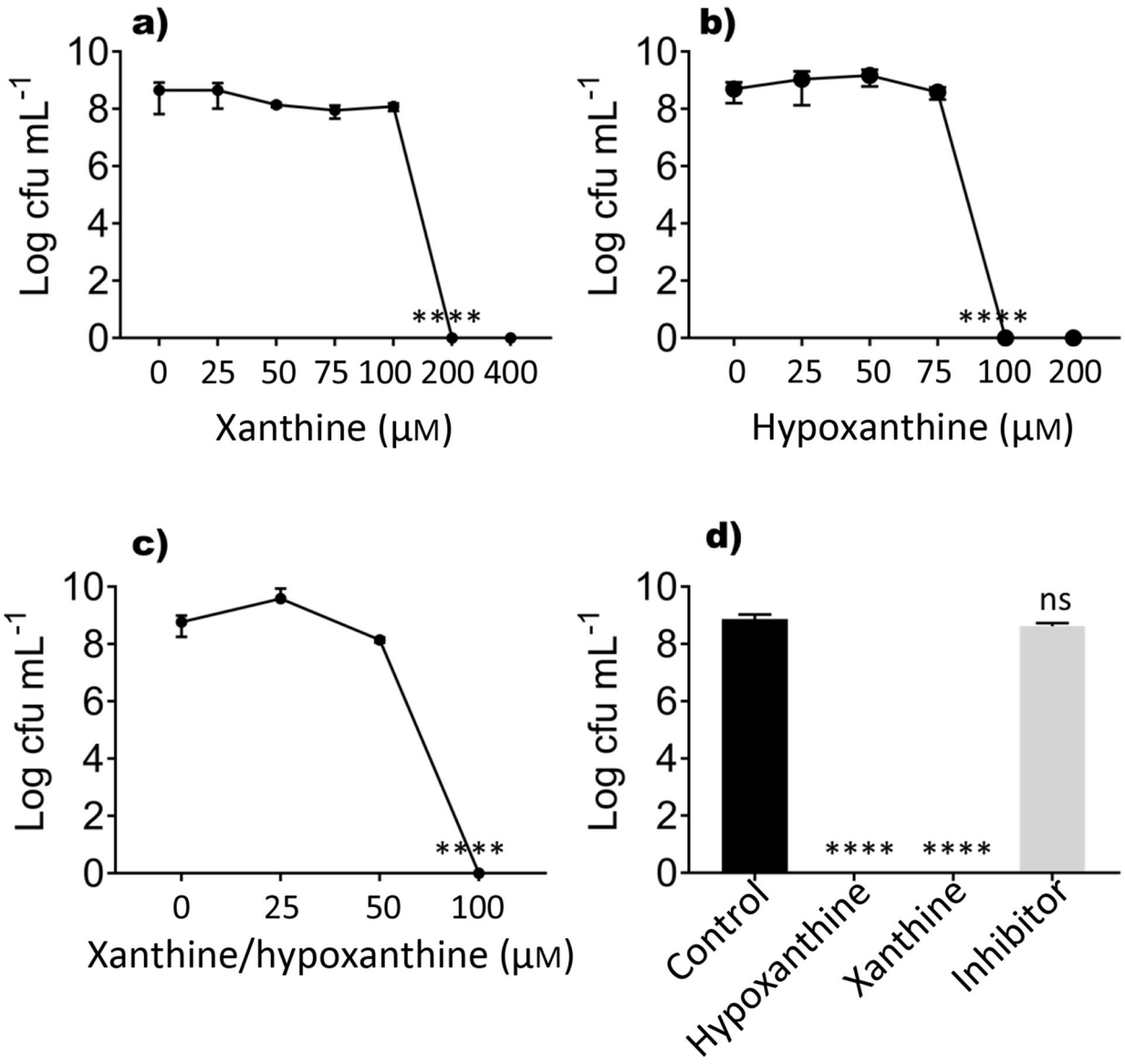

Fig. 4.

Bactericidal effects of H_2O_2 generated by bovine milk xanthine oxidase and increasing concentrations of (a) xanthine, (b) hypoxanthine or (c) xanthine and hypoxanthine (0–400 μM) and (d) hypoxanthine and allopurinol (inhibitor) for 24 h at 37 °C and then enumerated by sub-culturing onto agar plates. Values represent the mean ± SD (n = 3). Figure displays the concentration-dependent effect of hypoxanthine versus log cfu mL^{-1}; **** p <0.0001 t test was used to compare means.

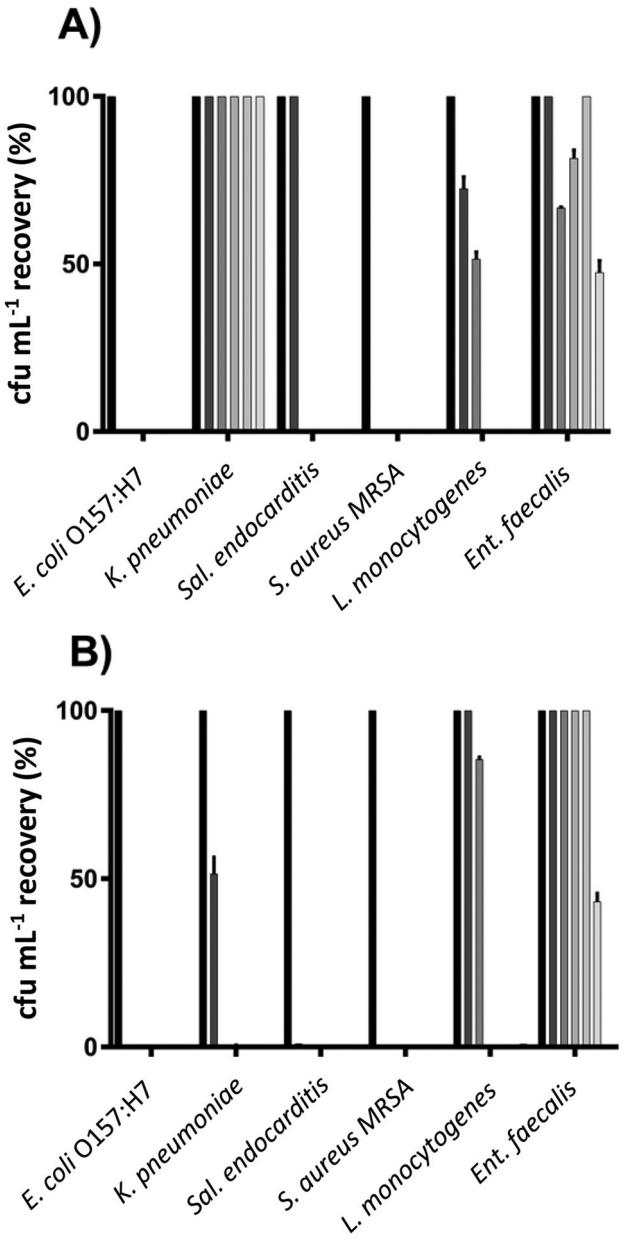

Fig. 5.

Inhibitory effects of H_2O_2 generated by bovine milk xanthine oxidase and increasing concentrations hypoxanthine (■, 0 μM; ■, 50 μM; ■, 100 μM; ■, 200 μM; ■, 300 μM; ■, 400 μM). Bacteria at high (A; 10^6 cfu mL^{-1}) and low concentrations (B; 10^3 cfu mL^{-1}) were incubated with bovine milk and hypoxanthine for 24 h at 37 °C and then enumerated by sub-culturing onto agar plates. Values represent the mean ± SD (n = 3); figure displays the concentration-dependent effect of hypoxanthine versus % cfu mL^{-1}.

Int Dairy J. Author manuscript; available in PMC 2021 March 01.

Table 1

Summary of studies reporting antimicrobial properties of xanthine oxidase.[a]

XO source	Conditions	Target bacteria	ROS/NOS	Inhibition	Reference
Purified enzyme (Diluted up to l:10^6)	Incubation at 37 °C for 18 h; hypoxanthine not added	*S. aureus* (Small inoculum) in broth culture possibly hypoxanthine in the broth	ROS	Yes	Lipmann & Owen (1943)
Pure enzyme (Diluted up to 1:10^6)	Incubation at 37 °C for 18 h; hypoxanthine added	*S. aureus* (Small inoculum) in peptone solution	ROS	Yes	Lipmann & Owen (1943)
Bovine milk	Incubation at 37 °C; hypoxanthine added	*S. aureus* Lightly	ROS	No, too heavy growth	Lipmann & Owen (1943)
Bovine milk	Incubation at 23–25 °C for 24 h; hypoxanthine added	*S. aureus* Lightly	ROS	A log reduction	Lipmann & Owen (1943)
Bovine milk	Incubation at 4 °C for 24 h; hypoxanthine added	Bacteria not added	ROS	A log reduction	Lipmann & Owen (1943)
Bovine milk			ROS +LPS		Björck & Claesson (1979)
Pasteurized semi skim milk	Incubation at 4–8 °C for 8 day; final concentration of 100 μM hypoxanthine added	Endogenous bacteria 10^2 cfu	ROS	Control increased: 10^5 cfu Sample - no change: 10 cfu	Blake et al. (2004)
Purified enzyme (14 μg)	Final concentration of 10,100 and 1000 μM hypoxanthine added	1.8×10^7 cfu *S. aureus 6571* and *Lb. casei 6375*	ROS	*Lactobacilli* was 50% of control; *Staphylococcus* was 10% of control	Blake et al. (2004)
Purified enzyme (30 μg)	Final concentration of 100 μM hypoxanthine added	1.8×10^7cfu *S. aureus*	ROS	The half maximal hypoxanthine dose was 103.5 μM	Blake et al. (2004)
Purified enzyme (30 μg)	Final concentration of 10,100 and 1000 μM hypoxanthine added	1.8×10^7 cfu *Bacillus, Micrococcus, Lactobacillus, Staphylococcus*	ROS	The half maximal hypoxanthine dose was 39.8, 25.1, 103.5 μM, respectively; *Staphylococcus* not	Blake et al. (2004)
	10^4–10^5 cells mL^{-1} in PBS; 0.01 to 100 μM $ONOO^-$ added and incubated at RT for 10 min	*E. coli* and *Sal. enteritidis*	NOS	being observed at these concentrations 1.402 and 2.026 μM $ONOO^-$ concentration reduced viability 50%	Blake et al. (2004)
Commercially produced bovine milk in semi skim form	Incubation at 4 °C for 4 days; portion-1, control; portion-2,100 μm of $ONOO^-$ added only day 1; portion-3, 100 μm of $ONOO^-$ daily for 4 days	Endogenous bacteria	NOS	Portion-1: 2000 cfu Portion 2: 600 cfu Portion 3: < 100 cfu	Blake et al. (2004)
Purified enzyme (53.2 $ugmL^{-1}$)	NADH (300 um), NaNO2 (1 mM), O_2 (0–30%) incubation at 37 °C for 30 min	10^4–10^5 cells mL^{-1} in PBS *E. coli*	NOS	The lowest viable cell at 3% of O_2	Blake et al. (2004)
Human milk	$O_2$5%, pH5.5, 37 °C, sodium nitrite (10 mmol L^{-1}), hypoxanthine (1, 10, 100, and 1000 μm) nitric oxide produced	*E. coli* and *Sal. enteritidis*	NOS	Inhibitory effect of nitic oxide, viable control 50%	Stevens et al. (2000)
Bovine and human milk	Luminescence monitoring of constructs expressing the luxCDABE genes; 0.63%	*E. coli* cells transformed with a plasmid, pLITE27, which carries the *luxCDABE* operon	NOS	Metabolic rates decrease by 70% in bovine milk and 40% in human milk	Hancock et al. (2002)

XO source	Conditions	Target bacteria	ROS/NOS	Inhibition	Reference
	oxygen, pterin (10 μm) and sodium nitrite (20 mM)				
Human milk (diluted 1:6)	Incubation at 37 °C for 24 h; 50 um hypoxanthine + xanthine added	*S. aureus, Salmonella, Lb. plantarum, E. coli*, 200 cfu	ROS	3, 1.5, 3, ns, log reduction, respectively	Al-Shehri et al. (2015)
Human milk (diluted 1:6)	Incubation at 37 °C for 24 h; hypoxanthine (27 μm) + xanthine (20 μm) added with inosine, adenosine, guanosine, uracil, uridine and saliva	*S. aureus, Salmonella, Lb. plantarum, E. coli*, 200 cfu	ROS +LPS	2, 2, ns, ns log reduction, respectively	Al-Shehri et al. (2015)
Human milk (diluted 1:6)	Incubation at 37 °C for 24 h; hypoxanthine (27 μm) + xanthine (20 μm) added with inosine, adenosine, guanosine, uracil, uridine and saliva	*S. epidermidis* (ATCC 14990), *S. aureus* (ATCC 29213; methicillin-sensitive; MSSA), *S. aureus* (ATCC 33591; methicillin-resistant; MRSA), *Str. pyogenes* (clinical isolate), *Ent. faecalis* (clinical isolate; vancomycin-sensitive), *Ent. faecium* (clinical isolate; vancomycin-resistant; VanA), *Ent.* faecalis (clinica isolate; vancomycin- and teicoplaninresistant; VanB), Ps. aeruginosa (ATCC27853), Kl. pneumoniae (ATCC 27736), *E. coli* (clinical isolate) and *C. albicans* (clinical isolate) tested at 200 or 10^7 cfu mL^{-1}	ROS +LPS	At low (200 cfu) concentrations *S. epidermidis, S. aureus* (MSSA), *Ps. aeruginosa*, and at high (10^7 cfu) concentrations *Str. pyogenes, S. aureus* (MSSA), and *Ps. aeruginosa*, after 24 h incubation, the decreases in viability were significant in the groups added hypoxanthine and xanthine	Sweeney et al. (2018)

[a]Abbreviations are: ROS, reactive oxygen species; NOS, reactive nitrogen species; LPS, lactoperoxide system; $ONOO^-$, peroxynitrite.