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RESEARCH ARTICLE

Increased milk concentration of nitric oxide and lysozyme is associated with listerial mastitis of different animal species

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Abstract

The aim of this study was to evaluate nitric oxide (NOx) (sum of nitrite and nitrate as indicators of endogenous nitric oxide production) and the indigenous enzymatic lysozyme concentrations in milk of lactating she-camel, buffaloes, cows, goats and ewes as markers on both mastitis severity and the causing pathogen *Listeria*. The relationship between *Listeria* species and NOx concentrations, as well as correlation between NOx concentrations and lysozyme was also assessed. Composite milk samples of all lactating animals in the selected farms were primarily tested for mastitis severity levels including clinical and subclinical mastitis using the California Mastitis Test. All composite milk samples were collected for NOx and the immunoactive protein lysozyme measurement and bacteriological analyses. Results showed that NOx and lysozyme in clinical mastitis was highest in comparison to sub-clinical mastitis and healthy udders. *Listeria* had a significant increasing impact on the level of NOx in she-camel, buffalo, cow, goat and ewe milk. On the other hand, *Listeria* had a significant increasing impact on the level of lysozyme in she-camel, cow and goat milk while in the buffalo and ewe milk the *Listeria* infection did not induce a significant influence on the level of lysozyme in the milk. A significant positive correlation was found between NOx and lysozyme levels in the she-camel ($r = 0.76$; $P < 0.01$), cow ($r = 0.86$; $P < 0.01$) and goat ($r = 0.28$; $P < 0.01$). In conclusion, differences in both severity of mastitis and mastitis pathogens were associated with differences of NOx and lysozyme products in infected udders

Introduction

Udder health is an essential component of quality milk production and cow well-being. Economy-wise, she-camel, buffalo, cattle, goat, and sheep are by far the most important for the dairy industry in the Middle East. Mastitis is a highly worldwide prevalent disease, is the most devastating disease of dairy animals and of major economic threat to the dairy farmers (Seegers et al., 2003). Mastitis is an inflammatory disease that most commonly results from bacterial infection of the mammary gland. A broad spectrum of bacteria can successfully establish an infection within the mammary gland, including gram-positive, gram-negative, and wall-less (*Mycoplasma bovis*) bacteria (Fox et al., 2005). *Staphylococcus aureus*, coagulase-negative *Staphylococci*, and *Streptococcus uberis, Streptococcus agalactiae*, *Streptococcus dysagalactiae*, *Streptococcus bovis*, *Corynebacterium pyogenes* and *Clostridium perfringens* (Radostits et al., 2000; Osman et al., 2010 a,b) are among the most prevalent gram-positive bacteria to cause this disease. Among the gram-negative bacteria that cause mastitis, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Enterobacter* spp. are the most common.

A great majority of animal species rely exclusively on innate immune responses while dealing with microbial insults (Kimbrell and Beutler, 2001) because it is poised to respond immediately to the earliest stages of infection and recognize pathogens that have not been encountered previously (Uthaisangsook et al., 2002). Intriguingly, various peptides, small proteins and enzymes with innate immune function are present in milk. Nitric oxide (NOx) is one of the components of the mammary innate immune system (Silanikove et al., 2012) and functions to create an effective bactericidal environment towards major mammary gland pathogens.

NOx is a gaseous diatomic radical that is involved in a wide range of physiological and pathological functions in biology. NOx is released from the endothelium, acting in an anti-inflammatory manner by inhibiting the adhesion of leukocytes and endothelial cells (Suhr et al., 2013). NOx is important in the host defense by destroying microbes. The antimicrobial effect of NOx on bacteria is due to peroxynitrite, reactive nitrogen metabolite, derived from oxidation of NOx. The macrophage and epithelial cells of mammary gland produce significant amount of nitric oxide that takes part in the inflammatory process (Boulanger et al., 2001). The level of NOx in mammary gland secretion increases significantly in subclinical and clinical mastitis (Komine et al., 2004; Osman et al., 2010 a,b). Lysozyme, a cell wall-lytic enzyme, is one of the first defense compounds induced in tissues after the onset of infection and undoubtedly one of the principal important enzymes of our innate immune system (Bera et al., 2006; Osman et al., 2010 a,b).

Although, diagnosis of clinical mastitis by visual inspection and palpation is relatively easy but, diagnostic problems arise when dealing with subclinical mastitis. Further, sub-clinical mastitis (SCM) possess a potential health threat to other cows for new infection. Therefore, efficient detection of SCM is very much required to reduce the incidence of mastitis in a well managed dairy herd. Bacteriological sampling is not always feasible as a routine test to identify subclinical mastitis as the procedure is both time consuming and uneconomical in large dairy herds. Presently, SCM is generally diagnosed by cow-side tests like the California Mastitis Test (CMT) or by Somatic Cell Count (SCC) in milk samples. These tests are less efficient in detecting chronic subclinical mastitis than for acute clinical cases (Nielen et al., 1995). Therefore, it is important to investigate alternative parameters/methods for detection of SCM.

In comparison with other foodborne diseases, the severity and high case fatality rate of listeriosis in pregnant women and immune-compromised people make listeriosis an important public health concern. It is important to point out that healthy animals are often carriers of *L. monocytogenes* and as such can be source of contamination of milk (Kasalica et al., 2011). Therefore there is a need to develop, implement, maintain and, where necessary, enhance programs for monitoring and controlling *Listeria* within the food industry. Every diagnosed listeriosis case should be reported and investigated in order to detect outbreaks early and identify risk factors, so that necessary prevention and control measures can be taken promptly. Within this context, the availability of rapid, specific and sensitive diagnostic tests capable of distinguishing *Listeria* mastitis is essential for the effective control of the disease and reduce unnecessary recalls of food products.

Hence, the present study was conducted to evaluate NOx (sum of nitrite and nitrate as indicators of endogenous nitric oxide production) and the indigenous enzymatic lysozyme concentrations in milk of lactating she-camel, buffaloes, cows, goats and ewes as markers on both mastitis severity and the causing pathogen *Listeria*. The relationship between *Listeria* species and NOx concentrations, as well as correlation between NOx concentrations and lysozyme was also assessed.

Materials and methods

Mastitis markers

The CMT was recommended by the American Public Health Association (APHA, 1992) as a method to detect subclinical mastitis from samples collected from apparently healthy quarters.

Milk samples

A total of 510 samples of raw fresh whole milk were obtained from randomly selected on-farm stores located around Cairo. Milk samples were collected in sterile universal bottles. The milk samples were quickly transported to the laboratory under chilled conditions and stored at 4°C till bacteriologically analyzed. Each sample was divided into two parts, each in a sterile McCartney bottles. One was incubated for 24 h for bacteriological examination, the second was used to separate milk serum and the extracted milk was stored at -20^{o}C until assayed for NOx and lysozyme.

Bacteriological examinations

Culturing

Conventional *Listeria* identification tests were done according to the procedures in the FDA Bacteriological Analytical Manual (Hitchins and Jinneman, 2013). Isolation and identification of *Listeria* was performed using the double enrichment procedure, the first (Half Fraser) and the second (Fraser) enrichment broths. A loopful of growth from Fraser II was subcultured onto Palcam selective agar supplemented with SR 0150E (polymyxin B 5 mg, acriflavine HCl 2.5 mg) (Oxoid) and incubated microaerobically (5% O_2, 10% CO_2 and 85% N_2). Presumptive black *Listeria* colonies were maintained at 4^oC on Trypticase soy agar slants with 0.6% yeast extract slants, incubated at 37^oC for 24 h and stored at 4^oC for PCR confirmation.

Molecular identification

Genomic DNA extraction. The *Listeria* genomic DNA used for PCR was prepared by resuspending and washing typical *Listeria*-like colonies (black colonies) collected from the surfaces of Palcam plates in 500 µl of PBS (pH 7.2). The washed bacterial pellets were resuspended in 400 µl of Tris-EDTA buffer (pH 8.0) and boiled for 10 min. After centrifugation for 15 min at 10,000 × *g*, 10 µl of supernatant was used as the DNA template and amplified for each PCR analysis. The following oligonucleotide primers were used. Set I consisted of primers Lis-1 and Lis-2, and set II consisted of primers U1 and LI1. Primers U1 (59- CAG-CMG-CCG-CGG-TAA-TWC-39, where M denotes A or C, and W denotes A or T) and LI1 (59-CTC-CAT-AAA-GGT-GACCCT-39) target a 938-bp 16S rRNA sequence in members of the genus *Listeria* (Wesley et al., 2002). Each PCR was carried out by using genomic DNA as the template in a 50 µl containing 10 µl (200 ng) of extracted DNA template from bacterial cultures, 5 µl 10X PCR buffer, 0.375 µl $MgCl_2$ (1.5 mM), 1.25 µl dNTPs (250 µM), 0.25 µl (1.25 Unit) AmpliTaq DNA polymerase, 0.25 µl (0.5 µM) from each primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW. The reaction mixture was overlaid with mineral oil, and the tubes were placed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The samples were subjected to an initial denaturation step of 94^oC for 4 min, followed by 25 amplification cycles of 1 min at 94^oC (denaturation), 1 min at 60^oC (primer annealing), and 1 min at 72^oC (primer extension) followed by a final extension step of 72^oC for 5 min. PCR reaction products were separated on 1.5% agarose gels (60 V for 1.5 h) in a horizontal gel bed (8.3 by 6.0 cm) with Tris-borate-EDTA as the running buffer. DNA molecular weight marker VI (Boehringer Mannheim) was included for base pair size comparison. The gel was then stained with ethidium bromide and visualized.

Detection of the effect of *Listeria* on nitric oxide and lysozyme concentration in milk

Assay of nitric oxide concentration in milk

NOx was assessed with the in-house Griess method. The NOx concentrations determined with the in-house Griess method was found to be higher than those determined with the commercial NOx assay and with accuracy that is comparable to widely accepted commercial NOx colorimetric assay (Yucel et al., 2012). It involves deproteinizing the plasma with 250 µL of 0.3M NaOH and 250 µL of 10% $ZnSO_4$ as a protein degenerative (solution clarification) (Sun et al., 2003; Yucel et al., 2012), and then converting nitrate into nitrite using 100 µL of 8% vanadium chloride ($VaCl_3$) (in 1 M HCl). Each milk sample was deproteinized prior to NOx analysis. In this assay, based on Griess reaction, 100 µL of each milk sample was added to 100 µl a mixture of solution, 50 µL of 0.1% NEDD (N-1-naphtylethylenediamine) and 50 µL of 2% sulphanomide (in 5% HCl) (1:1) to 100 µl of milk, to doing reaction and dye forming, solution mixture placed into incubator 37°C for 30 min. All the plates were incubated at 37°C for 30 min. After incubation, the OD of each reaction mix was read at 540 nm. The mean of duplicate wells for each sample and standard was taken as the ODs. The mean of duplicate wells for each sample and standard was taken as the ODs.

Lysoplate radial diffusion assay for lysozyme activity

The lysozyme is sensitive to the cell wall of *M. lysodeikticus*. The rate of breakdown of cell wall of the organism is directly proportional to the enzyme concentration.The lysozyme activities for milk samples were determined by lysoplates assay (Hikima et al., 2001). The *Micrococcus lysodeikticus* cells (Sigma, St. Louis, MO) was prepared in in 1% warm (50°C) melting agarose (0.067 M sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 6.24)) at room temperature. The concentration of *M. lysodeikticus* was adjusted to0.2 absorbance units at 600 nm (about 10 mg *M. lysodeikticus*to 100 ml 1% agarose). Working lysozyme standards were prepared freshly by diluting 3.0 ml of stock lysozyme

solution to 10.0 ml with 8.5 g/l sodium chloride to prepare 120 mg/ml. The wells were filled with a volume of 25 ml of skim milk samples. Each filled plate contained the 5 working lysozyme standards, the sample to be assayed, the negative control samples and 250 µg of hen egg-white lysozyme (Sigma) were put in individual wells in the agarose plates and incubated at 30°C for 24 h. After overnight incubation, the clear zone ring diameters were measured to the nearest 0.1 mm with an enlarger viewer. For each lysoplate, the lysozyme activity was determined by measuring the diameter of the clear zone relative to the standards.

Statistical analysis

Data were analyzed using statistical software package (SAS version 9.2 (SAS, Cary, NJ). Statistical significance among experimental periods and groups was determined with analysis of variance for multiple comparisons and bivariate correlation for relation between variables and regression for prediction was employed to note the differences of various parameters between milk samples of healthy and SCM infected animals. A *p*-value 0.05 or less was considered statistically significant. Results are reported as the means ± SEM. Correlation (*r*) analysis was performed to determine the relationship among nitric oxide and lysozyme.

Results

Nitric oxide level between animals/health condition

In this study we measured NOx by the dye assay. The animals (she-camel, buffaloes, cows, goats and ewes) were divided to six groups: apparent healthy negative for *Listeria,* subclinically negative for *Listeria*, clinically negative for *Listeria*, apparent healthy positive for *Listeria*, subclinically positive for *Listeria* and clinically positive for *Listeria*. The results showed a significant difference between groups (p value < 0.01). The results are summarized in Table 1. The analysis of variance of NOx level recorded in Table 2 show a significant effect of *Listeria* on the level of NOx in she-camel, buffalo, cow, goat and ewe milk.

Lysozyme level between animals/health condition

In this study, the lysozyme in the milk of the same groups of animals indicated previously was measured. The results showed a significant difference between groups (p value < 0.01) and the results are summarized in Table 1. The analysis of variance of the lysozyme level recorded in Table 3 how a significant effect of *Listeria* on the level of lysozyme in she-camel, cow and goat milk while in the buffalo and ewe milk the *Listeria* infection did not induce a significant influence on the level of lysozyme in the milk.

Correlation between nitric oxide and lysozyme

The correlation values (Table 4) reveal that there was a positive correlation in the she-camel, cow and goat. Meanwhile a negative correlation was indicated in the buffalo and ewe (Table 4). A significant positive correlation (Table 5) was found between NOx and lysozyme levels in the she-camel (r = 0.76; P < 0.01), cow (r = 0.86; P < 0.01) and goat (r = 0.28; P < 0.01).

Table 1. Prevalence of *Listeria* species in the udder milk after examination of the animals by the CMT

Health condition of the Animal	Animal species					Total
	She-camel n=100	Buffalo n=103	Cow n=103	Goat n=107	Ewe n=103	n=510
Apparently healthy animal (CMT negative)	2/46 (4.3%)	3/46 (6.5%)	3/30 (10.0%)	1/33 (3.0%)	1/38 (2.6%)	10/191 (5.2%)
Subclinicallymastitic animal (CMT positive)	1/34 (2.9%)	3/36 (8.3%)	2/45 (4.4%)	3/35 (8.6%)	2/41 (4.9%)	11/181 (6.1%)
Clinically mastitic animal (CMT positive)	1/30 (3.3%)	2/18 (11.1%)	4/28 (14.3%)	2/39 (5.1%)	1/23 (4.3%)	10/138 (7.2%)
Total	4/100 (4.0%)	8/100 (8.0%)	9/103 (8.7%)	6/107 (5.6%)	4/102 (3.9%)	31/512 (6.1%)

CMT, California Mastitis Test;
n= number of examined animals

Table2. Nitric oxide and lysozyme concentrations in the udder milk samples (Mean ± S.D.)

Animal Health Status	Animals									
	She-camel		Buffalo		Cow		Goat		Ewe	
	Nitric oxide (µMol)	Lysozyme (µg/ml)	Nitric oxide (µMol)	Lysozyme (µg/ml)	Nitric oxide (µMol)	Lysozyme (µg/ml)	Nitric oxide (µMol)	Lysozyme (µg/ml)	Nitric oxide (µMol)	Lysozyme (µg/ml)
Apparently healthy CMT negative and *Listeria* was not culturally isolated or PCR confirmed	4.9 [c] ±1.23	5.98 [b] ±1.347	5.55 [c] ±1.219	5.89 [b] ±1.609	6.5 [c] ±1.22	5.8 [b] ±1.61	4.01 [c] ±1.223	6.29 [b] ±1.607	4.01 [c] ±1.223	6.29 [b] ±1.607
Apparently healthy CMT negative but *Listeria* was culturally isolated and PCR confirmed	0	0	145.18 [c] ± 37.23	7.27 [b] ± 4.3	148.15 [a] ±51.5	8.75 [a] ± 0.23	130.15 [a] ±51.49	8.743 [a] ± 0.23	130.15 [a] ±51.49	8.743 [a] ± 0.23
Subclinically CMT positive but *Listeria* was not culturally isolated or PCR detected	49.18 [c] ± 35.23	6.07 [b] ± 4.24	55.18 [c] ± 37.23	6.27 [b] ± 4.3	52.18 [c] ± 38.2	5.57 [b] ± 4.37	49.18 [c] ± 38.23	5.07 [b] ± 4.3724	49.18 [c] ± 38.23	5.07 [b] ± 4.3724
Subclinically CMT positive and *Listeria* was culturally isolated and PCR confirmed	148.75 [a] ±20.66	8.34 [a] ±0.166	160.6 [a] ±22.60	8.08 [a] ±0.18	177.75 [a] ±21.66	8.37 [a] ±0.179	137.75 [a] ±21.66	8.08 [a] ±0.179	137.75 [a] ±21.66	8.08 [a] ±0.179
Clinically CMT positive but *Listeria* was not culturally isolated or PCR detected	78.69 [b] ± 40.05	7.01 [a] ± 0.16	88.69 [b] ± 42.05	7.79 [a] ± 0.1876	90.9 [b] ± 42.05	7.5 [a] ± 0.189	80.69 [b] ± 42.05	8.09 [a] ± 0.1876	80.69 [b] ± 42.05	8.09 [a] ± 0.1876
Clinically CMT positive and *Listeria* was culturally isolated and PCR confirmed	160.02 [a] ±21.6	8.81 [a] ±0.158	172.75 [a] ±22.6	8.01 [a] ±0.188	150.15 [a] ±51.4	8.43 [a] ± 0.24	142.75 [a] ±21.66	9.01 [a] ±0.188	142.75 [a] ±21.66	9.01 [a] ±0.188

Different superscripts a, b and c are significantly different at < 0.01P

Table 3. Analysis of variance of nitric oxide in appareant healthy, clinical and subclinical positive and negative *Listeria* udder milk samples

Parameters	Animals														
	She-camel			Buffalo			Cow			Goat			Ewe		
	Between	Within	Total	Between	Within	Total	Between	Within	Total	Between	Within	Total	Between	Within	Total
SS	198,741.289	37,859.615	236,600.904	219,335.055	50,070.188	269,405.243	218,808.371	80,930.668	299,739.039	158,145.185	61,386.862	219,532.047	228,714.292	40,361.435	269,075.727
df (n-1)	5	54	59	5	54	59	5	54	59	5	54	59	5	54	59
MS	39,748.258	701.104		43,867.011	927.226		43,761.674	1,498.716		31,629.037	1,136.794		45,742.858	747.434	
F	56.694			47.310			29.199			27.823			61.200		
P	0.000			0.000			0.0000			0.00			0.000		

Table 4. Analysis of variance of lysozyme **in apparent healthy, clinical and subclinical positive and negative *Listeria* udder milk samples**

Parameters	Animals														
	She-camel			Buffalo			Cow			Goat			Ewe		
	Between	Within	Total	Between	Within	Total	Between	Within	Total	Between	Within	Total	Between	Within	Total
SS	67.291	179.149	246.441	43.625	357.046	400.671	97.431	196.805	294.237	118.653	196.702	315.355	104.145	195.986	300.131
df (n-1)	5	54	59	5	54	59	5	54	59	5	54	59	5	54	59
MS	13.458	3.318		8.725	6.612		19.486	3.645		23.731	3.643		20.829	3.629	
F	4.057			1.320			5.347			6.515			5.739		
P	0.003			0.270			0.0000			0.000			0.000		

Table 5. **Pearson correlation and regression between nitric oxide and lysozyme**

Parameters	Animals									
	She-camel		Buffalo		Cow		Goat		Ewe	
	Nitric oxide and lysozyme	Significance	Nitric oxide and lysozyme	Significance	Nitric oxide and lysozyme	Significance	Nitric oxide and lysozyme	Significance	Nitric oxide and lysozyme	Significance
Pearson correlation	0.28	Positive correlation	-0.16	Negative correlation	0.63	Moderate positive correlation	-0.4	Negative correlation	0.51	Moderate positive correlation
Regression	14.171	0.000	-90.165	0.270	3.174	0.000	-7.645	0.100	1.337	0.000

Correlation is significant at the 0.01 level (2-tailed). Relationship between nitric oxide and lysozyme (Regression).

Table 6.Correlation (r=) between nitric oxide and lysozyme

Parameters	Animals									
	She-camel		Buffalo		Cow		Goat		Ewe	
	Lysozyme	Nitric oxide	Lysozyme	Nitric oxide	Lysozyme	Nitric oxide	Lysozyme	Nitric oxide	Lysozyme	Nitric oxide
Lysozyme **(n=60)**	1.000	0.76	1.000	-0.44	1.000	0.86	1.000	0.28	1.000	-0.37
Nitric oxide **(n=60)**	0.76	1.000	-0.44	1.000	0.86	1.000	0.28	1.000	0.37	1.000

Correlation is significant at the 0.01 level

Discussion

Foodborne diseases such as listeriosis are only now emerging as the role of food in their transmission has only recently been recognized responsible for about 50% of sporadic human cases world-wide and have been incriminated for all major foodborne outbreaks in Europe and North America since the 1980s (Ward et al., 2004).

Listeria monocytogenes isolated from the milk of buffaloes, cows and sheep suffering from subclinical mastitis can go undetected as the milk was characterized by persistent shedding of *Listeria* and by a normal appearance of the milk (Priyadarshini et al., 2002; Winter et al., 2004; Pintado et al., 2009; Hunt et al., 2012). Also, there were no apparent relationships between SCC and standard plate counts (SPC) and incidence of *L. monocytogenes* (Hariharan et al., 2004; Van Kessel et al., 2004).

Different factors are responsible for natural defense of mammary gland against invading microorganisms. Although clinical investigation does not result in visible signs of inflammatory process in SCM, however, elevated level of NOx activity could be a result of inflammation and increased immunological activity of mammary gland. Macrophage and epithelial cells of the mammary gland produce significant amounts of NOx, this inducible NOx mediates inflammation during mastitis (Bouchard et al., 1999). An increase in NOx concentrations in milk from mammary glands with subclinical mastitis also support the relationship between elevation of NOx levels and inflammation (Atakisi et al., 2010; Osman et al., 2010 a,b). NOx concentrations, however, did not correlate with SCC (Bastan et al., 2013). From the previous investigations, changes in NOx concentration were mainly dependent on the infectious status of the quarters with variations among the bacterial species. NOx production is considered as a primer defence system possessing antimicrobial properties implemented by an important powerful microbial oxidant, peroxynitrite, a reactive nitrogen metabolite derived from oxidation of NOx (Kell, 2010). The epithelial cells of mammary gland produce significant amounts of NOx (Bouchard et al., 1999) which takes part in the inflammatory process indicating a possible clinical relevance of NOx production during acute *E. coli*, *Staphylococcus* and *Clostridium perfringens* mastitis (Bouchard et al., 1999; Blum et al., 2000; Atakisi et al., 2010; Osman et al., 2010 a,b; De et al., 2011).

NOx cycles in milk through its auto-oxidation to nitrite and the conversion of nitrite into nitrate by LPO/H_2O_2/NO_2-/biological electron donor systems (Palumbo et al., 1999; Reszka et al., 1999; Silanikove et al., 2005) interacting with thiol-bearing groups on proteins to form thiyl radicals (Dalle-Donne et al., 2005; Silanikove et al., 2005) and with tyrosine on proteins to form nitrotyrosine (Silanikove et al., 2009). The interaction of NOx with thiyl radicals forms nitrosothiols, which serve as a pool that constantly delivers NOx into the system (Silanikove et al., 2005, 2009). The conversion of nitrite into nitrate causes excessive nitrosative stress (defined as the ratio of nitrosants to antioxidants as >1similarly to oxidative stress, but with involvement of reactive nitrogen species) in milk (Silanikove et al., 2005, 2009). Peroxynitrite can cause alterations in antioxidant balance in the organism when produced in excess (Kell, 2010). During inflammatory disease like mastitis, there is an increase in lipid peroxidation which causes a decrease in levels of some antioxidant molecules leading consequently to oxidative stress (Komine et al., 2004; Kell, 2010). During inflammatory diseases, high levels of NOx react with superoxide anions leading to formation of peroxinitrite radical (Pacher et al., 2007) and peroxinitrite radicals oxidize long chain fatty acids in cell membranes leading to increase in lipid peroxidation and formation of free radicals (Pacher et al., 2007). However, high levels of

nitrotyrosine, lipid peroxides and carbonyls in mastitic milk are associated with a high level of nitrite and nitrate in that milk. Nitrotyrosine, lipid peroxides and carbonyls are considered as causative agents and hallmarks of cancer, neurodegenerative diseases, Alzheimer's disease, Huntington's disease, Parkinson's disease, atherosclerosis and cardiovascular disease, septic shock, rheumatoid arthritis, celiac disease, atherosclerotic plaques and chronic renal failure (Pratico, 2000; Picklo et al., 2002; Ohshima et al., 2003; Dalle-Donne et al., 2005; Pacher et al., 2007; Yehet al., 2007; Sanyal et al., 2009; Kell, 2010; Reed, 2011; Daiber and Münzel, 2012).

Milk has a dual role of nourishment and immunological protection of the mammalian newborn and is a unique body fluid within the animal kingdom (Ward et al., 2004). Interestingly, both the immunological and the strong nutritional value of milk are largely due to contributions of the antimicrobial enzyme lysozyme, which is expressed in and secreted from the lactating mammary epithelium (Shahani et al., 1973). Semba et al. (1999) found that lysozyme concentrations in human milk are higher among women with mastitis than among women without mastitis.

Lysozyme is released as part of the non-specific immune response, Tissue macrophages discharge lysozyme into serum, nasal and lacrimal secretions along with various other bodily secretions. Lysozyme is among the minor milk proteins that has attracted increased attention recently due to its potent antimicrobial activity against a wide range of microorganisms and hence potential in food preservation and safety. The antibacterial activity of milk lysozyme as part of the unspecific innate defence mechanism is well established. It acts either independently by lysing sensitive bacteria or as a component of complex immunological reactions to enhance the phagocytosis of bacteria by macrophages (Cheema et al., 2011). It thus contributes to the innate protection from microbial infections while still in the udder.

In fact, the presence of lysozyme in some milk is controversial, and several authors have reported on the lack of lysozyme in bovine (Pahud and Widmer, 1982) and camel milks (Kappeler et al., 2003; ElHatmi et al., 2007). In contrast, other authors have confirmed its presence in these milks (Chandan et al., 1968; Barbour et al., 1984; ElAgamy et al., 1996; Parisien et al., 2008; Osman et al., 2010 a,b; Amena et al., 2011). Such controversy has been explained by the fact that lysozyme concentration in these milks is normally low and may fall, under certain conditions or periods of the year, to below the detection limit of the analytic methods used for lysozyme quantification (Benkerroum, 2008). Moreover, lysozyme concentration may reach abnormally high levels in mastitic milk as a response to infections of the mammary glands and has been considered as an indicator of the onset of clinical or subsclinical mastitis (Maroni and Cuccuri, 2001). The lactating mammary epithelium secretes the antimicrobial enzyme lysozyme for various protective functions (Bernt and Walker, 2001; Osman et al., 2010 a,b). The host defense status is a cardinal factor determining the outcome of the disease. Buffalo milk lysozyme, a 16 kDa basic protein found in buffalo milk has 10 times more specific activity than its bovine counterpart (Priyadarshini et al., 2002). This may be one of the causes of higher resistance of buffaloes to mastitis (White et al., 1988; Priyadarshini et al., 2002). A 10 to 50-fold increase in milk lysozyme activity was observed in mastitic cows. An assay of lysozyme activity in milk can be used to diagnose mastitis in cattle but not in buffaloes. Some buffaloes exhibited 1000 fold greater lysozyme activity and moderately raised somatic cell count in milk, but there was no sign of mastitis in these animals (Priyadarshini et al., 2002). However, there was no sign of mastitis in these animals. Buffaloes are much less susceptible to mastitis compared with bovines. A sharp rise in milk lysozyme activity could be the reason of some mild udder infection, and increased lysozyme activity might have protected buffaloes from development of mastitis (Priyadarshini et al., 2002). Lysozyme activity, however, does not correlate with SCC (Priyadarshini et al., 2002).

In conclusion, currently, very little is known about the presence of oxidative substances in commercial milk and about the health implications of their presence. Because the present experimental setup induced a very strong NOx response, it is still premature to draw conclusions pertaining to more common situations that prevailed in dairy farms. We also could not find more information regarding the effect of mastitis on milk safety in the scientific literature. Thus, more research at the basic cow/gland level and at the BMT level in different sites, both within and between countries, is required to clarify this issue (Silanikove et al., 2012). Clinical mastitis and SCC reflect the attitude of the farmers and advisors to use therapeutics or culling to meet quality goals while NOx and lysozyme are more an indication of the true udder health status of the animals. Therefore, alteration of NOx level in milk could be used as alternative diagnostic indicator to assess the inflammation during SCM of dairy animal. The diagnosis of SCM on the basis of a selected SCC value or a particular colony count of bacteria is problematic because workers have used different cut-off values (Hariharan et al., 2004). Mixing of milk from infected udders with milk from non-infected ones could not be detected by measures such as determination of SCC, proteose peptone content and % of casein, which worked well at the individual cow level as predictors of udder inflammation (White et al., 1988). Thus, the results of

this and former studies (White et al., 1988) indicate that it is important for the dairy industry to develop analytical tools that will allow to prevent combining low-quality milk, such as milk rich in somatic cells and nitrite, with high-quality milk in order to ensure optimal yield and quality of curd from milk designated for cheese production.

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