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Relationship between occurrence of mastitis pathogens in dairy cattle herds and raw-milk indicators of hygienic-sanitary quality

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Mastitis is an inflammation of the mammary glands and in most cases it is caused by the presence of microorganisms. High mastitis rates in dairy cattle herds can cause an increase in total microorganism counts of bulk tank milk. The present paper was aimed at verifying whether the occurrence of mastitis in dairy cattle herds is reflected in raw-milk indicators of hygienic-sanitary quality. To observe the correlation among the analysed variables, we performed a logarithmical transformation ($\log_{10}$) of different indicator counts of raw milk and compared them with the occurrence of mastitis in dairy cattle herds. Few correlations were observed among mastitis cases in dairy cattle herds and the raw-milk indicators of hygienic-sanitary quality. We observed a negative correlation between the $\log_{10}$ of mesophilic aerobic plate counts and psychotropic aerobic plate counts when compared with the occurrence of no bacterial growth. The $\log_{10}$ of thermophilic aerobic plate counts and yeasts and mould aerobic plate counts presented a positive correlation with the cases of infectious mastitis and mastitis caused by *Staphylococcus* spp.

Keywords: Mastitis, raw milk, microbial quality.

Mastitis, an inflammation of mammary glands, is the most frequent and costly disease of dairy cattle worldwide. Although stress and physical injuries may cause inflammation of mammary glands, infection by invading bacteria or other microorganisms (fungi, yeast and possibly viruses) is the primary cause of mastitis (Wattiaux, 1999; Ruegg, 2001).

Raw milk, as it leaves the udders of healthy animals, normally contains very low numbers of microorganisms. If lactating cows have mastitis, large numbers of infectious organisms may be shed in the milk and increase the total counts of bulk milk if the milk of infected cows is not kept separate (Hayes, 1995; Richter & Vedamuthu, 2001). Proteinases contribute significantly to the proteolysis in high cell count milk from *Streptococcus uberis* infected mammary quarters (Larsen et al. 2004).

The culture of bulk tank milk (BTM) samples for diagnosis of mastitis-causing bacteria began on dairy farms in the early 1970s. Nowadays, numerous systems of control are in use owing to the further development of BTM culture techniques; however, no standardized procedure is being used (Farnsworth, 1992; Jayarao & Wolfgang, 2003).

Some investigations showed that data obtained by means of microbiological analysis of raw milk are a good option to estimate the prevalence of mastitis in dairy cattle herds. Many papers indicate that the isolation of *Streptococcus* spp., *Staphylococcus* spp. and Enterobacteria of bulk tank milk are strong indicators of the presence of these microorganisms, causing mastitis (Felon et al. 1995; Hayes et al. 2001; Zadoks et al. 2004; Rysanek & Babak, 2005).

The aim of this paper was to ascertain whether there is a correlation between the occurrence of mastitis and raw-milk indicators of hygienic-sanitary quality. We analysed individually the mammary quarters in dairy cattle herds, according to the methodology for the detection of infected mammary quarters that was established by the National Mastitis Council (NMC, 1987) and we used raw-milk indicators of hygienic-sanitary quality, in accordance with the official methodologies adopted by the American Public Health Association (APHA, 2001) and by U.S. Food and Drugs Administration (U.S. FDA, 2001).

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Materials and Methods

Thirty-six dairy farms provided the convenience samples included in this study. All lactating cows were sampled to determine the presence of mastitis. The farms were located in fourteen cities of São Paulo State, Brazil. Samples were collected from April 2004 to September 2005. Among the 36 farms that took part in the study, 10 (27·78%) had an intensive system of production and 26 of them (72·22%) had a semi-intensive production system; 15 (41·67%) of the herds were pure Holstein-Friesian herds, 1 (2·78%) was a Jersey herd and 20 (55·55%) were herds with a mixed origin; the daily production ranged between 40 l and 1500 l; 15 farms (41·67%) produced up to 250 l/d, 15 (41·67%) produced between 251 l and 1000 l/d, and 6 (16·66%) produced between 1001 l and 1500 l/d; 16 properties (44·44%) were considered as producers of type B milk, which presupposes a limit of Bacteria Total Count of $5{\cdot}0 \times 10^5$ cfu/ml and 20 (66·66%) considered as producers of refrigerated raw milk with a maximum pattern of $1{\cdot}0 \times 10^6$ cfu/ml for the limit of Bacteria Total Count (Brazil, 2002).

Tests to detect mastitis

Each mammary quarter in the dairy production system was analysed only once for the presence of mastitis using Tamis Test (Blood & Radostits, 1991) and California Mastitis Test (CMT) (Schalm & Noorlander, 1957). Positive results to Tamis Test or CMT required one sample of ~5 ml collected in a sterile tube for the microbiological test. The samples were stored in isothermal boxes with ice and sent to the Laboratory of Infectious Diseases at the Department of Preventive Veterinary and Animal Health, Faculty of Veterinary Medicine, University of São Paulo.

The animals that were under antibiotic treatment at the time of collection were withdrawn from the sampling. All the animals presenting a positive result for the Tamis Test also presented positive results for CMT. Animals that presented any positive reactions to CMT test were considered as positive mastitis cases, both clinical or subclinical, and constituted the total mastitis group. Animals that were positive according to Tamis Test were considered as positive cases for the group of clinical mastitis.

In the laboratory, the samples were frozen until the moment of the analysis, which was performed within 30 d. Samples were then thawed at room temperature, homogenized in a vortex shake device and sowed with platinum handle (0·01 ml) in Blood Agar Base (Oxoid Ltd., Basingstoke, UK) with the addition of defibrinated sheep blood (5%) previously incubated for a period of 18 h at 37±1 °C (overnight) to verify the sterile conditions of the culture medium. To verify the growth of microorganisms, three readings were performed after 24, 48 and 72 h. Samples that did not present the growth (negative) of at least a colony of microorganisms were re-analysed, repeating the procedure that was described previously. Isolated microorganisms (bacteria and fungi) were identified and classified according to the National Mastitis Council (1987).

Raw milk indicators of hygienic-sanitary quality

For the analysis of raw-milk indicators of hygienic-sanitary quality, the only sample of ~200 ml of raw milk was collected into a sterile glass container on the same day of the analysis of mammary quarters of the herd for mastitis, and this sample was sent to the laboratory into an isothermal box with ice. Samples of raw milk were collected from the surface of the storage tank after homogenization, by raising the cover and taking out the predicted quantity, using sterile equipment. The samples were then stored under refrigeration for a maximum of 18 h before being subjected to microbiological analysis aimed at verifying the number of microorganisms present in the raw milk. Milk from mammary quarters of animals treated with antibiotics was discarded and milk from these animals was not added to the raw milk sample because Brazilian law does not permit milk containing antibiotic residues to be added to milk for human consumption.

The sample was subjected to serial decimal dilutions up to 10^{-6} in Peptone Water (0·1%; Difco, Becton, Dickinson, Sparks, USA) and the sowing was used in specific media as described by the American Public Health Association (2001) and by U.S. Food and Drugs Administration (2001).

Mesophilic aerobic plate count

For the mesophilic aerobic plate count (MAPC), we used the technique of sowing in depth with plaques in duplicate for the dilution in medium Plate Count Standard Methods, Agar (Difco) added to a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec Fine Chemistry Ltd, Taboão da Serra, São Paulo, Brazil) to 1% in the proportion of 1 : 100 to the culture medium. The plaques were incubated inverted in a bacteriological kiln at 37±1 °C for 42–48 h (American Public Health Association, 2001; U.S. Food and Drugs Administration, 2001).

Psychotropic aerobic plate count

For the psychotropic aerobic plate count (PAPC), we used the technique of sowing on the surface with plaques in duplicate for the dilution in Plate Count Standard Methods, Agar (Difco) added to a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec Fine Chemistry) to 1% in the proportion of 1 : 100 to the culture medium. The plaques were incubated inverted in a bacteriological kiln at 12±2 °C for 7 d (American Public Health Association, 2001; U.S. Food and Drugs Administration, 2001).

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Thermophilic aerobic plate count

For the thermophilic aerobic plate count (TAPC) we used the technique of sowing in depth with plaques in duplicates for each dilution in medium Potato Dextrose Agar (Merck, KgaA, Darmastadt, Germany) and added to a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec Fine Chemistry Ltd) to 1% in a proportion of 1 : 100 to the culture medium. The plaques were incubated inverted in a bacteriological kiln at 52±1 °C for 42–48 h (American Public Health Association, 2001; U.S. Food and Drugs Administration, 2001).

Yeast and mould counts (YMC)

For the yeast and mould count (YMC) we used a technique of sowing in depth, with plaques for each dilution in Potato Dextrose Agar (Merck) and added to a solution of L-tartaric acid (10%; Labysnth Laboratory Products Ltd, Diadema, São Paulo, Brazil) in the proportion of 14 ml per 1000 ml of the medium, and added to a solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec Fine Chemistry Ltd) to 1% in the proportion of 1 : 100 to the culture medium. The plaques were incubated inverted, protected from light, at room temperature (about 25 °C) for 5 d (American Public Health Association, 2001; U.S. Food and Drugs Administration, 2001).

Most probable number of total coliforms

The technique used to determine the most probable number (MPN) of total coliforms (MPN-TC) was developed according to the American Public Health Association (2001) and U.S. Food and Drugs Administration (2001). After homogenization and decimal dilution (until 10^{-5}) of the sample, 1 ml of each dilution, in triplicate, was put into tubes with a Durham inverted tube each containing 9 ml of Brila Broth (Merck). The tubes were incubated in a hot bath with stirring at 36±1 °C for 42–48 h. Following this period of time, one aliquot of each tube, which was considered as positive, was sowed on MacConckey Agar plaques (Difco) to confirm the growth of microorganisms. The plaques were incubated in an inverted position at 37±1 °C for 18–24 h.

Most probable number of faecal coliforms

The technique was used to determine the most probable number of faecal coliforms (MPN-FC) was devised according to the American Public Health Association (2001) and U.S. Food and Drugs Administration (2001). Each tube, considered as positive in agreement with the MPN-TC technique, was sowed into a 9-ml tube of EC Broth (Difco) and into a 3-ml tube of Bacto Tryptone (Difco).

The tubes with EC Broth had a Durhan inverted tube each. All the tubes were incubated in hot bath with stirring at 44·5±1 °C for 42–48 h. Tubes with growth and gas production in EC Broth and positive Indol proof in Bacto Tryptone were considered as positive for NPM-FC count.

Table 1. Distribution of cases of mastitis prevalence and their respective percentages (in relation to the mammary quarters) found in the 36 analysed properties

Dairy herds data	Total number	Percentage, %
Sampled cows	1180	
Sampled quarters	4662	
Tamis Test positive (clinical mastitis)	125	2·68
CMT positive (total mastitis)	1824	39·12
No bacterial growth	379	8·13
Mastitis infection	1445	31·00
Staphylococcus spp.	429	9·20
Streptoccus spp.	249	5·34
Corynebacterium spp.	524	11·24
Staphylococcus spp.+ *Streptococcus* spp.	58	1·24
Staphylococcus spp.+ *Corynebacterium* spp.	94	2·02
Streptococcus spp.+ *Corynebacterium* spp.	63	1·35
Staphylococcus spp.+*Streptococcus* spp.+*Corynebacterium* spp.	7	0·15
Yeasts	2	0·04
Other microorganisms	19	0·41

Statistical analysis

To calculate the correlation between the occurrence of mastitis and the counts, we performed a logarithmical transformation ($\log_{10}$) of the microorganism counts in the raw milk, by using the OpenOffice 2.0 Calc software (2005).

Pearson's correlation coefficient was calculated in order to compare the frequency of mastitis (percentage) in each dairy herd with the respective counts of microorganisms in the raw milk, using the Minitab 14 Statistical Software.

Results

Samples of 4662 (100%) mammary quarters of the 1180 (100%) animals in lactation were collected from 36 farms. Among all the animals analysed, we observed the functional loss of 58 (1·24%) mammary quarters. Among the 4662 mammary quarters analysed, 1824 (39·12%) showed mastitis, of which 125 (2·68%) were cases of clinical mastitis. The majority of the detected mastitis cases were caused by some kind of microorganism. *Corynebacterium* spp. was the most frequently isolated microorganism, followed by *Staphylococcus* spp. and *Streptococcus* spp. Combinations of two or three of these microorganisms were detected as causing infection of mammary quarters (Table 1).

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Table 2. Lowest and highest values, averages and median of aspects related to mastitis in dairy bovine herds (number of mammary quarters)

Dairy herds data	Minimum	Maximum	Average	Median
Sampled cows	9	92	32·78	27
Sampled quarters	36	364	129·50	107·5
Tamil Test positive (clinical mastitis)	0	19	3·47	1
CMT positive (total mastitis)	7	148	50·67	46
No bacterial growth	0	42	10·53	6
Mastitis infection	6	126	40·14	34·5
Staphylococcus spp.	1	37	11·92	9
Streptoccus spp.	0	34	6·92	5
Corynebacterium spp.	0	64	14·56	9
Staphylococcus spp.+*Streptococcus* spp.	0	12	1·61	1
Staphylococcus spp.+*Corynebacterium* spp.	0	12	2·61	2
Streptococcus spp.+*Corynebacterium* spp.	0	8	1·75	1
Staphylococcus spp.+*Streptococcus* spp.+ *Corynebacterium* spp.	0	2	0·19	0
Yeasts	0	1	0·06	0
Other microorganisms	0	7	0·53	0

Table 3. Lowest, highest, average and median values of various microorganism counts in raw milk

Microorganisms Counts of raw milk in all dairy herds	Minimum	Maximum	Average	Median
Mesophilic Aerobic Plate Counts†	$1{\cdot}5\times10^{3}$	$1{\cdot}4\times10^{8}$	$8{\cdot}1\times10^{6}$	$2{\cdot}2\times10^{5}$
Psychrotrophic Aerobic Plate Count†	$2{\cdot}2\times10^{2}$	$1{\cdot}4\times10^{8}$	$9{\cdot}3\times10^{6}$	$4{\cdot}1\times10^{5}$
Thermophilic Aerobic Plate Count†	$<1{\cdot}0\times100$§	$9{\cdot}5\times10^{1}$	$1{\cdot}6\times10^{1}$	$5{\cdot}3\times10^{0}$
Yeasts and Moulds Count†	$3{\cdot}0\times10^{0}$	$1{\cdot}6\times10^{5}$	$1{\cdot}4\times10^{4}$	$2{\cdot}7\times10^{3}$
MPN–Total Coliform‡	$9{\cdot}3\times10^{0}$	$>1{\cdot}1\times10^{5}$¶	$2{\cdot}0\times10^{4}$	$2{\cdot}3\times10^{3}$
MPN–Faecal Coliform‡	$<3{\cdot}0\times10-1$§	$9{\cdot}3\times103$	$4{\cdot}9\times10^{2}$	$1{\cdot}5\times10^{0}$

† Plate count agar (cfu/ml)
‡ Most probable number (MPN/ml)
§ Minimum value detected
¶ Maximum value detected

The properties that were studied had between 36 and 364 mammary quarters. The variation of results for positive mammary quarters in CMT was 7–148, for the Tamis Test it was 0–19. Table 2 presents the minimum, maximum, average and median values for aspects related to mastitis prevalence by mammary quarter in the dairy cattle herds analysed. Counts of microorganisms in raw milk showed a large variation among the properties, MAPC and PAPC were the counts that presented the highest values and TAPC and MPN–FC presented the lowest ones (Table 3).

Table 4 shows the results of Pearson's correlation coefficient between the microorganism log counts and the main indicators of mastitis occurrence among the dairy herds studied. Log_{10} of the MAPC and PAPC presented a negative correlation with the cases of no-bacterial-growth mastitis ($P\leqslant0{\cdot}01$). Log_{10} of the TAPC and YMC presented a positive correlation with the cases of infectious mastitis and mastitis caused by *Staphylococcus* spp. ($P\leqslant0{\cdot}05$). There were no correlations between all other counts of microorganisms and mastitis cases.

Discussion

The low number of correlations among the variables presented in this experiment indicates the slight influence of bacteria that cause mastitis on the raw milk produced on farms and sent to dairy industries.

Staphylococcus spp., *Streptococcus* spp. and *Corynebacterium* spp. accounted for the majority of microorganisms that caused infectious mastitis. The incidence of mastitis caused by yeasts (0·04%) and other microorganisms (0·41%), including *Nocardia* spp. and Enterobacteria, was very low (Table 1). Piepers et al. (2007) isolated only 0·1% of coliforms in cases of subclinical mastitis. Other studies found a higher incidence of these microorganisms causing mammary gland inflammation (Costa et al. 1995; Vaarst & Enevoldsen, 1997; Langoni et al. 1998). Enterobacteria are generally described in association with clinical mastitis cases. The low incidence of clinical mastitis caused by yeasts and coliforms that was observed in this paper could have been due to the methodology of collection termed 'convenience samples' in the choice of

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Table 4. Values of Pearson's correlation test and *P*-value among the various $\log_{10}$ of microorganism counts in raw milk and the mastitis occurrence in dairy bovine herds

Correlation Test (Pearson)	MAPC†	PAPC‡	TAPC§	YMC¶	MPN-TC††	MPN-FC‡‡
CMT (total mastitis)	−0·084	−0·082	0·192	0·235	0·066	−0·070
No bacterial growth	−0·4821*	−0·454**	−0·286	−0·202	−0·351	−0·306
Infectious mastitis	0·113	0·104	0·329*	0·340*	0·220	0·054
Staphylococcus spp.	0·289	0·276	0·522**	0·369*	0·283	0·064
Streptococcus spp.	−0·133	−0·049	−0·249	0·232	−0·005	−0·162
Corynebacterium spp.	−0·125	−0·219	0·122	−0·109	−0·056	0·075

† Mesophilic Aerobic Plate Count, cfu/ml
‡ Psychrotrophic Aerobic Plate Count, cfu/ml
§ Thermophilic Aerobic Plate Count, cfu/ml
¶ Yeasts and Moulds Count, cfu/ml
†† Most Probable Number (MPN) Total Coliform, MPN/ml
‡‡ Most Probable Number (MPN) Faecal Coliform, MPN/ml
* $P<0·05$
** $P<0·01$

the properties, and not including the ones considered as problematic, with outbreaks or a high number of chronic cases of mastitis.

Several investigations point to a relation between the increase of mastitis incidence and the elevation in the counts of microorganisms in BTM. These investigations also show that the main microorganisms that cause mastitis can be isolated from BTM, and this fact indicates the presence of the infectious agent in the herd (Godkin & Leslie, 1993; Adesiyun, 1994; Felon et al. 1995; Hogan et al. 1988; Zadoks et al. 2004; Rysanek & Babak, 2005). In spite of several investigations showing that mastitis is directly related to the number of microorganisms present in BTM, under the conditions established for the present study it cannot be stated that the bacteria causing mastitis in dairy cattle herds also provoked the increase in numbers of microorganisms present in raw milk from the dairy farms.

Log_{10} of MAPC and PAPC presented a significant negative correlation ($P\leqslant 0·01$), when compared with the data on the occurrence of no-bacterial-growth mastitis (Table 4), despite factors related to no-bacterial-growth mastitis seeming to have influence on the decreasing of microorganisms in MAPC and PAPC. It was not the aim of this paper to evaluate these factors. A study of the evaluation of production factors influencing these relations might explain better the causes of these associations. Log_{10} of TAPC and YMC showed a positive correlation with the occurrence of infectious mastitis and mastitis caused by *Staphylococcus* spp. Factors related to the increase of $\log_{10}$ TAPC and YMC seem to be associated with causes that have influence on the increase of infectious mastitis and of *Staphylococcus* spp. mastitis in dairy herds under the conditions of this experiment.

Teat washing and drying prior to milking significantly reduce the bacterial contamination of milk. Bacterial counts increase at each stage as the milk passes through the milking equipament (McKinnon et al. 1990). The limited shedding of *Staph. aureus* from infected quarters means that results do not accurately estimate herd prevalence. Increased numbers of environmental organisms and *Staphylococcus* spp. on BTM suggest that equipment sanitation and/or udder preparation procedures are insufficient (Goldberg et al. 1991). After it leaves the udder, milk may become contaminated with microorganisms from the surfaces of the cow, from the environment and from unclean milking systems (Hayes, 1995; Richter & Vedamuthu, 2001). Characteristics associated with the lack of hygiene and contamination during the process of milk production must influence even more the raw-milk indicators of hygienic-sanitary quality than the contamination by microorganisms that cause the inflammatory process in the mammary gland under the conditions established in this experiment. The wide variation of microorganism counts that was observed could indicate that the hygiene conditions of the visited properties were very different (Table 3). The use of dairy herds with low microorganism counts in raw milk possibly increases the correlation level in comparison with the parameters of mastitis occurrence in dairy herds.

Rysanek & Babak (2005) observed that herds with more elevated bulk tank milk somatic cell count presented a better correlation with bulk tank milk total bacteria count and coliform bacteria count. On the other hand, Gonzales et al. (1986) asserted that despite the number of bacterial colonies isolated from BTM being a good indicator of the percentage of cows infected in the herd when the number of infected animals is very high, these correlations were not sufficiently large to provide perfect predictability. In this investigation, the somatic cell count (SCC) was not a considered parameter for the selection or the evaluation of the properties because the aim was to ascertain whether the microorganisms that cause mastitis influence the number of microorganisms that are present in raw milk.

Jayarao & Wolfgang (2003) declared that the individual analysis on the quality of milk from animals infected by mastitis offers more accurate data when compared with BTM analysis, but this second procedure is less expensive.

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A reason for the different results obtained in the present study might be the methodology adopted. Many studies limit themselves to researching factors that indicate mastitis directly from BTM. The search for microorganisms that cause mastitis by means of the analysis of mammary quarters directly from the cattle herds can present a more accurate result on the lack of evidence for the correlation between microorganisms that cause mastitis and raw-milk indicators of hygienic-sanitary quality.

Monitoring BTM may be an effective means for detecting management changes in herds with low bacterial and milk SCC (Hogan et al. 1988). The low correlation between the $\log_{10}$ of raw-milk indicators of hygienic-sanitary quality and the parameters of mastitis frequency observed in the present experiment may have been due to the large variability observed in the counts of microorganisms on the different farms (Table 3).

The present experiment did not find a high level of correlation between raw-milk indicators of hygienic-sanitary quality and mastitis occurrence in dairy herds, but this does not mean that the inflammatory process of the mammary glands does not influence milk quality. Many investigations show that high mastitis frequency in dairy herds or high SCC in raw milk have a negative influence on the durability and quality of dairy products, causing a shortening of shelf life. The increase of SCC causes the increase of proteolysis and lipolysis of raw milk, processed milk and dairy products. Cheese, yogurts and other dairy products produced from milk with high SCC show an inferior quality (Philport, 1967; Rogers & Mitchell, 1994; Kelly & Foley, 1997; Santos et al. 2003; Larsen et al. 2004; Leitner et al. 2006; Fernandes et al. 2007).

The analysis of the productive process should be carefully studied in order to identify the possible causes that cause an increase of the microorganisms count in BTM. Standardized production processes and a systematic methodology that provides indicators of the raw-milk quality must be created for specific conditions according to the social-economic-cultural situations of each region.

Conclusion

In this study we observed a low correlation between the raw-milk indicators of hygienic-sanitary quality and the occurrence of mastitis in bovine dairy herds. The high variability in the counts of microorganisms from the raw milk is the probable cause of the low correlation between the analysed variables. Factors related to the lack of hygiene and environmental contaminations in productive systems seem to have more influence on the microbiological quality of the raw milk than the mastitis frequency in dairy herds.

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