Casein hydrolysate for mammary quarters drying-off during lactation in cows with chronic mastitis

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Supplementary File

Material and Methods

Herd Characteristics, Inclusion Criteria and Cases Definition

The farms were located in Minas Gerais (n = 2) and São Paulo (n = 5) states, with an average of 232 lactating cows (ranging from 100 to 500 cows). Cows were housed in compost bedded pack barn (n = 3), free stall (n = 1) and grazing (n = 3) systems, and nutritional management was balanced according to stage of lactation and milk yield. Lactating cows were milked twice (n = 6) or three times/day (n = 1), and five farms had an automatic milk yield meter.

Clinical chronic mastitis cows were considered when she presented three or more cases of clinical mastitis in the same quarter within the same lactation, with an interval of at least 14 days between clinical cases. For subclinical chronic mastitis, cows were considered chronic when the SCC >1,000,000 cells/mL in composite milk samples were observed for at least two consecutive months. All selected farms recorded the clinical cases of mastitis in on farm spreadsheets.

Clinical mastitis was identified by trained milkers, based on visible changes of the appearance of the first streams of milk before milking (presence of lumps, pus, clots, blood or aqueous milk) and/or in the udder (edema, redness).

For subclinical mastitis cows which were selected based on the SCC criteria, the California mastitis test (CMT) was performed to identify the affected MQ. Only cows that had only one affected QM (score 3 on CMT) were selected. Cows that had clinical signs and recent antimicrobial treatment (systemic or intramammary) up to 30 days before the start of the experiment were not included in the study.

Milk Samples and Analysis

Prior to collection, the MQ were immersed in pre-milking disinfectant solution and, after 30 sec, dried with disposable paper towels. Teat ends asepsis was performed with 70% alcohol, and the first milk strips were discarded before milk collection. After teat end disinfection, 10 mL of milk from the MQ were collected in sterile tube previously identified and transported refrigerated in an isothermal box to the laboratory, where they were kept frozen (-20 ° C) until the mastitis-causing pathogen identification.

Microbiological Identification by MALDI-TOF MS.

An aliquot of milk (0.01 mL) was inoculated in blood agar plate supplemented with 5% bovine blood and aerobically incubated at 37 °C for 48 h. For identification, a bacterial colony was transferred with a sterile wood applicator to an MSP 96-spot plate (Bruker Daltonics Inc.) for ribosomal protein extraction and kept at room temperature for drying. Then, 1 μL of matrix solution (α-cyano-4-hydroxycinnamic acid matrix- saturated solution in 50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid) were added to each plate spot

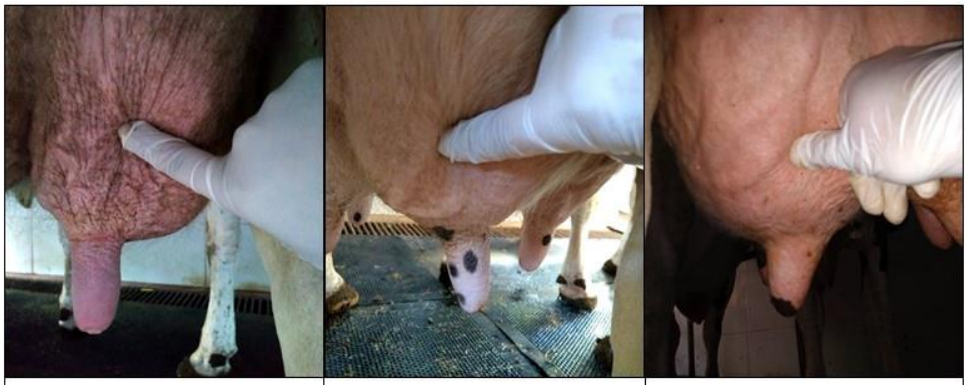
over the colonies. All plates were calibrated with bacterial test standard (BTS, Bruker Daltonics Inc). The spectra were generated by Microflex LT Bruker equipment from ± 250 randomly emitted laser shots. Flex Control software (version 3.4) was used to automatically capture the mass spectra of each sample, generating a fingerprint (protein peak set) that was later compared to the MBT Compass software database (MALDI BioTyper® Compass, version 4.1.7, User Manual, Bruker Daltonics Inc.) for the classification of spectra.

The degree of similarity between sample spectrum and reference spectrum was interpreted according to the manufacturer's recommendations (Bruker Daltonics Inc.), and a score ≥ 2 was considered for species-level identification, between 1.7 and 2 at genus level and score <1.7 when there was no reliable identification.

Cessation of Lactation

Udder pressure was assessed by a single evaluator in all CH-treated cows, by pressing the mammary gland about five centimeters above the MQ base. A scale from 0 to 3 was used, where: (0) represents no pressure; (1) mean pressure; (2) high pressure; and (3) extremely high pressure, characteristic of inflammatory process. An intermediate score was included between scores 1 and 2, since the cessation of lactation of some mammary quarters occurred more gradually (Supplementary Figure S1).

Supplementary Figure S1. Involution degrees of dry mammary quarters with casein hydrolysate



Degree 1	Degree 1.5	Degree 2

Statistical Analysis

Data were analyzed using the Statistical Analysis System® (SAS, version 9.4) software. The normality of residues and homogeneity of variances were verified by PROC UNIVARIATE. The degrees of freedom were calculated according to the Satterthwaite method (DDFM = Satterth) and the adjusted means were calculated by the DIFF option of the LSMEANS statement. For all statistical analyses, statistical significance was defined at *P*-value ≤ 0.05. The adjusted means of the descriptive characteristics of the selected cows (parity, average milk yield, SCC, number of clinical mastitis cases and DIM) before infusion of CH, as well as the results of milk SCC were compared between treatments by Tukey test. For statistical analysis of cessation of lactation index, the PROC GLIMMIX was used according to the model:

$$logit(pi) = \beta 0 + \beta 1 \times Treat + \beta 2 \times day + \beta 3 \times (Treat \times day) + Re$$

Where logit (pi) is the function of the UPI score probability (0, 1, 1.5, 2 and 3); β0 is the intercept; β1 regression coefficient for intramammary treatment (Trat); β2 is the regression

coefficient of the observation day (day); β3 is the regression coefficient for Trat × day interaction. First order autoregressive error structure was used and Re is the term residual of the model.

Somatic cell count (× 10^3 cells/mL) was transformed to logarithmic scale according to the formula $LOG_{SCC} = Log_2 (SCC/100) + 3$, as suggested by Schukken *et al*. (2003). Analysis of milk yield data at D0 and D7 and SCC of untreated quarters (D30, D60 and D90) was performed by PROC MIXED according to the following model:

$$Y_{ijkl} = \mu + T_i + Dj + Dj \times T_i + LogSCC1 + CMC + DIM + Pt + \text{Farm} + e_{ijkl},$$

Where Y_{ijkl} = is the observed value; μ = overall mean; Ti = fixed treatment effect i; Dj = fixed effect of observation day; Dj × Ti = interaction between Dj and Ti; LogSCC1 = fixed effect of composed SCC log prior to treatment; CMC = fixed effect of number of clinical mastitis cases; DIM = Fixed effect of days in milk at drying-off; Pt = fixed effect of parity; Farm = random farm effect; eijkl = random error associated with each observation. The days of observation were included in the model as repeated measures over time.

Results

Cows' Characteristics

Supplementary Table S1 - Adjusted means of cows' characteristics with chronic mastitis before treatments with one or three intramammary infusion of casein hydrolysate

Characteristics	Single infusion (n = 30)	Three infusions (n = 30)	Total (n = 60)	SEM[1]	*P value*[*]
Parity	2.60	3.23	2.91	0.20	0.130
Milk yield, Kg/d	20.70	18.40	19.57	0.94	0.230
Previous cases of clinical mastitis	2.23	1.86	2.05	0.15	0.220
Days in milk	239.10	266.10	252.60	14.10	0.340
Cows' logSCC[2]	6.59	6.14	6.37	0.23	0.340
MQ's logSCC[3]	7.32	7.41	7.36	0.31	0.880

[*] Means compared by Tukey test and significative values <0.05

[1]SEM: standard error mean.

[2]Cows' logSCC: Composed SCC logarithmic scale before infusion of CH.

[3]MQ's logSCC: Mammary quarter SCC logarithmic scale before infusion of CH.

Frequency of Mastitis Causing Pathogens

Supplementary Table S2 - Frequency of mastitis causing pathogens isolated from chronic mastitis before infusion of casein hydrolysate

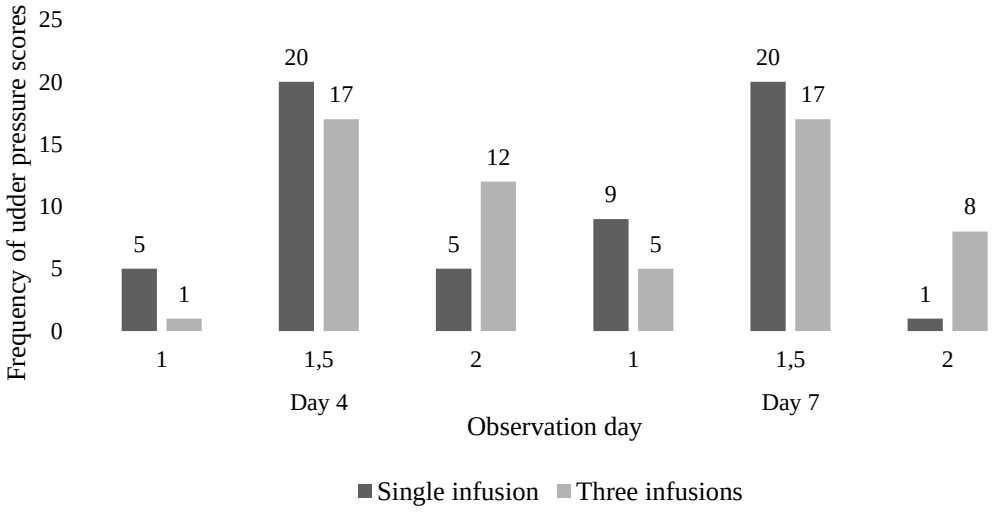
Microorganisms	Single infusion		Three infusions		Total	
	N	%	N	%	N	%

Negative culture	8	26.67	9	30.00	17	28.33
Positive culture	22	73.33	21	70.00	43	71.66
Gram-positive						
Strep. uberis	11	50.00	6	28.57	17	39.53
Staph. aureus	2	9.09	4	19.04	6	13.95
Corynebacterium spp.	2	9.09	2	9.52	4	9.30
Staph. chromogenes	2	9.09	2	9.52	4	9.30
Staph. sciuri	0	0	2	9.52	2	4.65
Strep. dysgalactiae	1	4.54	2	9.52	3	6.97
Strep. agalactiae	1	4.54	0	0	1	2.32
Enterococcus faecalis	0	0	1	4.76	1	2.32
Strep. gallolyticus	1	4.54	0	0	1	2.32
Strept. pluranimalium	1	4.54	0	0	1	2.32
S. aureus/ S. dysgalactiae	1	4.54	0	0	1	2.32
Gram-negative						
Pseudomonas aeruginosa	0	0	1	4.76	1	2.32
Stenotrophomonas maltophilia	0	0	1	4.76	1	2.32
Total	30		30		60	

After intramammary CH infusions (D7), 10 MQ (16.67%) of the 60 MQ evaluated, presented bacteriological cure, of which six (20%) were treated with three intramammary infusions of CH. initially identified as *Staph. sciuri* (n = 2), *Strep. uberis* (n = 1), *Corynebacterium bovis* (n = 1), *Enterococcus faecalis* (n = 1) and *Stenotrophomonas maltophilia* (n = 1)*;* and four (13,33%) with single infusion of CH, initially identified as *Strep. uberis* (n = 2), *Corynebacterium bovis* (n = 1) and *Staph. chromogenes* (n = 1).

Evaluation of Udder Pressure

Supplementary Figure S2 - Frequency of udder pressure scores of the mammary quarters treated with one or three intramammary casein hydrolysate infusions according to the evaluation days after treatments



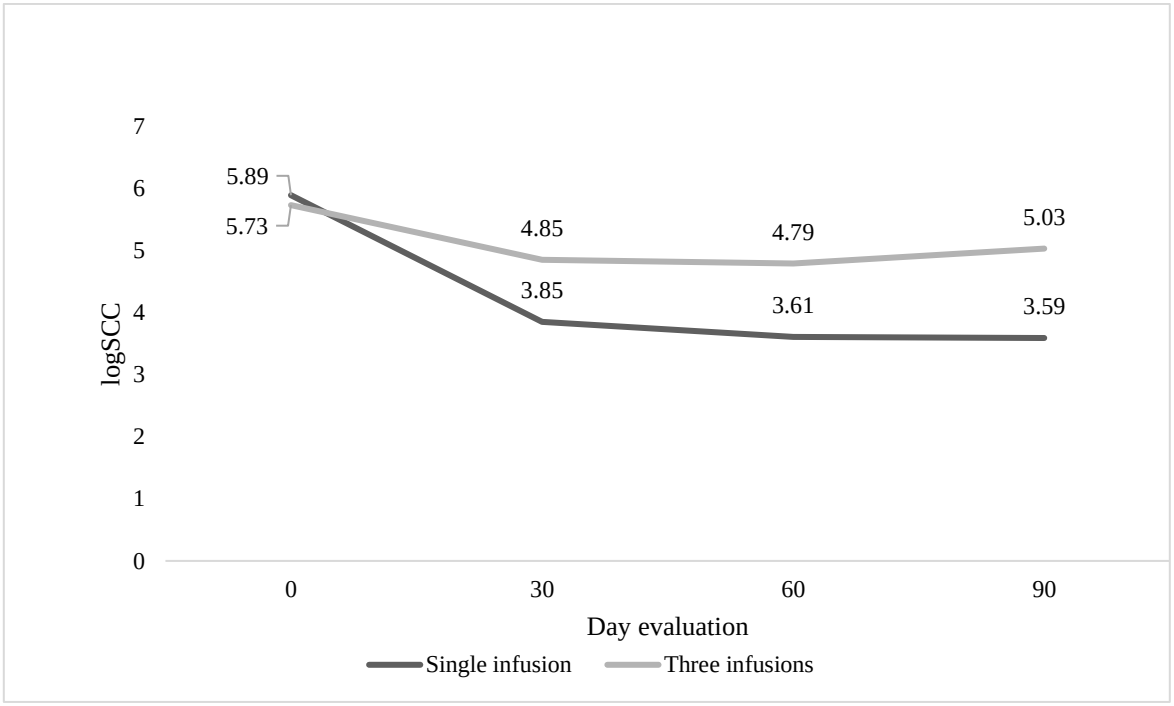
$P_{treatement}$ = 0,0018; P_{day} = 0,0146; $P_{treatment \times day\ interaction}$ = 0,9751

Milk Yield

P_{treat} = 0,2657; P_{day} = 0,0004; $P_{treat\ x\ day}$ = 0,5289; P_{DIM} = 0,0007; P_{LogSCC} = 0,8584; P_{CMH} = 0,3250; P_{Pt} = 0,1229

SCC of Untreated Mammary Quarters

Supplementary Figure S3 - Effect of casein hydrolysate infusion in mammary quarters with chronic mastitis on logSCC composed of functional quarters



$P_{treatment}$ = 0.6440; P_{day} = 0.0002; $P_{treatment \times day}$ = 0.5428; P_{DIM} = 0.1381; P_{LogSCC} = 0.3828; $P_{number\ of\ clinical\ mastitis\ case}$ = 0.6133; P_{Parity}= 0.0007

P value within treatment: One-CH: $P_{day\ 0 \times day\ 30}$ = 0.0035; $P_{day\ 0 \times day\ 60}$ = <0.0001; $P_{day\ 0 \times day\ 90}$ = <0.0001; $P_{day\ 30 \times day\ 60}$ = 0.4295; $P_{day\ 30 \times day\ 90}$ = 0.4610; $P_{day\ 60 \times day\ 90}$ = 0.9440. Three-CH: $P_{day\ 0 \times day\ 30}$ = 0.1630; $P_{day\ 0 \times day\ 60}$ = 0.0482; $P_{day\ 0 \times day\ 90}$ = 0.1679; $P_{day\ 30 \times day\ 60}$ = 0.8547; $P_{day\ 30 \times day\ 90}$ = 0.5633; $P_{day\ 60 \times day\ 90}$ = 0.4162

P value between treatment: $P_{day\ 0}$ = 0.6444; $P_{day\ 30}$ = 0.2789; $P_{day\ 60}$ = 0.0837; $P_{day\ 90}$ = 0.0533

D0 = logSCC of four quarters; D30 = logSCC of three functional quarters; D60 = logSCC of three functional quarters; D90 = logSCC of three functional quarters.

SCC data were transformed on logarithmic scale according to Schukken et al. (2003), based on the following equation: $LogSCC = Log_2(SCC/100) + 3$. For example, SCC values of 200 and 700 (x 10^3 cells / mL) corresponding to the logSCC of 4.0 and 5.8, respectively.