mRNA expression of immune factors and milk proteins in mammary tissue and milk cells and their concentration in milk during subclinical mastitis

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Mastitis causes changes of milk protein composition and synthesis of various immune factors. We have investigated if changes take place during subclinical mastitis with somatic cell counts (SCC) between 150,000 and 300,000 cells/ml. The experiment included 8 control animals with a SCC <150,000 cells/ml (C) and 5 cows with partially elevated quarter SCC showing at least 1 quarter with SCC >150,000 and ≤300,000 cells/ml (H) and 1 quarter with <150,000 cells/ml (L).The milk from 1 quarter of control cows and from 2 quarters of cows with partially elevated SCC was collected at one milking. At slaughter mammary tissue of the respective quarters was sampled. mRNA expression of κ-CN was reduced in H compared to L quarter whereas mRNA expression of all other investigated parameters showed no differences between groups in mammary tissue. All immunological factors measured showed elevated mRNA expression in milk cells of H quarters. In H quarters the milk protein content was elevated. Changes occurred despite only mild subclinical mastitis.

mRNA Expression von immunologischen Faktoren und Milchproteinen in Milchdrüsengewebe und Milchzellen und ihre Konzentration in der Milch während einer subklinischen Mastitis

Mastitis bedingt Veränderungen der Milchproteinzusammensetzung und der Synthese immunologischer Faktoren. In dieser Arbeit wurde untersucht, ob Veränderungen bei einer subklinischen Mastitis mit einer somatischen Zellzahl (SCC) zwischen 150.000 und 300.000 Zellen/ml stattfinden. Das Experiment umfasste 8 Kontrolltiere mit einer SCC unter 150.000 Zellen/ml (C) und 5 Kühe mit teilweise erhöhten Zellzahlen. Deren SCC war wenigstens in einem Viertel >150.000 und ≤300.000 Zellen/ml (H) und in einem Viertel <150.000 Zellen/ml (L). Die Gemelke eines Viertels der Kontrolltiere und zweier Viertel der Tiere mit teilweise erhöhtem SCC wurden am Tag vor der Schlachtung gesammelt. Am Tag der Schlachtung wurde das Milchdrüsengewebe der jeweiligen Viertel entnommen. Die κ-CN-mRNA-Expression im Milchdrüsengewebe war gegenüber den L-Vierteln in den H-Vierteln erniedrigt, während bei allen anderen Parametern keine Expressionsunterschiede zwischen den Gruppen auftraten. Die mRNA-Expression der untersuchten immunologischen Faktoren war in den Milchzellen der H-Viertel ebenso erhöht wie der Gesamtproteingehalt. Die beschriebenen Veränderungen traten trotz einer nur leichten subklinischen Mastitis auf.

06 Mastitis (immunological factors) **06 Mastitis** (immunologische Faktoren)

1. Introduction

The immune defence of the mammary gland is mainly based on unspecific mechanisms but also on specific components. The concentration of somatic cells reflects the activity of the cellular immune system in response to microorganisms and hence the health status of the mammary gland.

The soluble immune components within the nonspecific immune response comprise inflammatory mediators like cytokines, platelet activating factor (PAF) and nitric oxide (NO) as well as antimicrobial factors. In addition, milk proteins and bioactive peptides derived from them supposedly contribute to the defence mechanisms of the mammary gland (19).

The concentration of immune mediators changes during mastitis (24, 15, 20). Proteins from the circulation and antimicrobial proteins like lactoferrin (Lf) and lysozyme (Lz) have been reported to increase during mastitis whereas major milk proteins decrease (6, 5, 9).

In the present study, the mRNA expression of different milk proteins and immunological factors in mammary tissue and the protein composition of milk within the lowest subclinical range of mastitis were investigated. The mRNA expression of immunological factors was determined in milk cells, too.

2. Materials and methods

2.1 Animals and sample collection

Thirteen dairy cows free of clinical mastitis were selected. Cows (n=8) with a foremilk SCC below 150,000 cells/ml in all quarters were defined as control group (C); cows with at least one quarter with SCC from 150,000 to 300,000 cells/ml (H=high) and one with <150,000 cells/ml (L=low) were defined as group with partially elevated quarter SCC (n=5). The milk from one quarter of C cows and from 2 quarters of cows with partially elevated SCC (1 of H and 1 of L) was collected at one milking. On the next day, cows were slaughtered and mammary tissue from the respective quarters was collected, snap frozen in liquid nitrogen and stored at –80 °C until analysis.

2.2 RNA preparation and cDNA synthesis

Milk samples were centrifuged for 30 min at 4 °C and 220 g. Thereafter the cell pellet was washed in phosphate buffered saline pH 7.4 and centrifuged again. Total RNA of milk cells and mammary tissue was isolated using TriPure (Roche Diagnostics, Mannheim, Germany). To quantify the amount of total RNA extracted, optical density (OD) of the RNA stock solution was determined

at 260 nm. In addition, optical density of the OD_{260nm}/OD_{280nm} (nucleic acid/protein) absorption ratio was measured and shown to be in the optimum range between 1.8 and 2.0. RNA integrity was verified by ethidium bromide stained gel electrophoresis. Complementary DNA (cDNA) was synthetized with reverse transcriptase (MMLV-RT H -, Promega, Madison, WI, USA) and random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany).

2.3 Quantification by real-time PCR

Primer pairs were either newly designed using published bovine or human nucleic acid sequences or previously published primer sequences were used (1, 16, 14). Sense and antisense primers of each target gene were designed to produce an amplification product which spanned two exons. All relevant primer information is listed in Table 1.

PCR was performed in the LightCycler (Roche Diagnostics) with 25 ng and accordingly 10 ng (for highly expressed milk proteins) reverse transcribed total RNA (ng/µl). Further reaction components for the LightCycler reactions were 1.0 µl LightCycler DNA Master SYBR Green I (Roche Diagnostics) and 1.2 µl $MgCl_2$ (4 mM), 0.2 µl forward primer (0.4 µM), 0.2 µl reverse primer (0.4 µM) and water up to a final volume of 10 µl. All PCR reactions were performed with 40 cycles and product specific PCR cycle conditions are listed in Table 2. To verify the specificity of each PCR quantification real-time amplified RT-PCR products were sequenced and a nucleotide sequence identity to the bovine sequence of at least 98–100% could be confirmed (HUSAR program, DKFZ, Heidelberg, Germany).

In order to confirm a constant housekeeping gene expression level in the investigated total RNA extractions derived from the different samples, additionally an Ubiquitin (UbC) real-time RT-PCR was performed.

2.4 Enzyme linked immunosorbent assay (ELISA) for Lf determination in milk

Bovine Lf (Sigma Aldrich, Taufkirchen, Germany) was used to generate a polyclonal antiserum in rabbits. The specificity of the serum was confirmed by Western Blot. Using the double antibody technique (17), a competitive approach with biotinylated bLF as tracer was realised. In brief, microtiter plates were coated with Fc fragment from sheep anti-rabbit IgG. After an 1 h preincubation of standard bLF or a milk sample with the antiserum (1:630,000 final dilution), biotinylated Lf was added and incubated for 1 h. Bound tracer was quantified via streptavidin-peroxidase as described earlier (11). The assay´s limit of detection was 5 ng/ml, parallelism between bLF standard curve and different dilutions of milk samples was confirmed and the recovery of bLF spiked into milk samples was 101.2±12.1 % (n=6). Intra- and inter-assay of variation were 3.9 and 7.5%, respectively.

2.5 Compositional analysis

Total quarter milk SCC was measured on a Fossomatic 5000 (Foss Electric, Hillerød, Denmark) and the determination of lactose and milk fat percentages was accomplished with a Milko-Scan 605 (Foss Electric) in the laboratory of the Milchprüfring Bayern e.V.. Total nitrogen, non-casein nitrogen, non-protein nitrogen were determined by standard laboratory procedures (22). Components of the casein (CN) and whey protein were separated and quantified by reversed phase HPLC methods (8, 23).

Table 1: Sequence of PCR primers (forward: for and reverse: rev), PCR product length and GenBank and EMBL accession number (species in brackets) of the used published nucleic acid sequences

Primer	Sequence (5'→3')	Length	EMBL ac. no.
UbC for UbC rev	AGATCCAGGATAAGGAAGGCAT GCTCCACCTCCAGGGTGAT	198 bp	Z18245 (bovine)
IGF-1 for IGF-1 rev	TCGCATCTCTTCTATCTGGCCCTGT GCAGTACATCTCCAGCCTCCTCAGA	240 bp	X15726 (bovine)
INOS for iNOS rev	ACCTACCAGCTGACGGGAGAT TGGCAGGGTCCCCTCTGATG	316 bp	U14640 (bovine) L09210 (human)
Lz for Lz rev	GAGACCAAAGCACTGATTATGGGA TCCATGCCACCCATGCTCTAA	195 bp	U25810 (bovine)
PAF-AH for PAF-AH rev	CGATTTATTCTGCTATTGGCATTG GGCTCCACCAAAAGAATGTCC	362 bp	U34247 (bovine)
α_{s1}-CN for α_{s1}-CN rev	GAACTGAGCAAGGATATTGGGA TAGGCATCCAGCTGGTAGAAT	362 bp	M33123 (bovine)
α_{s2}-CN for α_{s2}-CN rev	GGACGATAAGCACTACCAGA TGGCTTCATAGCTTTCTGATGC	358 bp	M16644 (bovine)
β-CN for β-CN rev	TCCCTAAATATCCAGTTGAGCC TCCTGGTACAGCAGAAAGGC	253 bp	M16645 (bovine)
κ-CN for κ-CN rev	ACCAACAGAAACCAGTTGCAC CTACAGTGCTCTCTACTGCTT	303 bp	M36641 (bovine)
α-LA for α-LA rev	ACCAGTGGTTATGACACACAAGC AGTGCTTTATGGGCCAACCAG T	233 bp	M18780 (bovine)
β-LG for β-LG rev	AGATCGATGCCTTGAACG AGAA TGTCGAATTTCTCCAGGGCCT	165 bp	X14712 (bovine)
Lf for Lf rev	GGCCTTTGCCTTGGAATGTATC ATTTAGCCACAGCTCCCTGGAG	338 bp	L08604 (bovine)

Table 2: Product specific LightCycler PCR conditions

Factor	Denaturation		Primer annealing		Elongation		Fluorescence acquisition	
	(°C)	(s)	(°C)	(s)	(°C)	(s)	(°C)	(s)
UbC	95	5	62	10	72	20	85	3
IGF-I	95	5	62	10	72	20	86	3
iNOS	95	5	62	10	72	20	88	3
Lz	95	5	62	10	72	20	84	3
PAF-AH	95	5	62	10	72	20	83	3
α_{s1}-CN	95	5	62	10	72	20	81	3
α_{s2}-CN	95	5	62	10	72	20	80	3
β-CN	95	5	62	10	72	20	85	3
κ-CN	95	5	62	10	72	20	81	3
α-LA	95	5	62	10	72	20	78	3
β-LG	95	5	62	10	72	20	83	3
Lf	95	5	62	10	72	20	86	3

UbC, ubiquitin; IGF-I, insulin-like growth factor I; iNOS, inducible nitric oxide synthase; Lz, lysozyme; PAF-AH, platelet activating factor- acetylhydrolase; CN, casein; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; Lf, lactoferrin

2.6 Data acquisition and statistical evaluations

mRNA expression was evaluated by amplification curve analysis of the LightCycler real-time RT-PCR. For the determination of expression levels only the crossing point (CP) values obtained by the LightCycler software (3.1) based on the *"Fit Point Method"* fluorescence acquisition were calculated. The CP value is a virtual cycle number and the more highly the less template molecules are contained in a sample and vice versa. Therefore values determined were subtracted from the highest cycle number (= 40) used to amplify PCR products

Table 3: Relative mRNA expression (mean±SEM) of immunologically relevant factors in milk cells and mammary tissue and correlation with the SCC within the lowest range of subclinical mastitis

	Rel. mRNA expression (log_2) in milk cells			Correlation		Rel. mRNA expression (log_2) in mammary tissue			Correlation	
	C	L	H	R	P	C	L	H	R	P
IGF-I	9.7±0.8	11.3±0.5	12.9±0.4^x	0.62	***	12.9±0.1	12.2±0.3	12.9±0.5	0.21	0.38
iNOS	13.8±1.0	15.9±0.8	17.6±1.3^x	0.53	**	9.3±0.5	9.0±0.7	9.3±0.7	-0.03	0.91
Lz	17.4±0.9	17.9±1.4	20.0±0.6	0.46	**	13.2±0.6	13.7±0.4	13.3±0.4	0.06	0.81
PAF-AH	9.8±0.8	11.6±0.9	12.8±1.1	0.51	**	8.7±0.4	8.9±0.5	9.5±0.3	0.27	0.26

^{x}Means of quarters "L" and "H" are significantly different (p<0.1); **p<0.05; ***p<0.01. Abbreviations see Table 2

and thus ratios represented in reverse. The logarithm dualis (log_2) was used to realise the difference between two CP values. Data are presented as means±SEM. Differences between C and L quarters were non-parametrically tested for significance (p<0.1) using Wilcoxon's rank sum test of SAS version 8.01 (18). Differences between L and H quarters (within animals) were tested for significance (p<0.1) by Wilcoxon's signed rank test of SAS. In addition, Pearson's correlation coefficients were calculated.

3. Results

3.1 IGF-I, iNOS, Lz and PAF mRNA expression in mammary parenchyma and in milk cells (Table 3)

The mRNA expressions both in tissue and milk cells were similar in L and C quarters for all factors tested. The same applied to their expression in mammary tissue of H and L quarters. Significant positive correlations between SCC and mRNA expressions were only determined in milk cells. mRNA expressions of insulin-like growth factor I (IGF-I) and inducible nitric oxide synthase (iNOS) in milk cells were higher (p<0.1) in H compared to L quarters whereas those of Lz and PAF-acetylhydrolase (AH) were only numerically and not significantly higher (P=0.19; P=0.25). mRNA expression of IGF-I and iNOS as well as PAF-AH and Lz were positively correlated with the SCC.

With exception of IGF-I mRNA, all factors tested were expressed at a higher level in milk cells than in udder tissue.

3,2 Milk proteins and other milk constituents (Tables 4, 5)

κ-CN concentration in milk was different (p<0.1) between C and L quarters whereas the other parameters referring to mRNA expression and concentration in milk were not different between quarters. mRNA expression of κ-CN in tissue was smaller (p<0.1) in H than in L quarters. Furthermore, correlation of α-LA mRNA and SCC was negative. Concentration of lactose in milk was negatively correlated with SCC. The protein content in the milk from H was higher (p<0.1) than in L quarters and positively correlated with SCC. Beside Lf concentration within the whey proteins only serum albumin (BSA) was numerically increased (P=0.31) in milk of H in relation to L quarters and positively correlated with SCC. Proportion of α_{s1}-CN and SCC were negatively correlated, while proportions of α_{s2}-, β- and κ-CN showed no correlation with SCC.

Table 4: Relative mRNA expression (mean±SEM) of milk proteins in mammary tissue and correlation with the SCC within the lowest range of subclinical mastitis

	Rel. mRNA expression (log_2)			Correlation	
	C	L	H	R	P
α_{s1}-CN	29.2±0.3	29.4±0.2	29.1±0.1	-0.30	0.20
α_{s2}-CN	26.2±0.3	26.6±0.2	26.2±0.3	-0.21	0.37
β-CN	28.7±0.4	29.1±0.3	28.7±0.3	-0.17	0.47
κ-CN	28.4±0.2	28.6±0.1	28.2±0.2^x	-0.28	0.23
α-LA	22.1±0.7	21.8±0.3	21.6±0.5	-0.39	*
β-LG	23.3±0.4	24.1±0.4	23.4±0.4	-0.28	0.23
Lf	24.4±0.7	24.6±0.6	25.2±0.8	0.43	*

^{x}Means of quarters "L" and "H" are significantly different (p<0.1); *p<0.1. Abbreviations see Table 2

Table 5: Contents and fractions of milk proteins and other milk parameters (means±SEM) in milk as well as their correlation with SCC within the lowest range of subclinical mastitis

Parameters	Contents C	L	H	Correlation R	P
SCC (1000/ml)	44±17	64±18	231±26^x	–	–
Fat (%)	3.0±0.4	2.9±0.3	2.7±0.3	0.14	0.55
Lactose (%)	4.6±0.1	4.7±0.1	4.5±0.1	-0.40	*
Total protein (%)	3.5±0.2	3.6±0.2	3.7±0.2^x	0.45	**
NPN[1] (%)	0.02±0.00	0.03±0.00	0.03±0.00	0.33	0.16
Casein (%)	2.8±0.1	2.9±0.2	2.8±0.2	0.38	**
CN[2]:total protein ratio (%)	79.3±0.6	79.0±0.6	77.3±0.8	-0.52	**
α_{s1}-CN (%)[3]	37.3±0.6	35.9±1.2	35.2±1.4	-0.57	***
α_{s2}-CN (%)[3]	4.8±0.6	4.7±0.4	4.1±0.3	-0.27	0.24
β-CN (%)[3]	42.2±1.2	42.6±0.7	42.1±1.0	-0.32	0.17
κ-CN (%)[3]	10.9±0.5$^+$	9.3±0.7	9.5±0.9	0.12	0.61
Whey protein (%)	0.58±0.05	0.59±0.05	0.67±0.08	0.53	**
β-LG (%)[4]	67.0±2.1	64.0±2.3	65.0±3.3	-0.16	0.50
α-LA (%)[4]	22.1±1.3	24.8±1.7	22.5±2.2	-0.33	0.16
BSA[5] (%)[4]	3.6±0.5	3.9±0.6	4.5±0.8	0.52	**

[1]Non protein nitrogen; [2]casein; [3]% of total casein; [4]% of total whey protein; [5]bovine serum albumin. ^{x}Means of quarters "L" and "H" are significantly different (p<0.1). $^+$Means of quarters "K" and "L" are significantly different (p<0.1). *p<0.1; **p<0.05; ***p<0.01

mRNA expression of Lf in mammary tissue of H and L quarters was not different whereas Lf concentration in milk was increased (p<0.1) in H as compared to L quarters (Fig. 1a). Both, Lf mRNA and Lf protein concentration was positively correlated with SCC (Table 4, Fig. 1b). Also the correlation between Lf concentration in milk

and Lf mRNA expression in mammary tissue was highly significant (Fig. 1c).

UbC (housekeeping gene) mRNA expression was almost similar in all groups without detectable differences ($p > 0.1$; data not shown).

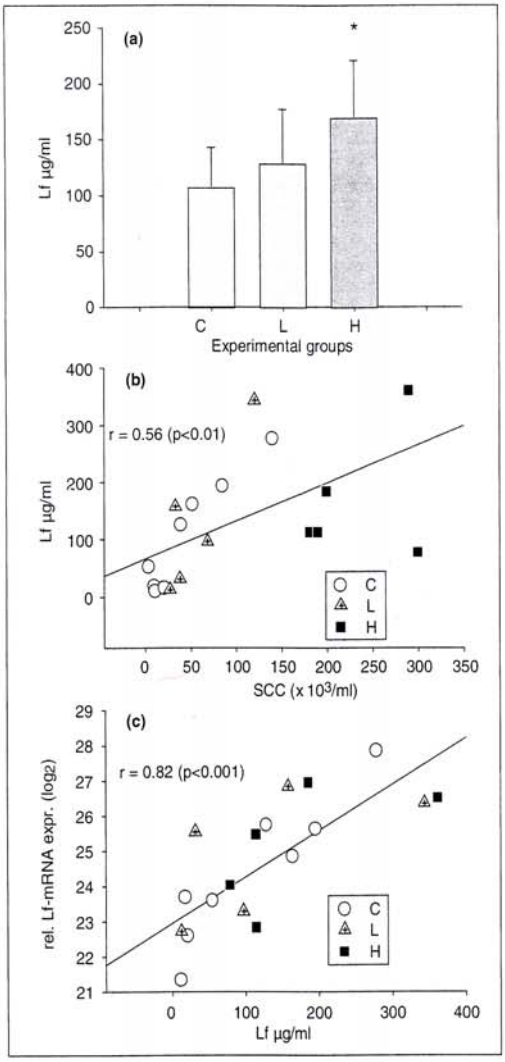

Fig. 1: (a) Lf concentration in milk of experimental groups. *, means of quarters "L" and "H" are significantly different. (b) Correlation between SCC and Lf concentration in milk. (c) Correlation between Lf concentration in milk and rel. Lf mRNA expression in mammary tissue

4. Discussion

During mammary infection, changes of the protein fraction as well as increases of immunological factors are likely important in the defence system of lactating mammary glands as documented in earlier studies (6, 15).

Despite of an only slight subclinical mastitis in this study, mRNA expression of all immunological factors investigated was elevated only in milk cells of H quarters thus showing their importance in the regulation of immune response. iNOS is an antimicrobial enzyme catalyzing synthesis of nitric oxide (NO). BOULANGER et al. (3) suggested that mammary epithelial cells and monocytes were responsible for NO release. An enhanced intramammary production of NO during clinical mastitis was previously shown (2). Accordingly NO is, like Lz, of relevance for the natural defence system of the mammary gland also during subclinical mastitis. Lz is known to increase during mastitis and the higher Lz mRNA expression in milk cells than in udder tissue confirmed assumptions of PERSSON et al. (13) that leukocytes are the most likely source of Lz during mastitis. The synthesis of PAF which can be concluded from our data is in line with earlier reports (15) demonstrating that the increased leukotriene- and prostaglandin biosynthesis is closely related to PAF synthesis via phospholipase A_2. A key mechanism for the removal of PAF is hydrolysis catalyzed by PAF-AH, which converts PAF to the biologically inactive lyso-PAF. Since PAF is a lipidmediator it could only be estimated via the mRNA expression of a key enzyme of its degradation, PAF-AH. CAO *et al.* (4) showed that PAF stimulates the expression of its own inactivating enzyme. Therefore, elevated PAF-AH expression allows the assumption of an increased PAF synthesis. These results could clarify that at least during subclinical mastitis somatic milk cells are important for the synthesis of immunologically relevant factors what is supported by the significant correlation coefficients between SCC and their mRNA expression.

Increased SCC and altered contents of milk proteins are among the principal features of mastitic milk (6, 7, 10). This study showed that changes already take place within the subclinical SCC range. As shown before (21) and herein, the total protein content in milk from quarters with subclinical mastitis increased significantly. Contrary to URECH *et al.* (21) the concentration of CN and whey protein underwent only numerical changes.

Antimicrobial defence proteins such as Lf are known to increase in milk during clinical mastitis (7). Even quarters with subclinical mastitis showed a significant elevation of Lf level compared with normal quarters (21).

Also in this study Lf concentration in milk was elevated in H quarters. These changes are in general paralleled by changes in Lf mRNA expression (12), being manifested in the highly positive correlation between Lf concentration in milk and Lf mRNA expression in mammary tissue.

In this work differences of SCC between healthy quarters and quarters with subclinical mastitis were smaller than in an other study (21) what might be the reason for less clear changes in our study as compared to URECH *et al.* (21).

CN content as well as the CN/total protein ratio did not differ in milk of H and L quarters. Nevertheless there was a decrease of κ-CN mRNA expression, not to be measured on a protein level. A decrease of κ-CN was already shown during LPS-induced mastitis in a previous study (20).

α-LA is a part of lactose synthetase and its reduction agrees with reduced lactose synthesis during mastitis. In this study both α-LA and lactose showed a negative correlation with SCC. Even if lactose level tended to be depressed in H as compared with L quarters, significant differences of α-LA between experimental groups could

not be found. Therefore the reduced amount of lactose seems to result from alterations in blood milk barrier permeability.

In conclusion, the present study shows that changes in the composition of milk as well as changes of mRNA expression in milk cells and mammary tissue occur during very mild forms of subclinical mastitis.

Changes in mRNA expression of immunologically relevant factors as well as bactericidally active substances with exception of Lf in quarters with subclinical mastitis as compared to healthy quarters were only observed in milk cells, clarifying that the cells are necessary to ensure an early and effective immunological reaction.

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5. References

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Milk and whey protein-stabilized O/W emulsions with increasing oil content

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Model emulsions were prepared with increasing sunflower oil concentration at the homogenization pressure of 30/9 MPa and temperature 60°C using two-stage homogenizer. Milk protein concentrate (MPC, 75% proteins) and whey protein concentrate (WPC, 60% proteins) were used as stabilizers. Samples were submitted to measurements of rheology, conductivity, size distribution and stability in order to estimate the effect of oil concentration on the physicochemical properties of the emulsions. In the range of oil concentrations used (10, 15, 20, 25, 30 vol%) the emulsions exhibited non-Newtonian behavior. The most concentrated emulsions (30% oil content) were described by the Herschel-Bulkley model and others by the Bingham model. The volume-surface diameters (Dvs) for WPC-stabilized emulsions were lower (1.81–2.43 µm) compared to the emulsions with MPC (2.38–2.84 µm). Lower Dvs for WPC emulsions did not respond to the higher emulsion stability. Increasing oil concentration increased viscosity, stability and decreased conductivity and volume-surface diameters. Protein to fat ratio appeared to be fundamental in obtaining stable emulsions.

Milch- und Molkenprotein stabilisierte O/W-Emulsionen mit steigendem Ölgehalt

Es wurden Modellemulsionen mit zunehmender Sonnenblumenöl-Konzentration bei einem Homogenisationsdruck von 30/9 MPa und einer Temperatur von 60°C mit einem 2-Stufen-Homogenisator hergestellt. Das Milchproteinkonzentrat (MPC, 75% Protein) und das Molkenproteinkonzentrat (WPC, 60% Protein) wurden als Stabilisatoren verwendet. Proben wurden auf Rheologie, Leitfähigkeit, Größenverteilung und Stabilität untersucht, um die Wirkung der Ölkonzentrationen