*Institut für Ernährungs- und Lebensmittelwissenschaften*

---

# Generation, enrichment and characterization of bioactive oligosaccharides and peptides from milk

**Inaugural Dissertation**

zur

Erlangung des Grades

Doktorin der Ingenieurwissenschaften

(Dr.-Ing.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt im November 2015

von

Karina Altmann

aus

Oppeln

**Referent: Herr Prof. Dr. Andreas Schieber**

**Korreferent: Herr Prof. Dr. Peter Chr. Lorenzen**

**Tag der mündlichen Prüfung:12.02.2016**

**Erscheinungsjahr: 2016**

*to my family*

# Danksagung

An dieser Stelle sei all jenen gedankt, die zum Gelingen dieser Arbeit beigetragen haben.

Mein großer Dank gebührt **Herrn Prof. Dr. Lorenzen** für die hervorragende und motivierte Betreuung, die Einführung in das wissenschaftliche Arbeiten, die wertvollen Ratschläge, für die wissenschaftliche und persönliche Förderung und für die mir gewidmete Zeit. Als Leiter des Teilprojektes „LactoPep“ des Kompetenznetzwerkes „Focus- Food Chain Plus“ möchte ich Herrn Prof. Lorenzen für die wissenschaftliche Leitung danken.

**Herrn Prof. Dr. Schieber** möchte ich für die engagierte Betreuung und Förderung meines wissenschaftlichen Vorhabens, die Ermöglichung der Aufnahme eines Promotionsstudiums an der Universität Bonn sowie die persönliche Förderung danken.

**Frau Dr. Clawin-Rädecker** möchte ich als Leiterin des Teilprojekts „LactoCarb“ im Kompetenznetzwerk „FoCus-Food Chain Plus“ für die Durchführung der Analytik und Beratung bei lebensmittelchemischen Fragestellungen danken. **Frau Jacobsen, Frau Schwall und Herrn Neumann** möchte ich für die analytische Untersuchung der zahlreichen Proben danken. **Herrn Dr. Wutkowski** möchte ich für die Durchführung der Analytik an der LC-MS und HPAEC, für den wissenschaftlichen Austausch und die gute Zusammenarbeit danken.

**Herrn Dr. Klempt** möchte ich besonders für die Ermöglichung der Durchführung von Zelltests zur Bestimmung bioaktiver Eigenschaften, für die Einführung in der Zellchemie, für die angeregten Diskussionen und die hilfreichen Ratschläge danken.

Meinen Arbeitskollegen **Frau Böttcher, Herrn Johannsen, Frau Splitzer** und **Frau Köpke** möchte ich für die Durchführung von Versuchen, die gute Zusammenarbeit, das tolle Arbeitsklima, die enorme Unterstützung und den großen Rückhalt danken, der mir entgegengebracht wurde.

**Herrn Kämpfer**, Unternehmensgruppe Theo Müller, möchte ich für die Kooperation und die Ermöglichung der Durchführung von Nanofiltrationen im Industriemaßstab danken.

**Meiner Familie** gilt unendlich tiefer Dank. So viel bedingungslose Unterstützung und Zuspruch wurde mir zugetragen.

# Content

Danksagung IV

1. Abbreviations IX
2. Figures XII
3. Tables XV
4. Definitions XVII
5. Amino acid code XVIII
6. Summary XIX
7. Kurzdarstellung XXI
8. Publications related to the PhD thesis XXIII
9. Contributions of the co-authors XXV

**Chapter I General Introduction** 1

I.1 Theoretical background 1

I.1.1 Bioactive substances from milk 1

I.1.2 Bioactive oligosaccharides 4

I.1.2.1 Structure of milk oligosaccharides (MOS) 4

I.1.2.2 Biological activities of MOS 9

I.1.2.3 Availability of MOS as functional food ingredients 13

I.1.2.4 Membrane filtration procedures 17

I.1.2.5 Whey as a raw material for the enrichment of MOS 19

I.1.2.6 Nanofiltration (NF) process for the enrichment of MOS 22

I.1.2.7 Structure, production and biological activity of galactooligosaccharides (GOS) 24

I.1.3 Bioactive peptides from milk 27

I.1.3.1 Anti-inflammatory peptides from milk and other sources 27

I.1.3.2 β-Casein as a precursor of bioactive peptides 33

I.1.3.3 Isolation of β-casein and generation of bioactive peptides 38

I.1.4 Determination of NFκB activation in $HEK^{nfκb\text{-}RE}$ cells 41

I.2 Motivation 44

I.2.1 Development of procedures for the enrichment milk oligosaccharides (MOS) by nanofiltration (NF) 44

I.2.2 Development of procedures for the generation of anti-inflammatory peptides 45

I.2.3 Characterization of the biological activity of enriched MOS concentrates and generated β-casein hydrolysates 46

I.2 References 47

I.4 Objectives 61

**Chapter II Comparison of the efficiency of different NF membranes for the enrichment of milk oligosaccharides from bovine milk** 62

II.1 Abstract 63

II.2 Introduction 64

II.3 Material and methods 66

II.3.1 Nanofiltration assays 65

II.3.2 Analytical methods 70

II.3.2.1 Quantification of MOS, mono- and disaccharides 70

II.3.2.1.1 Sample pretreatment 70

II.3.2.1.2 Determination of milk oligosaccharides by HPAEC-PAD/MS 70

II.3.2.1.3 Quantification of other components 71

II.3.3 Effect of NF MOS concentrates on NFκB activation in $HEK^{nfκb\text{-}RE}$ cells and followed Luciferase assay 72

II.4 Results and discussion 74

II.4.1 Efficiency of different NF membranes for the enrichment of MOS 74

II.4.1.1 Laboratory scale NF 74

II.4.1.2 Pilot plant scale NF 78

II.4.1.3 Industrial scale NF 81

II.4.2 Composition of sugars in MOS concentrates 86

II.4.3 The effect of MOS concentrates on the NFκB activity in TNF-α induced $HEK^{nfκb\text{-}RE}$ cells 88

II.5 Conclusion 91

II.6 References 93

**Chapter III NF-enrichment of milk oligosaccharides (MOS) in relation to process parameters** 97

III.1 Abstract 98

III.2 Introduction 99

III.3 Material and methods 101

III.3.1 Nanofiltration assays 101

III.3.2 Analytical methods 107

III.3.2.1 Quantification of MOS, mono- and disaccharides 107

III.3.2.1.1 Sample pretreatment 107

III.3.2.1.2 Determination of milk oligosaccharides by HPAEC-PAD/MS 107

III.3.2.1.3 Quantification of other components 107

III.4 Results and discussion 108

III.4.1 Enrichment of MOS from bovine milk 108

III.4.2 Enrichment of MOS from caprine milk 114

III.4.3 Composition of sugars in MOS concentrates 118

III.5 Conclusion 123

III.6 References 125

**Chapter IV Generation and identification of anti-inflammatory peptides from bovine β-casein using enzyme preparations from cod and hog** 129

IV.1 Abstract 130

IV.2 Introduction 131

IV.3 Material and methods 133

IV.3.1 Isolation of β-casein from rennet casein 133

IV.3.2 Enzymes and hydrolysates 133

IV.3.3 Separation and identification of peptides by HPLC-ESI-$MS^n$ (LC-MS) 135

IV.3.4 Evaluation of anti-inflammatory effect via NFκB activation in $HEK^{nf\kappa b\text{-}RE}$ cells 136

IV.4 Results and discussion 138

IV.4.1 Identification of peptides in tryptic/ chymotryptic hydrolysates 138

IV.4.2 Potentially anti-inflammatory effect of peptide fractions in HEK$^{nf\kappa b\text{-}RE}$ cells 142

IV.5 Conclusion 149

IV.6 References 151

**Chapter V Conclusions** 154

V.1 Development of procedures for the enrichment of MOS by NF 154

V.2 Development of procedures for the generation of anti-inflammatory peptides 157

V.3 Characterization of biological activity of enriched MOS concentrates and generated β-casein hydrolysates 159

V.4 Outlook 160

V.5 References 162

# 1. Abbreviations

| | |
|---|---|
| 2’-FL | 2’-Fucosyllactose |
| 3’-GL | 3’-Galactosyllactose |
| 3’-SL | 3’-Sialyllactose |
| 4’-GL | 4’-Galactosyllactose |
| 6’-GL | 6’-Galactosyllactose |
| 6’-SL | 6’-Sialyllactose |
| BCL-$X_L$ | B-cell lymphoma-extra large |
| Caco-2 cells | Colon adenocarcinoma cell line |
| CBMC | Cord blood mononuclear cells |
| COX-2 | Cyclooxygenase 2 |
| DDS | Dextran sodium sulphate |
| DF | Diafiltration |
| DV | Diavolumes |
| Fuc | Fucose |
| Gal | Galactose |
| GalNAc | N-Acetylgalactosamine |
| GalNAc-Gal-Glc | N-Acetylgalactosaminyl-lactose |
| GH | Growth hormone |
| GIP | Glucose-dependent insulinotropic polypeptide |
| Glc | Glucose |
| GlcNAc | N-Acetylglucosamine |
| GLP-1 | Glucagone-like peptide-1 |
| GMP | Glycomacropeptide |
| GOS | Galactooligosaccharides |
| GRO α | Growth related oncogene-α |

| | |
|---|---|
| $HEK^{nfκb\text{-}RE}$ cells | Human embryonic kidney cells (transfected with the NFκB response element luciferase) |
| HUVEC | Human umbilical vein endothelial cells |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IEC 18 | Intestinal epithelial cells from rat |
| IFN-γ | Interferon-γ |
| IGF-1 | Insulin-like growth factor-1 |
| IKK | IκB-kinase |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| IκB | Inhibitor of κ B |
| LNDFH I | Lacto-N-difucohexaose I |
| LNFP I | lacto-N-fucopentaose I |
| LNT | Lacto-N-tetraose |
| LPS | Lipopolysaccharide |
| MCP1 | Monocyte chemoattractant protein-1 |
| MF | Microfiltration |
| MnSOD | Manganese superoxide dismutase |
| MOS | Milk oligosaccharides |
| mTOCRC1 | Mechanistic target of rapamycin complex 1 |
| Neu5Ac | N-Acetylneuramic acid |
| Neu5Gc | N-Glycolylneuramic acid |
| NF | Nanofiltration |
| NFκB | Nuclear factor κ B |
| NMWCO | Nominal molecular weight cut-off |
| NO | Nitric oxide |
| NPN | Non-protein nitrogen |

| | |
|---|---|
| NSAID | Nonsteroidal anti-inflammatory drugs |
| PG $E_2$ | Prostaglandin $E_2$ |
| PHA | Phytohemagglutinin |
| $PLA_2$ | Phospholipase A2 |
| RO | Reverse osmosis |
| ROS | Reactive oxygen species |
| SM-UF permeate | Skimmed and ultrafiltered milk permeate |
| TBNS | Trinitrobenzene sulfonic acid |
| TG | Transglutaminase |
| TGF-β1 | Transforming growth factor-β1 |
| TLR4 | Toll-like receptor 4 |
| TNFR | Tumor necrosis factor receptor |
| TNF-α | Tumor necrosis factor-α |
| TPCK | Tosyl phenylalanyl chloromethyl ketone |
| UF | Ultrafiltration |
| WPC | Whey protein concentrate |
| WPI | Whey protein isolate |
| β-CN | β-Casein |

# 2. Figures

**Fig. 1.1** Bioactive milk derived components.

**Fig. 1.2 a** Structure of the main sugars components of MOS and of the oligosaccharides 3'-SL, 6'-SL and GalNAc-Gal-Glc.

**Fig. 1.2 b** MOS structures.

**Fig. 1.3** Schematic drawing of a cross-flow filtration process.

**Figure 1.4** Separation behavior of the different membrane separation processes by application of milk as feed solution.

**Fig. 1.5** Production of skimmed and ultrafiltered milk permeate as raw material for the enrichment of MOS.

**Fig. 1.6** Whey streams for enrichment of MOS.

**Fig. 1.7** Flow chart of the industrial scale NF applying the membrane module DOW NF-3840-30-FF at 10°C and 10-15 bar.

**Fig. 1.8** Primary structure of bovine β-casein $A^2$-5P (*bos taurus*) with bioactive peptides encrypted within the sequence.

**Fig. 1.9** Isolation of β-casein from rennet casein.

**Fig. 1.10** Hydrolysis of β-casein with tryptic/ chymotryptic enzyme preparations.

**Fig. 1.11** Canonical NFκB signaling pathway.

**Fig. 2.1** Retention of MOS by NF on laboratory scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate).

**Fig. 2.2** Retention of MOS by NF on pilot plant scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate).

**Fig. 2.3** Retention of MOS by NF on industrial scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate).

**Fig. 2.4** HPAEC chromatograms of the SM-UF-permeate before lactose hydrolysis (I) lactose hydrolyzed SM-UF-permeate (II), and NF retentate after diafiltration of the membrane DOW at 10-15 bar (III, industrial scale).

**Fig. 2.5** Potentially immunostimulatory activity of NF retentates in $HEK^{nfkB\text{-}RE}$ cells measured as luciferase activity.

**Fig. 3.1 a)** Process for the enrichment of MOS by NF from bovine milk.

**Fig. 3.1 b)** Process for the enrichment of MOS by NF from caprine milk.

**Fig. 3.2** Retention of MOS by NF of bovine milk at pH 5 and pH 7 and MOS content in relation to the total sugar content.

**Fig. 3.3** Retention of MOS by NF of caprine milk produced by application of the membrane modules SR 50 and DL and MOS content in relation to the total sugar content.

**Fig. 3.4** HPAEC chromatograms of the initial samples and the retentates of the NFs for the enrichment of MOS from bovine and caprine milk.

a) Chromatograms of the initial sample (bovine NF retentate DOW) (I) and the bovine NF retentate (DL) after diafiltration at pH 5 (II).

b) Chromatograms of the caprine SM-UF permeate before lactose hydrolysis (I), the caprine lactose hydrolyzed SM-UF permeate (II) and the caprine NF retentate generated by the membrane DL.

**Fig. 4.1 a).** LC-MS profiles of total β-casein hydrolysates (fraction I) generated with the enzyme preparations cod trypsin (CT), trypsin TPCK-treated (T), PTN 6.0S (P), and Cryotin F (CF).

**Fig.4.1 b)** LC-MS profiles of retentates 5 kDa (fraction II) generated with the enzyme preparations cod trypsin (CT), porcine trypsin (TPCK) (T), PTN 6.0S (P), and Cryotin F (CF).

**Fig. 4.1 c)** LC-MS profiles of permeates 5 kDa (fraction III) generated with the enzyme preparations cod trypsin (CT), porcine trypsin (TPCK) (T), PTN 6.0S (P), and Cryotin F (CF).

**Fig. 4.2.** Potential anti-inflammatory activity of (a) total β-casein hydrolysate (fraction I), (b) 5 kDa retentates (fraction II), and (c) 5 kDa permeates (fraction III), generated with porcine trypsin, cod trypsin, PTN 6.0 S, Cryotin, and Cryotin F in TNF-α-induced $HEK^{nfκB\text{-}RE}$ cells measured as luciferase activity.

# 3. Tables

**Table 1.1** Literature overview about the immunostimulatory effect of MOS.

**Table 1.2** Literature overview about membrane separation processes for the enrichment of MOS.

**Table 1.3** Classification of membrane filtration technology.

**Table 1.4** Food-derived anti-inflammatory proteins and peptides.

**Table 1.5** Bioactive peptides derived from β-casein.

**Table 1.6** Classification of amino acids according to their degree of hydrophobicity.

**Table 2.1** Properties of NF membranes applied for the enrichment of MOS on laboratory, pilot plant and industrial scale and corresponding experimental set-ups.

**Table 2.2** Concentration of MOS, mono- and disaccharides and dry mass in lactose hydrolyzed SM-UF-permeate and retentates of laboratory scale NF.

**Table 2.3** Concentration of MOS, mono- and disaccharides and dry mass in lactose hydrolyzed SM-UF-permeate and retentates of pilot plant scale NF.

**Table 2.4 (a)** Concentration of MOS, mono- and disaccharides and dry mass in lactose hydrolyzed SM-UF-permeate and retentates of industrial scale NF.

**Table 2.4 (b)** Protein, non-protein nitrogen, ash and salt content of NF retentate after diafiltration generated on industrial scale NF by using the membrane DOW.

**Table 3.1** Properties of NF membranes applied for the enrichment of MOS on pilot plant scale and corresponding experimental set-ups.

**Table 3.2 a)** Concentration of MOS, mono- and disaccharides, dry mass, citric acid, total nitrogen and ash in bovine NF retentates DOW, NF retentates DL after concentration and NF retentates DL after diafiltration at pH 5 and pH 7.

**Table 3.2 b)** Composition of mineral compounds in bovine NF retentates DOW and bovine NF retentates DL after diafiltration at pH 5 and pH 7.

**Table 3.3 a)** Concentration of MOS, mono- and disaccharides, dry mass, citric acid, total nitrogen and ash in caprine lactose hydrolyzed SM-UF-permeate and caprine retentates produced by application of the membrane modules SR 50 and DL.

**Table 3.3 b)** Composition of mineral compounds in caprine lactose hydrolyzed SM-UF-permeate and caprine retentates produced by application of the membrane modules SR 50 and DL.

**Table 4.1** Sources of enzyme preparations and enzyme activities applied for hydrolysis of a 5% β-casein solution at pH 7.8 and 37 °C for 3- 4 h.

**Table 4.2** Identification of the main peptides of total β-casein hydrolysate (I), peptides > 5 kDa (II) and peptides < 5 kDa (III) generated with different proteolytic enzymes by HPLC-ESI-$MS^n$.

# 4. Definitions

| | | |
|---|---|---|
| $A$ | Active membrane area | [$m^2$] |
| $CF$ | Concentration factor | |
| $C_{f,i}$ | Concentration of the component i in feed | [mg/L] |
| $C_{r,i}$ | Concentration of the component i in retentate | [mg/L] |
| $C_{r,MOS}$ | Concentration of MOS in retentate | [mg/L] |
| $C_{r,total\ sugar}$ | Concentration of total sugar in retentate | [mg/L] |
| $DV$ | Number of diavolumes | |
| $F$ | Permeate flux | [L $m^{-2}$ $h^{-1}$] |
| $M_{MOS/total\ sugar}$ | MOS content in relation to total sugar content | [%] |
| $R_i$ | Retention of a component i | [%] |
| $t$ | Time | [h] |
| $V_f$ | Initial feed volume | [L] |
| $V_p$ | Permeate volume | [L] |
| $V_r$ | Retentate volume | [L] |
| $p$ | Pressure | [bar] |
| $T$ | Temperature | [°C] |

## 5. Amino acid code (IUPAC)

| | | |
|---|---|---|
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartic acid |
| E | Glu | Glutamic acid |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asn | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| Y | Tyr | Tyrosine |

## 6. Summary

Milk is considered to be a rich and one of the most important sources of bioactive compounds containing various milk-borne biologically active constituents or precursor substances for bioactive components. The present work was focused on the production of functional food ingredients of the carbohydrate and protein fraction of milk. The aim of the thesis was the development of procedures for the generation, enrichment and characterization of bioactive oligosaccharides and peptides from milk. Enrichment of bioactive milk-derived oligosaccharides (MOS) was performed by nanofiltration (NF). For generation and identification of anti-inflammatory peptides from bovine β-casein, enzyme preparations with different tryptic and chymotryptic activities were applied. Characterization of the biological activities of the MOS concentrates and the β-casein hydrolysates was performed by the evaluation of the activity of the transcription factor NFκB in human embryonic kidney cells (HEK$^{nfκb\text{-}RE}$ cells) *in vitro*.

*Manuscript 1:* For the enrichment of MOS (6'-sialyllactose, 3'-sialyllactose, N-acetylgalactosaminyl lactose) by membrane filtration initially a screening and a comparison of the efficiency of different NF membranes was performed on laboratory scale. Successful retention of MOS of 49 % to 84 % in retentate was achieved by application of membranes with a nominal molecular weight cut-off (NMWCO) of 150-400 Da. The transfer of the NF process from laboratory to pilot plant and industrial scale resulted in a comparable retention of MOS in retentate. A 100-fold increase of the MOS content in relation to total sugar content in retentate (10.6 %) compared to the initial sample (0.1 %) was realized after NF on industrial scale using the membrane DOW. This NF retentate was composed of 2.6 % MOS, 28.3 % mono- and disaccharides, 23.5 % citric acid and 24.7 % ash in dry mass. The MOS retentates and the standards (3'-sialyl-lactose, 6'-sialyl-lactose) exhibited increased NFκB activity in HEK$^{nfκb\text{-}RE}$ cells. A screening for the efficiency of different NF membranes for the enrichment of MOS on different scales of production was conducted for the first time.

*Manuscript 2:* The NF process for the enrichment of MOS was optimized realizing a better permeation of milk salts and residual sugars during NF at pH 5 and pH 7. As initial sample, the already concentrated NF retentate with a 100-fold increase of MOS content in relation to total sugar content was applied. A high retention of MOS of 68 % to 79 % was realized after

NF and diafiltration at acidic and neutral milieu. The high MOS content in relation to total sugar content of 87.3 % to 92.7 % in the retentate at pH 5 and 7 (9-fold higher than in the first NF retentate, 900-fold higher than in the initial sample) indicated a nearly complete permeation of mono- and disaccharides. A higher enrichment of MOS in dry mass (5-fold higher than in the first NF retentate, 140-fold higher than in the initial sample) and an increased permeation of salts were achieved by NF at pH 5. The MOS concentrate at pH 5 was composed of 14.1 % MOS, 1.2 % mono- and disaccharides, 13.3 % citric acid and 21.8 % ash in dry mass. Furthermore, first investigations for the transfer of the procedure to caprine milk resulted in an increased MOS content in relation to total sugar content in retentate (23- to 31-fold higher than in the initial sample) of NF at neutral milieu. For the first time, the influence of the pH on the degree of enrichment of MOS by NF was evaluated.

*Manuscript 3:* Potential anti-inflammatory peptides were generated by hydrolysis of β-casein with the tryptic enzyme preparations Cod Trypsin, porcine Trypsin (tosyl phenylalanyl chloromethyl ketone (TPCK)-treated) and the tryptic and chymotryptic preparation PTN 6.0 S. The β-casein hydrolysates exhibited a reduced NFκB activity measured as luciferase activity in human embryonic kidney cells ($HEK^{nfκb\text{-}RE}$ cells). β-casein hydrolysates produced by application of enzyme preparations with mainly chymotryptic activity (Cryotin, Cryotin F) did not reveal any effect. The higher the chymotryptic activity present, the lower the anti-inflammatory activity in $HEK^{nfκb\text{-}RE}$ cells. Hydrolysis of β-casein with Cod Trypsin and porcine Trypsin (TPCK) resulted in comparable peptides. Thus, the enzyme preparation Cod Trypsin can replace the non-food grade porcine enzyme preparation Trypsin (TPCK) for the generation of potential anti-inflammatory β-casein derived peptides.

# 7. Kurzdarstellung

Milch zählt zu den wichtigsten Quellen für bioaktive Substanzen und enthält zahlreiche milchoriginäre bioaktive Komponenten sowie Precursor für bioaktive Komponenten. Der Focus der Arbeit lag auf der Produktion von funktionellen Lebensmittelinhaltsstoffen aus der Kohlenhydrat- und Proteinfraktion von Milch. Das Ziel der Arbeit war die Entwicklung von Verfahren zur Generierung, Anreicherung und Charakterisierung von bioaktiven Oligosacchariden und Peptiden aus Milch. Die Anreicherung von milchoriginären Oligosacchariden (MOS) erfolgte mit Hilfe der Nanofiltration (NF). Für die Generierung und Identifizierung von anti-inflammatorischen Peptiden aus bovinem β-Casein wurden Enzympräparate mit unterschiedlichen tryptischen und chymotryptischen Aktivitäten angewendet. Eine Charakterisierung der biologischen Aktivität der MOS Konzentrate und der β-Casein Hydrolysate erfolgte durch die Bestimmung der Aktivität des Transkriptionsfaktors NFκB in humanen embryonalen Nierenzellen ($HEK^{nfκb\text{-}RE}$–Zellen) *in vitro*.

*Manuskript 1:* Für die Anreicherung der MOS (3‘-Sialyllactose, 6‘-Sialyllactose, N-Acetylgalactosaminyl-Lactose) mit Hilfe der Membranfiltration wurde zu Beginn ein Screening und ein Vergleich der Effizienz unterschiedlicher NF-Membranen im Labormaßstab durchgeführt. Eine erfolgreiche Wiederfindung der MOS im Retentat von 49 % bis 84 % wurde bei Einsatz von Membranen mit einem NMWCO (nominal molecular weight cut-off) von 150-400 Da erzielt. Eine Übertragung des NF-Prozesses vom Labor in den Technikum- und Industriemaßstab erzielte eine vergleichbar hohe Wiederfindung der MOS im Retentat. Die NF im Industriemaßstab bei Einsatz der Membran DOW erzielte einen 100-fachen Anstieg des MOS Gehaltes bezogen auf den Gesamtzuckergehalt im Retentat (10.6 %) im Vergleich zur Ausgangsprobe (0.1 %). Das NF-Retentat setzte sich aus 2,6 % MOS, 28,3 % Mono- und Disacchariden, 23,5 % Zitronensäure und 24,7 % Asche in der Trockenmasse zusammen. Die MOS Konzentrate und die Standards (3‘-Sialyllactose, 6‘-Sialyllactose) zeigten eine erhöhte NFκB Aktivität in $HEK^{nfκb\text{-}RE}$–Zellen. Ein Screening bezüglich der Effizient verschiedener NF-Membranen für die Anreicherung der MOS in unterschiedlichen Produktionsmaßstäben erfolgte zum ersten Mal.

*Manuskript 2:* Der NF-Prozess für die Anreicherung der MOS wurde optimiert. Eine höhere Permeation der Milchsalze und restlichen Zucker während der NF bei pH 5 und 7 wurde

erzielt. Als Ausgangsprobe wurde das zuvor konzentrierte NF-Retentat mit einem 100-fach erhöhten MOS Gehalt bezogen auf den Gesamtzuckergehalt verwendet. Eine hohe Wiederfindung der MOS von 68 % bis 79 % wurde nach NF und Diafiltration bei saurem und neutralem Milieu erzielt. Der hohe MOS Gehalt bezogen auf den Gesamtzuckergehalt von 87,3 % bis 92,7 % im Retentat bei pH 5 und pH 7 (9-fach höher als im ersten NF-Retentat, 900-fach höher als in der Ausgangsprobe) zeigte eine nahezu komplette Permeation der Mono- und Disaccharide. Eine höhere Anreicherung der MOS in der Trockenmasse (5-fach höher als im ersten NF-Retentat, 140-fach höher als in der Ausgangsprobe) und eine höhere Permeation der Salze wurden mit der NF bei pH 5 erzielt. Das MOS Konzentrat bei pH 5 setzte sich aus 14,1 % MOS, 1,2 % Mono- und Disacchariden, 13,3 % Zitronensäure und 21,8 % Asche in Trockenmasse zusammen. Des Weiteren führten erste Studien zur Übertragung des Prozesses auf Ziegenmilch zu einem erhöhten MOS Gehalt bezogen auf den Gesamtzuckergehalt im Retentat (23- bis 31-fach höher als in der Ausgangsprobe) der NF bei neutralem Milieu. Der Einfluss des pH-Wertes auf den Grad der Anreicherung der MOS mit Hilfe der NF wurde zum ersten Mal untersucht.

*Manuskript 3:* Potentiell anti-inflammatorische Peptide wurden durch die Hydrolyse von β-Casein mit den tryptischen Enzympräparaten Cod Trypsin, Schweinetrypsin (Tosyl-Phenylalanyl-Chlormethyl-Keton (TPCK)-behandelt) und dem tryptischen und chymotryptischen Präparat PTN 6.0 S generiert. Die β-Casein Hydrolysate zeigten eine reduzierte NFκB Aktivität gemessen als Luciferase Aktivität in $HEK^{nf\kappa b\text{-}RE}$–Zellen. β-Casein Hydrolysate generiert durch den Einsatz von Enzympräparaten mit hauptsächlich chymotryptischer Aktivität (Cryotin, Cryotin F) zeigten keinen Effekt. Je höher die chymotryptische Aktivität desto geringer war der anti-inflammatorische Effekt in $HEK^{nf\kappa B\text{-}RE}$–Zellen. Die Hydrolyse von β-Casein mit Cod Trypsin und Schweinetrypsin (TPCK) führten zu vergleichbaren Peptiden. Folglich kann das Enzympräparat Cod Trypsin das „non-food grade“ Präparat Trypsin (TPCK) für die Generierung von potentiell anti-inflammatorischen Peptiden aus β-Casein ersetzen.

# 8. Publications related to the PhD thesis

The work presented in this thesis is a selection of papers published or submitted in international peer reviewed journals, which are listed below.

**Manuscripts:**

1. Altmann, K., Wutkowski, A., Kämpfer, S., Klempt, M., Lorenzen, P. Chr., Clawin-Rädecker, I. (2015) Comparison of the efficiency of different NF membranes for the enrichment of milk oligosaccharides from bovine milk. European Food Research and Technology. 241(6), 803-815.

2. Altmann, K., Clawin-Rädecker, I., Hoffmann, W., Lorenzen, P. Chr. (2015) NF-enrichment of milk oligosaccharides (MOS) in relation to process parameters, Food and Bioprocess Technology, under review.

3. Altmann, K., Wutkowski, A., Klempt, M., Clawin-Rädecker, I., Meisel, H., Lorenzen, P. Chr. (2016) Generation and identification of anti-inflammatory peptides from bovine β-casein using enzyme preparations from cod and hog. Journal of the Science of Food and Agriculture, 96(3), 868-877.

**Poster Presentations:**

4. Altmann K., Zerge K., Hoffmann W., Clawin-Rädecker I., Lorenzen P. Chr., (2013) Anreicherung von Milcholigosacchariden aus Kuhmilch mit Hilfe der Nanofiltration, 6. Symposium funktionelle Lebensmittel, 4.-5. Juni 2013, Kiel

5. Altmann K., Zerge K., Clawin-Rädecker I., Hoffmann W., Lorenzen P. Chr., (2013) Efficiency of different NF-Membranes for the enrichment of milk oligosaccharides, Dairy Conference, 16.-17. September 2013, Hohenheim

6. Altmann K., Zerge K., Clawin-Rädecker I., Hoffmann W., Lorenzen P. Chr., (2014) Efficiency of different NF-membranes for the enrichment of milk oligosaccharides, 3rd Kiel Food Science Symposium, 20.-21. May 2014, Kiel

7. Altmann K., Wutkowski A., Clawin-Rädecker I., Klempt M., Meisel H., Lorenzen P. Chr., (2014) Generation of anti-inflammatory peptides from β-casein on pilot plant scale, 3rd Kiel Food Science Symposium, 20.-21. May 2014, Kiel

8. Altmann K., Zerge K., Clawin-Rädecker I., Hoffmann W., Lorenzen P.Chr., (2014) Concentration of milk oligosaccharides, 7th International Whey Conference, 7.-9. September 2014, Rotterdam

9. Altmann K., Wutkowski A., Clawin-Rädecker I., Klempt M., Meisel H., Lorenzen P. Chr., (2014) Comparison of tryptic/chymotryptic enzyme preparations of different origin for generation of potentially anti-inflammatory peptides from bovine β-casein, 7th International Whey Conference, 7.-9.September 2014, Rotterdam

10. Altmann K., Wutkowski A., Clawin-Rädecker I., Klempt M., Meisel H., Lorenzen P. Chr. (2015) Generation of anti-inflammatory peptides from β-casein by tryptic/chymotryptic enzyme preparations, Food Nutrition Health Symposium, 12.-13. May 2015, Kiel

**Oral Presentation**

11. Altmann K., Clawin-Rädecker I., Wutkowski A., Kämpfer S., Klempt M., Lorenzen P Chr. (2015) Anreicherung von Milcholigosacchariden (MOS) aus Kuhmilch mit Hilfe der Nanofiltration (NF), Food Nutrition Health Symposium, 12.-13. May 2015, Kiel

## 9. Contributions of the co-authors

The co-authors' contributions to the papers present in Chapter II-IV are specified as follows:

The work presented in this thesis was carried out under the supervision of **Prof. Dr. Peter Chr. Lorenzen** at the Max Rubner-Institut Kiel, Department of Safety and Quality of Milk and Fish Products. Prof. Lorenzen contributed to the conception of the scientific program (FoCous- Food Chain Plus).

**Prof. Dr. Hans Meisel,** as a specialist in bioactive protein and peptide research, had a role as advisor in the performance of investigations for the generation of potential anti-inflammatory peptides from β-casein *(Chapter IV)*.

**Dr. Ingrid Clawin-Rädecker**, Max Rubner-Intitut Kiel, Department of Safety and Quality of Milk and Fish Products, carried out the identification and quantification of MOS by HPAEC-PAD/MS *(Chapter II, III)*.

**Dr. Adam Wutkowski** and **Dr. Ingrid Clawin-Rädecker**, Max Rubner-Intitut Kiel, Department of Safety and Quality of Milk and Fish Products, performed the identification of peptides in β-casein hydrolysates by HPLC-ESI-$MS^n$ (LC-MS) *(Chapter IV)*.

In cooperation with **Sebastian Kämpfer**, Central Production and Engineering, Müller Service GmbH, the NF on industrial scale was conducted at the Theo Müller Group in Leppersdorf, Germay *(Chapter II)*.

In cooperation with **Dr. Wolfgang Hoffmann**, Max Rubner-Intitut Kiel, Department of Safety and Quality of Milk and Fish Products, the NF on pilot plant scale for the enrichment of MOS in caprine milk was realized *(Chapter III)*.

**Dr. Martin Klempt** supervised in the performance of the cell assay for the evaluation of the activity of the transcription factor NFκB in $HEK^{nfκb\text{-}RE}$ cells *(Chapter II, IV)*.

The work was integral part of subprojects of the competence network "FoCus-Food Chain Plus", which was funded by the Federal Ministry for Education and Research (BMBF).

# Chapter I General introduction

## I.1 Theoretical background

### *I.1.1 Bioactive substances from milk*

Milk and milk-derived products represent an essential and valuable contribution to the nutrition of countless people all over the world (Hartmann et al. 2002). Beside their nutritional value based on the principal ingredients water, fat, protein, carbohydrates, vitamins and minerals, milk contains various milk-borne biologically active constituents or precursor substances for bioactive components (Ebringer et al. 2008; Hartmann and Meisel 2002; Park 2009). The amount, the potentially and the importance of bioactive compounds in milk is greater than previously thought (Ebringer et al. 2008). In fact, bovine milk is considered a rich and the most important source of bioactive components with significant impact on human nutrition and health (Korhonen 2009; Mills et al. 2011; Park 2009; Walstra et al. 1999). Extensive studies have been conducted to evaluate the significance of these substances for human nutrition and health (Hartmann et al. 2002, Park et al. 2009). **Fig. 1.1** gives an overview of the bioactive components which are naturally present in milk or can be generated of milk components.

Carbohydrates in milk encode functional health benefits to the consumers far beyond the nutritional content (Tamime 2009). The principal carbohydrate in mammalian milk is lactose (Gal(β1-4)-Glc). This reducing disaccharide is unique to milk and has been found in milks of most mammals (Fox and McSweeney 2009; Walstra et al. 1999; Tamime 2009). Lactose is the precursor of various different biologically active compounds synthesized by chemical, physical or enzymatic conversion (Fox and McSweeney 2009; Gänzle et al. 2008). Except for some trace amounts, these compounds do not occur naturally in milk.

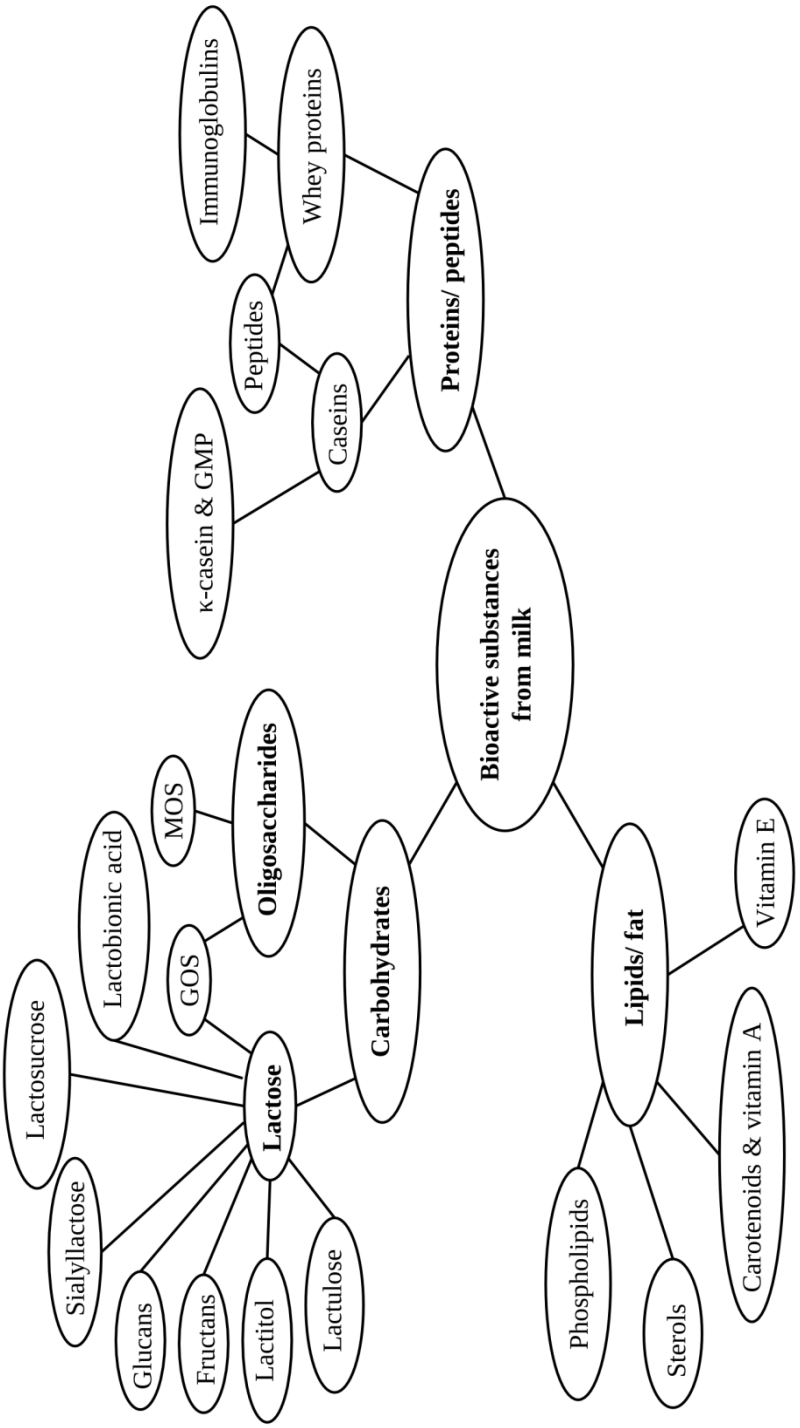



**Fig. 1.1** Bioactive milk-derived components. GMP = glycomacropeptide

For instance, the disaccharide lactulose is a well-known lactose derivative (Moreno et al. 2014), composed of galactose and fructose (Fru(β1-4)Gal). This disaccharide is formed in small amounts by heating of milk and is therefore an indicator of milk heat treatment (Walstra et al. 1999; Gänzle et al. 2008). Lactulose is industrially produced by isomerization of lactose in alkaline solution (Gänzle et al. 2008). This sugar has also been produced enzymatically by application of β-galactosidases and providing fructose as galactosyl acceptor on laboratory scale (Gänzle et al. 2008). The lactose derivative is used as a pharmaceutical for treatment of hepatic encephalopathy and, due to its laxative and prebiotic effects, as a bioactive component in food (Fox and McSweeney 2009; Gänzle et al. 2008). Various other lactose derivatives with biological activities such as lactitol, glucans, fructans, sialyllactose, lactosucrose or lactobionic acid exhibit biological activities.

Oligosaccharides are defined as "glycosides composed of 2-10 covalently linked monomer sugar units" (Fox and McSweeney 2009). Although this definition is dominant, disaccharides such as lactose or lactulose are not regarded as oligosaccharides, whereas saccharides with more than 10 monomers are also called oligosaccharides (Fox and McSweeney 2009). The biologically active galactooligosaccharides (GOS) are synthesized by the transgalactosylation activity of β-galactosidases from lactose, while milk oligosaccharides (MOS) represent the milk-borne bioactive components of the carbohydrate fraction in milk. The structure, biological activity, production or enrichment and availability are discussed in chapter I.1.2.

Milk provides various bioactive proteins such as glycosylated proteins, κ-casein, immunoglobulins as well as casein and whey derived peptides. Biologically active peptides derived from β-casein are presented in chapter I.1.3.2. Furthermore, anti-inflammatory hydrolysates were produced by tryptic proteolysis of β-casein *(Manuscript 3, chapter IV)*. Moreover, milk lipids such as phospholipids, carotenoids, conjugated linoleic acids and vitamins exhibit biological activities (Korhonen 2009).

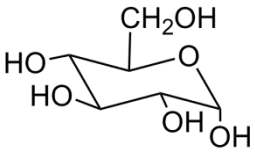
In contrast to the various studies about bioactive components in milk, a review from Melnik et al. (2013) pointed out that milk consumption in adulthood promotes the development of chronic diseases that are associated with increased cell growth regulating mechanistic target of rapamycin complex 1 (mTOCRC1) signaling such as acne, obesity, type-2 diabetes, arterial hypertension, Alzheimer's disease and cancer. The research group described that milk favors increased plasma levels of glucose-dependent insulinotropic polypeptide (GIP), glucagone-

like peptide-1 (GLP-1), insulin, growth hormone (GH) and insulin-like growth factor-1 (IGF-1), which enhance mTORIC-driven metabolic processes. They stated that while human milk is the ideal food for infants, the continued cow's milk consumption during adolescence and adulthood may promote the mTORC-1-driven diseases of civilization (Melnik et al. 2013). This theory was controversially discussed. In consideration of the various bioactive compounds in milk, which are beneficial for human health, this basic research needs further investigation. The critical aspects about milk consumption in adulthood should be regarded carefully but not be forgotten.

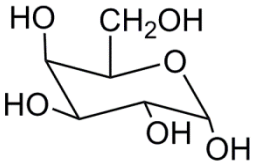
### *I.1.2 Bioactive oligosaccharides*

#### *I.1.2.1 Structure of milk oligosaccharides (MOS)*

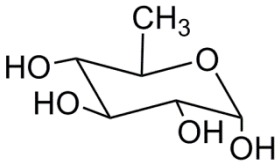
Milk oligosaccharides (MOS) are a family of structurally diverse complex glycans (Bode 2012, 2009). These oligosaccharides are composed of the core structure lactose, which is modified by the addition of the sugars glucose (Glc), galactose (Gal), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), N-acetylneuramic acid (=sialic acid) (Neu5Ac) and in milk of domestic animals also N-glycolylneuramic acid (=sialic acid) (Neu5Gc) **(Fig. 1.2 a)** (Kunz and Rudloff 2006; Bode 2012). All MOS contain lactose at the reducing end. On the one hand, lactose can be sialylated in α2-3 or α2-6 glycosidic linkage, resulting in the formation of 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL), respectively (**Fig. 1.2 b, Fig. 1.2 c** (1)). In addition, lactose can be fucosylated in α1-2 or α1-3 or α1-4 linkages representing, 2'-fucosyllactose (2'-FL) and 3'-fucosyllactose (3'-FL) (**Fig 1.2 c** (1)). On the other hand, β1-3- or β1-6-linked lacto-N-biose (Galβ1-3GlcNAc-, type 1 chain) or N-acetyllactosamine (Galβ1-4GlcNAc-, type 2 chain) (**Fig. 1.2 c** (2)) can be added to lactose resulting in lacto-N-tetraose (LNT) and lacto-N-neotetratose (LNnT), respectively (**Fig 1.2 c** (3)). The fucosylation of type I and type II chains lead to neutral MOS, while several isomeric forms can be synthesized such as lacto-N-fucopentaose (LNFP) I-III (**Fig. 1.2 c** (4)). Sialylation of type I chain and type II chain results in acidic MOS, which can show different isomeric forms, too, e.g., sialyllacto-N-tetraose (LST a-c) (**Fig. 1.2 c** (5)) (Bode 2012). Due to various different possible combinations of the monomers, a wide range of MOS with different degree of polymerization and glycosidic linkages can be formed (Bode 2012; Fox and McSweeney 2009; Mehra and Kelly 2006; Bode 2006).



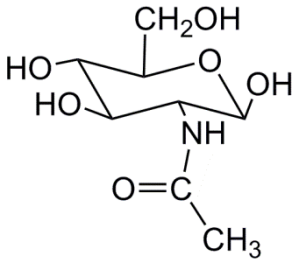

glucose
(Glc)



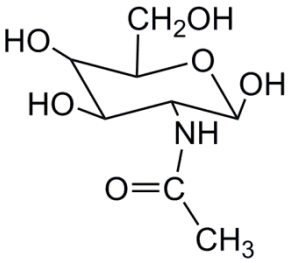

galactose
(Gal)



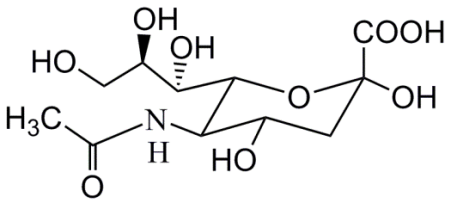

fucose
(Fuc)



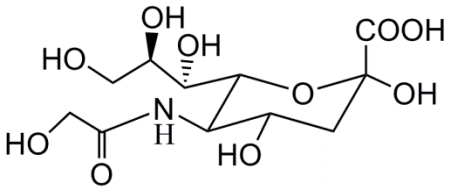

N-acetylglucosamine
(GlcNAc)




N-acetylgalactosamine
(GalNAc)

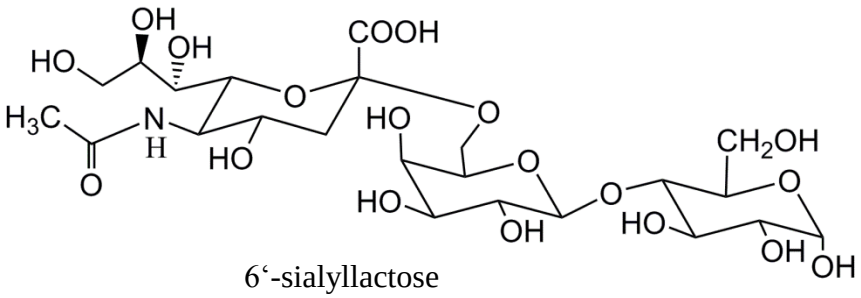



N-acetylneuramic acid
(Neu5NAc)

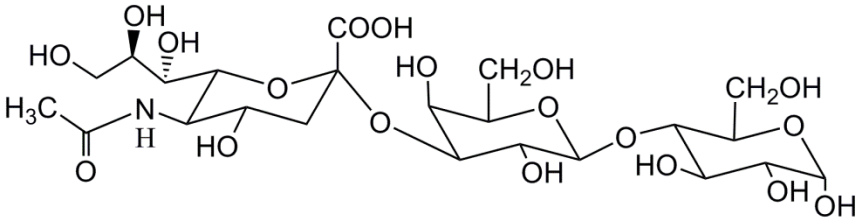



N-glycolylneuramic acid
(Neu5Gc)

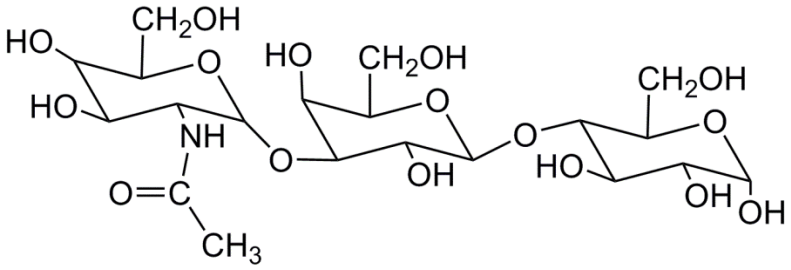
**Fig. 1.2 a** Structure of the main sugar components of MOS.



6‘-sialyllactose
(6‘-SL)
Neu5Ac-α(2-6)Gal-β(1-4)Glc



3‘-sialyllactose
(3‘-SL)
Neu5Ac-α(2-3)Gal-β(1-4)Glc



N-acetylgalactosaminyl-lactose
(GalNAc-Gal-Glc)
GalNAc-α(1-3)Gal-β(1-4)Glc

**Fig. 1.2 b** Structure of the dominant acidic (6’-SL, 3’-SL) and neutral (GalNAc-Gal-Glc) MOS present in bovine milk.

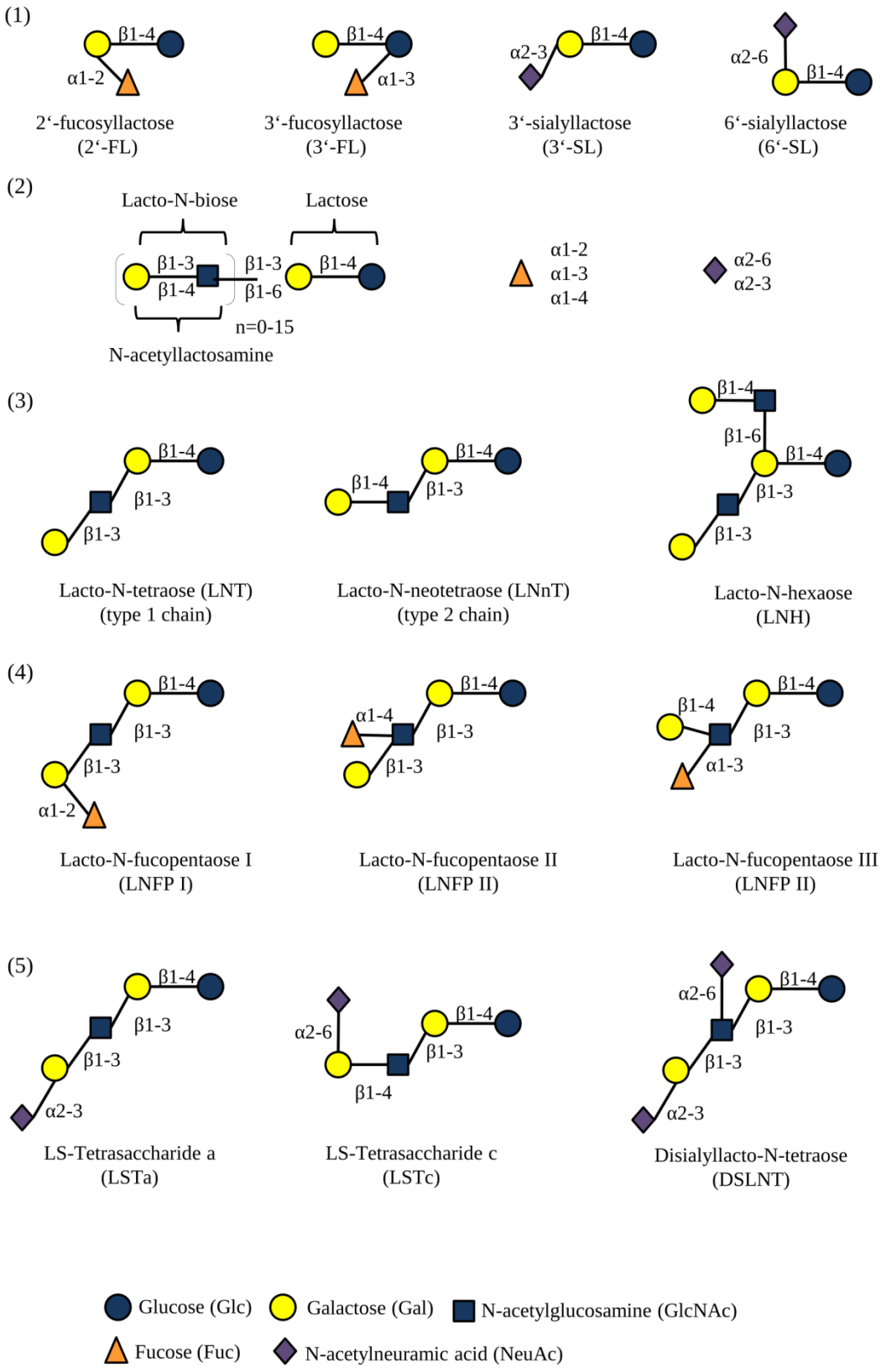



**Fig. 1.2 c** MOS structures. Bode et al. (2012) modified.

*Concentration of MOS in human milk and milk of domestic animals*

MOS represent the third most predominant solid component after lactose and lipids in human milk (Tamime 2009; Mehra and Kelly 2006; Fox and McSweeney 2009; ten Bruggencate et al. 2014). Over 200 different oligosaccharides have been identified in human milk so far (Bode 2012, 2006; Fox and McSweeney 2009; Mehra and Kelly 2006). The concentration of MOS in human colostrum is 13-25 g/L (Eiwegger et al. 2004; Fox and McSweeney 2009; Bode 2012) and decreases to 5-8 g/L in mature milk (Martinez-Ferez et al. 2006a; Bode 2012; Oliveira et al. 2015). 50 % to 80 % of MOS are neutral fucosylated oligosaccharides (Bode 2012; ten Bruggencate et al. 2014), composed mainly of 2'-fucosyllactose (2'-FL); lacto-N-fucopentaose I (LNFP I), lacto-N-difucohexaose I (LNDFH I) and lacto-N-tetraose (LNT). 10 % to 30 % of MOS are acidic sialylated sugars (ten Bruggencate et al. 2014; Bode 2012). The acidic fraction is mainly composed of lacto-N-neotetraose (LST c), disialyl lacto-N-tetraose (DSLNT), 3'-SL and 6'-SL. The most prominent MOS in colostrum are DSLNT and LST, while in mature milk DSLNT is the most abundant oligosaccharide. Human milk is the only milk of mammals which contains more lacto-N-biose (Galβ1-3GlcNAc-, type 1 chain) than N-acetyllactosamine (Galβ1-4GlcNAc-, type 2 chain) (Fox and McSweeney 2009).

In bovine milk, 35 to 50 MOS were detected with a concentration of 1 g/L in colostrum and 0.03-0.06 g/L in mature milk (Fox and McSweeney 2009; Albrecht et al. 2014; ten Bruggencate et al. 2014; Martinez-Ferez et al. 2006b). Bovine MOS are composed of 91 % acidic and 9 % neutral oligosaccharides. 12 different neutral MOS, thereof 3 fucosylated sugars (2'-FL, 3'-FL), and 21 different acidic MOS as well as 2 phosphorylated structures were identified (Albrecht et al. 2014; ten Bruggencate et al. 2014). The most dominant acidic bovine MOS are 3'-SL followed by 6'-sialyllactosamine, 6'-SL and disialyllactose (NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc) (Gopal and Gill 2000). Among the neutral oligosaccharide fraction N-acetylgalactosaminyl-lactose (GalNAc-α(1-3)-Gal-β(1-4)Glc) is prominent (Fox and McSweeney 2009). While 3'-SL and 6'-SL as well as 3'-GL and 6'-GL are present in bovine and human milk, the structures of the other sialylated oligosaccharides are different (Mehra et al. 2006). 3'-SL is the predominant sialyllactose, representing 70 % of all MOS in bovine milk, whereas 6'-SL is the dominant sialyllactose in human milk (Fox and McSweeney 2009; ten Bruggencate et al. 2014). MOS in bovine colostrum contain the two different sialic acids Neu5Ac and Neu5Gc (Mehra and Kelly 2006). Amog the sialylated

bovine oligosaccharides, the majority (97 %) contains Neu5Ac and only a low part (3 %) incorporates Neu5Gc (Albrecht et al. 2014). In human milk and colostrum only Neu5Ac is present (Mehra and Kelly 2006).

In caprine milk 40, MOS have been identified (Albrecht et al. 2014) with a concentration of 0.25-0.30 g/L in mature milk (Oliveira et al. 2015; Martinez-Ferez et al. 2006b). Caprine oligosaccharides are composed of 95 % acidic and 5 % neutral MOS. Albrecht et al. (2014) identified 23 different acidic and 16 neutral oligosaccharides including 3 fucosylated sugars and one phosphorylated sugar (Albrecht et al. 2014). The most prevalent acidic MOS in caprine milk are 6'-SL followed by 3'-SL, disialyllactose and N-glycolylneuraminyl-lactose (Martinez-Ferez et al. 2006b). In contrast to bovine milk, the sialylated sugar fraction of caprine milk contains higher amounts of Neu5Glc (64 %) than Neu5Ac (36 %) (Albrecht et al. 2014). 3'-GL, lacto-N-hexaose (LNH) and N-acetylglucosamilyl-lactose (GlcNAc-Gal-Glc) are mainly present in the neutral oligosaccharide fraction (Martinez-Ferez et al. 2006b). The profile of MOS distributed in caprine milk is described to be closer to human milk oligosaccharides than the pattern in bovine MOS (Oliveira et al. 2015).

In ovine milk, 39 different oligosaccharides were identified (Albrecht et al. 2014) presenting a concentration of 0.02-0.04 g/L in mature milk (Martinez-Ferez et al. 2006b; Oliveira et al. 2015). Similar to bovine and caprine milk, the MOS in ovine milk are mainly composed of acidic sugars (86 %) and only a small fraction of neutral sugars (14 %) (Albrecht et al. 2014).

In camel milk, 45 different MOS were identified (Albrecht et al. 2014) composed mainly of acidic oligosaccharides (84 %) and neutral oligosaccharides (16 %) (Albrecht et al. 2014).

### *I.1.2.2 Biological activities of MOS*

After ingestion, MOS resist the low pH of the stomach and the pancreatic digestion, reaching the small intestine and the colon in intact form (Bode 2012; Mehra and Kelly 2006; ten Bruggencate et al. 2014). Most gastrointestinal enzymes are not capable of cleaving fucose, N-acetyl-neuramic acid (Neu5Ac, sialic acid), or N-acetyl-glucosamine (GlcNAc) from oligosaccharides (ten Bruggencate et al. 2014; Fox and McSweeney 2009; Urashima et al. 2001). The MOS are excreted with the infant's feces (Bode 2012). In the first two months of

life, the feces contains many acidic and neutral MOS similar to the human MOS followed by a phase with various different degradation products of MOS, resulting in complete disappearance of MOS and their degradation products in feces when other feedings than human milk are induced (Bode 2012). Moreover, about 1 % of human MOS are absorbed via the paracellular route (acidic and neutral MOS) and transcellular route (neutral MOS), circulate long enough to display their biological effect at the target sides and finally are excreted in the infant's urine (Bode 2012; Kunz and Rudloff 2006; ten Bruggencate et al. 2014; Gnoth et al. 2001; Fox and McSweeney 2009).

Because MOS reach the colon in intact form, they are mainly described to exhibit prebiotic activity (Bode and Rudloff 2002; ten Bruggencate et al. 2014; Zivkovic and Barile 2011; Kunz et al. 2000; Kunz and Rudloff 2008). Prebiotics are defined in 1995 as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson and Roberfroid 1995). The definition of prebiotics implies that the compounds must resist the small intestinal digestion such as gastric acidity, hydrolysis by gastrointestinal enzymes as well as gastrointestinal absorption to exert the positive effect in the large intestine (Bode 2012; Moreno et al. 2014). MOS meet these requirements when the absorption rate of 1 % is neglected in this relation and the high amount of intact MOS reaching the colon is considered (Bode 2012). The oligosaccharides promote the growth of commensal bacteria. *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* exhibited a high consumption of MOS (Fox and McSweeney 2009; Bode 2012), while *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium breve* hardly grow with this carbohydrate source and metabolize only lacto-N-tetraose (LNnT) (Bode 2012). These commensal bacteria compete with potential pathogens for the carbohydrate source as nutrient. Moreover, the beneficial microbiota produce short chain fatty acids, which are responsible for a decrease in pH and a development of an environment favoring the growth of commensals over harmful bacteria (Bode 2012; Zivkovic and Barile 2011).

Aside of prebiotic activity, MOS exert further host-microbial interactions by acting as anti-adhesive antimicrobials (Bode and Rudloff 2002; Fox and McSweeney 2009). To cause an infection, pathogenic bacteria and viruses need to adhere to specific carbohydrate structures of glycoconjugates on the surface of the colonic epithelial cells. The adhesion is mediated by

bacterial proteins termed lectins, carbohydrate-binding proteins which bind to the oligosaccharides on the epithelial cell surface. MOS contain structural units that are homologous to the epithelial oligosaccharides and therefore belong to the carbohydrate-binding determinants of the bacteria. The oligosaccharides in milk, especially sialylated MOS, compete with the epithelial ligands for bacterial binding. Thus, MOS act as soluble receptor analogues, inhibit the adhesion of pathogens and reduce the risk of infection (Bode 2009; Zivkovic and Barile 2011; Kunz et al. 2000; ten Bruggencate et al. 2014; Fox and McSweeney 2009).

In addition, MOS can be modulators of intestinal epithelial cell responses (Bode 2012). An incubation of human intestinal epithelial colon adenocarcinoma cell line (Caco-2 cells) with 3'-SL resulted in decreased α2-3- and α2-6-sialylation of the epithelial cell surface oligosaccharides. Adhesion of enteropathogenic *Escherichia coli* was reduced about 90 %, because these bacteria need sialylated glycans to bind at epithelial cell surfaces. It can be concluded that MOS are capable of influencing the gene expression by modulating or altering the cell surface glycosylation (Bode 2009).

Furthermore, MOS can modulate the immune response locally at the mucosa of lymphoid tissue or systemically as 1 % of the oligosaccharides are absorbed and appear in the systemic circulation (Bode 2012). On the one hand, MOS were described to elicit an anti-inflammatory effect. Incubation of Caco-2 cells with 3'-SL resulted in reduction of cytokine secretion (IL-12) and cytokine gene expression (IL-8 and TNF-α). In addition, the translocation of the immune modulating transcription factor nuclear factor κ B (NFκB) was decreased by this MOS (Zenhom et al. 2011).

On the other hand, immunostimulatory effects of MOS have been described in a few investigations **(Table 1.1)**. For instance, the incubation of cord blood-derived mononuclear cells (CBMC) with acidic MOS increased the interferon-γ producing CD3+CD4+ and CD3+CD8+ lymphocytes and the interleukin-13 (IL-13) synthesis in CD3+CD8+ lymphocytes *in vitro* (Eiwegger et al. 2004) **(Table 1.1)**. In addition, Ortega-González et al. (2014) reported about an increased secretion of chemokines (GROα = growth related oncogene-α, MCP1= monocyte chemoattractant protein-1) as inflammatory markers in intestinal epithelial cells (IEC 18) by a goat MOS product containing 6'-SL, 3'-SL, disialyllactose, N-glycolylneuraminyl-lactose, 3'-GL, lacto-N-hexaose (LNH), N-

acetylglucosaminyllactose. These oligosaccharides are described to up-regulate the activity of the immune regulatory transcription factor nuclear factor κ B (NFκB) as ligand for the TLR4 receptor in intestinal epithelial cells by triggering the NFκB-signaling pathway *(chapter I.1.4)* (Ortega-González et al. 2014). Moreover, the immunostimulatory activity of MOS by increased cytokine secretion (IFN-γ, IL-13, TNF-α, IL-6, IL-10) was determined in colonic epithelial cells (HT-29) (Lane et al.2013), primary mouse splenocytes (Capintan-Canadas et al. 2014) and human cord blood-derived T-cells (Eiwegger et al 2014). Studies investigating the immunostimulatory effect of MOS are listed in **Table 1.1**.

**Table 1.1** Literature overview about the immunostimulatory effect of MOS. (IEC18 = intestinal epithelial cells from rat, GRO α = growth related oncogene-α, MCP1 = monocyte chemoattractant protein-1, TLR4 = Toll-like receptor 4, NFκB = nuclear factor κ B, CBMC = cord blood mononuclear cells)

| Cells | Sample | Concentration | Results | Reference |
|---|---|---|---|---|
| IEC18 | Goat MOS | 5 mg/ml | ○ increased secretion of chemokines (GROα, MCP1)<br>○ MOS act as TLR4 ligand by up-regulation of the transcription factor NFκB | (Ortega-González et al. 2014) |
| Mouse splenocytes | Goat MOS | 5 g/L | ○ Goat MOS showed increased secretion of TNF-α, IL-6, and IL-10 in mouse splenocytes in comparison to the control without sample.<br>○ Goat MOS showed no effect in secretion of TNF-α, IL-6, and IL-10 in LPS induced mouse splenocytes in comparison to the control (with LPS).<br>○ Goat MOS reduce secretion of IFN-γ and IL-17 in concanavalin A stimulated mouse splenocytes in comparison to the control (with concanavalin A) **(Fig.2)**.<br>○ MOS bind and activate TLR4 receptors in mice splenocytes. | (Capitán-Cañadas et al. 2014) |
| CBMC | Acidic human MOS,<br>neutral human MOS | 10 µg/mL neutral human MOS,<br>1 µg/mL acidic human MOS | ○ Acidic MOS fraction increased the IFN-γ producing CD3+CD4+ and CD3+CD8+ lymphocytes and the IL-13 production in CD3+CD8+ lymphocytes. | (Eiwegger et al. 2004) |

It requires further investigation to explain the influence of MOS on the immune response and especially to determine which receptors and signaling pathways HMO employ to trigger gene expression. Generally, the evaluation of the influence of MOS on activation of NFκB (nuclear factor κ B) in cells is a valuable approach, as this transcription factor is a key mediator in regulation and induction of various chronic inflammatory diseases such as cancer, diabetes and asthma. Therefore, the immunostimulatory effect of bovine MOS was determined by analyzing their effect on the activity of the transcription factor NFκB in human embryonic kidney cells ($HEK^{nfκb\text{-}RE}$ cells) *(chapter II.4.3).* The NFκB-signaling pathway is described in *chapter I.1.4*.

### *I.1.2.3 Availability of MOS as functional food ingredients*

MOS play a key role in supporting and maintaining health in breast-fed infants. It would be beneficial to provide MOS to formula-fed infants as well as to humans of all ages (Mehra et al. 2014). There exist various studies about the identification, quantification and the biological activities of MOS from different milks (Tao et al. 2008; Tao et al. 2010; Wu et al. 2010; Albrecht et al. 2014). Research is required to develop an efficient process to provide a MOS product for the industrial application as functional ingredient for food.

On the one hand, investigations were performed on biotechnological synthesis of MOS with microbial and enzymatic systems (Han et al. 2012; Luo et al. 2014; Michalak et al. 2014; Zeuner et al. 2014; Fierfort and Samain 2008; Priem et al. 2002; Guo et al. 2014; Choi et al. 2014). Cell methods have been developed using recombinant *Escherichia coli* strains, in which sialyllactose or complex sialylated oligosaccharides can be produced either with synthesis of N-acetylneuramic acid (Neu5Ac) by internalizing and sialylation of lactose with glycosyltransferase (CMP-Neu5Ac) (novo synthetic pathway) or with uptake of sialic acid from medium (salvage method) (Han et al. 2012). Moreover, some researchers were able to produce fucosylated oligosaccharides as well as some complex fucosylated and sialylated oligosaccharides by enzymatic or microbial methods. So far, sialyllactoses (3'-SL, 6'-SL), fucosyllactoses (2'-FL, 3'-FL), lacto-N-biose and lacto-N-tetraose (LNT) are commercially available but rather as a standard (Han et al. 2012). Due to the high diversity of human MOS, a lot of research has to be done to create a MOS composition similar to human milk by these

biotechnological processes. The development of cost effective biotechnological processes on industrial scale and the requirement to meet the criteria of novel food are further barriers.

On the other hand, only few studies were conducted to enrich the naturally presented MOS from domestic animal milk by membrane filtration procedures. So far, there exist only three studies about the enrichment of MOS by membrane separation on laboratory and pilot plant scale and only one study realized the concentration of MOS on industrial scale **(Table 1.2)**. In all studies, a two-stage cross-flow membrane filtration was applied. For the first filtration either ultrafiltration (UF, NMWCO 10-50 kDa) or microfiltration (MF, NMWCO 1 mm) was applied to separate the protein fraction of skimmed milk or whey from the carbohydrate moiety. The permeate (UF permeate, MF permeate) was composed of lactose or the hydrolyzed lactose products glucose and galactose and MOS as well as milk salts. The permeate of the first filtration step was used as the initial sample for the enrichment of MOS. In the second filtration step, the NF membrane module as well as the conditions and the process parameters have to be chosen carefully. During this step, the MOS should remain in the retentate and the other sugars should pass the membrane. Moreover, diafiltration (DF) supports the permeation of the residual sugars (lactose, glucose, galactose) and salts. However, DF might lead to a higher permeation or loss of MOS. It depends on the target whether a high recovery of MOS and/or a high purity of the retentate wants to be achieved. The NF on laboratory scale resulted in a 84 % to 90 % retention of MOS in the retentate (Martinez-Ferez et al. 2006a; Sarney et al. 2000). On pilot plant scale UF, a 10 % retention of total carbohydrates (lactose, oligosaccharides) was achieved, composed of about 90 % oligosaccharides (Oliveira et al. 2014). Due to different methods of quantification **(Table 1.2 a-c)**, the results are difficult to compare. Within these studies, the content of the oligosaccharide fraction after subtraction of the residual sugar (lactose, glucose, galactose) concentration was determined. A more precise quantification and identification of individual sialyllactoses (3'-Sl and 6'-SL) by HPAEC were conducted in the UF retentate on industrial scale NF by Mehra et al. (2014). As a result, a 40-fold enrichment of the sialyllactose-to-lactose ratio of 14 % with 2.8 % MOS in dry mass was achieved (Mehra et al. 2014). However, no process has been efficiently implemented at industrial scale so far due to high capital costs, operational complexity and low productivity.

**Table 1.2** Literature overview of membrane separation processes for the enrichment of MOS (p = pressure, T = temperature, Ref. = reference, DV = diavolumes *(chapter II, III)*, NMWCO = nominal molecular weight cut-off, UF = ultrafiltration, NF = nanofiltration, DF = diafiltration, MF = microfiltration).

| Method | NF | | | | Origin of MOS | Results | Ref. |
|---|---|---|---|---|---|---|---|
| | Membrane type /manufacturer | p [bar] | T [°C] | pH | | | |
| Laboratory scale<br>two-stage cross-flow UF-NF process<br>1. UF of skimmed caprine milk, 4 DV<br>2. NF of UF-permeate for enrichment of MOS in retentate, 3 DV | Tubular ceramic membranes<br>1. NMWCO 50 kDa module<br>2. NMWCO 1 kDa module | 2,5 | 30 | - | Pasteurized skimmed caprine milk | 84 %[a] retention of MOS in NF retentate, 94 % permeation of lactose and proteins | (Martinez-Ferez et al. 2006a) |
| Laboratory scale<br>two-stage cross-flow UF-NF process<br>1. Skimmed milk was lactose hydrolyzed and UF<br>2. NF of UF permeate; 36-54 DV | UF membrane:<br>PM 10, NMWCO 10 kDa<br>NF membranes:<br>RO-CA-96, cellulose acetate;<br>NF-CA-50, cellulose acetate;<br>NF-TEC-50 trilaminate polyethersulphone | 30 | - | 5.2 | Human milk,<br>Caprine milk,<br>Bovine milk,<br>Ovine milk | About 90 %[b] retention of MOS in human and caprine milk after NF and DF with 36-54 DV;<br>NF-CA-50 most suitable,<br>retention of MOS in bovine and ovine milk was too low | (Sarney et al. 2000) |

*Continued.*

| Method | NF | | | | Origin of MOS | Results | Ref. |
|---|---|---|---|---|---|---|---|
| | Membrane type /manufacturer | p [bar] | T [°C] | pH | | | |
| Laboratory and pilot plant scale<br>two-stage cross-flow UF- tight UF process<br>1. UF of caprine whey<br>2. tight UF of UF permeate without DF | UF membrane:<br>ES625 NMWCO 25 kDa (ITT PCi Membranes)<br>tight UF membrane:<br>CéRAM™ NMWCO 1 kDa | 14 | 25-30 | 4-5 | Caprine whey | 10 % retention of total carbohydrates in UF retentate which contain about 90 %[c] oligosaccharides | (Oliveira et al. 2014) |
| Mother liquor[d] was centrifuged,<br>industrial scale two-stage cross-flow MF-NF process<br>1. MF of clarified mother liquor<br>2. UF of MF permeate and DF | MF membrane:<br>0.1 mm NMWCO,<br>UF membrane:<br>1 kDa NMWCO | 5 | 10 | - | Bovine milk | Sialyllactose to lactose ratio of 14 % in UF retentate= 40-fold enrichment, 2.8 % sialyllactose[e] (3'-SL, 6'-SL) in dry mass of UF retentate | (Mehra et al. 2014) |

(a = relation of total area under the HPAEC chromatogram of initial sample to NF retentate, the samples were purified and contain the oligosaccharide fraction due to removal of fat, proteins salts and lactose; b = the percentage of oligosaccharides in the retentate as determined by integration of peaks on HPLC chromatograms relative to lactose; c = determined with capillary electrophoresis by the difference of total sugar and galactose, glucose and lactose, d = the solution remaining after lactose crystallization and separation of a whey permeate, e = sialyllactoses were quantified by HPEC)

*I.1.2.4 Membrane filtration procedures*

Every membrane separation process is characterized by the use of a semi-permeable membrane capable of a selective separation of molecules of different sizes and characteristics (Ramaswamy and Marcotte 2005; Nielsen 2000; Mulder 1992; Tamime 2009). The two basic membrane processing categories are the dead-end filtration and the cross-flow filtration. In dead-end filtration the separation of molecules in the liquid occurs perpendicular to the membrane surface using gravity or vacuum as driving force. In cross-flow filtration the feed stream flows parallel or tangentially to the membrane surface using hydrostatic pressure as driving force **(Fig. 1.3)**. In this pressure-driven filtration process, the feed overflows the membrane. Compounds smaller than the nominal molecular weight cut-off (NMWCO) of the membrane pass predominantly the membrane into the permeate. Constituents which are bigger than the NMWCO are mainly retained in the retentate. For industrial processes, cross-flow filtration is preferred because of the lower membrane fouling tendency in comparison to dead-end filtration (Ramaswamy and Marcotte 2005; Mulder 1992; Nielsen 2000). In this work, cross-flow filtration technique was applied.

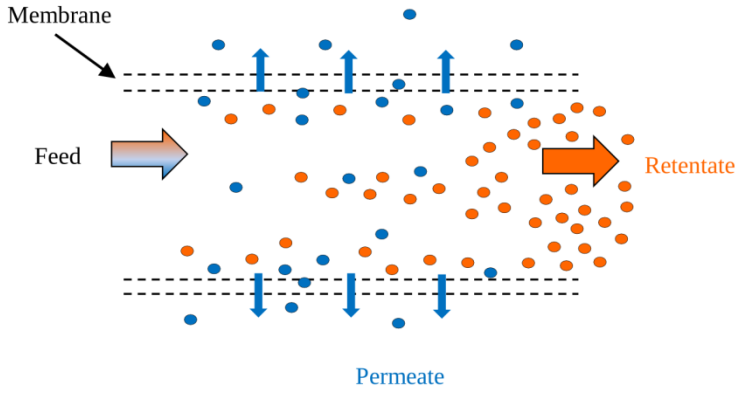



**Fig. 1.3:** Schematic drawing of a cross-flow filtration process.

Membrane filtration procedures mainly applied in the food industry can be classified by the NMWCO of the membranes into microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Mulder 1992; Nielsen 2000; Ramaswamy and Marcotte 2005). **Table 1.3** shows the classification of the membrane separation processes according to the

NMWCO. There is no sharp division between the membrane filtration procedures (Tamime 2009).

**Table 1.3** Classification of membrane filtration technology (Tamime 2009). MF = microfiltration, UF = ultrafiltration, NF = nanofiltration, RO= reverse osmosis

| Membrane separation process | NMWCO [Da] |
|---|---|
| MF | > 100,000 |
| UF | 1,000 -500,000 |
| NF | 100 – 1,000 |
| RO | < 100 |

**Figure 1.4** shows the different applications of membrane filtration processes in the dairy industry. During MF of milk whey proteins, lactose, mono- and divalent ions and non-protein nitrogen (NPN) permeate with water while the caseins, milk fat and bacteria are retained. By performance of UF, additionally the whey proteins are retained from the UF permeate containing lactose, mono- and divalent ions and non-protein nitrogen (NPN). In NF process, only monovalent ions permeate with water, while lactose, divalent ions and NPN are concentrated in the retentate. RO retains all compounds except water.

According to the configuration, membranes can be classified in tubular and flat-sheet modules. Tubular modules include hollow-fiber, tubular and capillary systems. Flat-sheet configurations are used in plate-and-frame modules also called flat-sheet modules and spiral-wound modules (Mulder 1992). In plate-and-frame configurations, also called flat-sheet membranes, two flat membranes build a “sandwich” around a support plate, which contain permeate channels allowing the separation of permeate and retentate. The spiral-wound module is an extension of the flat sheet module, whereas the membrane and spacer system is rolled into a spiral wound around a central porous tube. The feed solution is distributed at one end of the cylindrical module. The retentate flows axial through the module parallel to the central tube, while the permeate flows spirally to the central porous pipe, where it can run out of the system (Ramaswamy and Marcotte 2005; Mulder 1992). In our investigations, flat-sheet and spiral-wound modules were used.

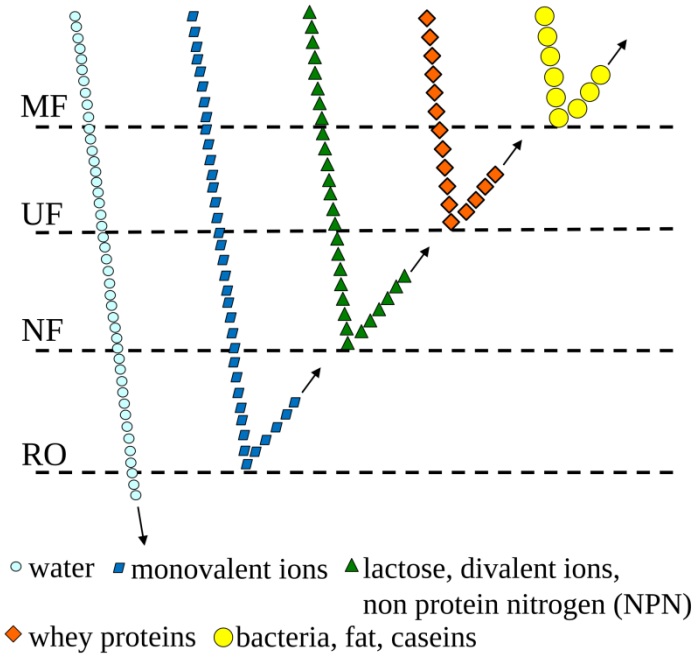



**Figure 1.4** Separation behavior of the different membrane separation processes by application of milk as feed solution (Tamime 2009). MF= microfiltration, UF= ultrafiltration, NF= nanofiltration, RO = reverse osmosis

*I.1.2.5 Whey as a raw material for the enrichment of MOS*

Whey streams are regarded as the key source of MOS (Mehra et al. 2014). For the enrichment of oligosaccharides from bovine and caprine milk, skimmed and ultrafiltered milk permeate (SM-UF-permeate) was applied as a raw material in our investigations *(Manuscript 1, chapter II, Manuscript 2, chapter III)*. The production of SM-UF-permeate is shown in **Fig. 1.5**. Raw milk was heated at 45 °C and skimmed in a centrifuge for separation of the milk fat. The skim milk (SM) was ultrafiltered by application of a 5 kDa membrane module *(Manuscript 1, chapter II, Manuscript 2, chapter III)* to remove the protein fraction. During the UF process, compounds with a molecular mass < 5 kDa passed through the membrane into permeate. Because MOS are soluble in the milk serum and exhibit a molecular size of 600 to 1600 Da depending on the degree of polymerization of 3 to 8 (Albrecht et al. 2014), the sugars passed the membrane. Beside the MOS, the resulting SM-UF permeate contained lactose as main component, milk salts and trace amounts of non-protein nitrogen *(Manuscript 1, chapter II, Manuscript 2, chapter III)*. This SM-UF permeate is also called “ideal whey”, as it is

produced from skim milk without any by-products of protein coagulation, e.g. during cheese processing such as rennet or fermentation products (Morison and She 2003). The protein fraction, which contains the caseins and whey proteins, remained in the retentate > 5 kDa. Also other studies (Martinez-Ferez et al. 2006a; Sarney et al. 2000; Oliveira et al. 2014) dealing with the enrichment of MOS by membrane separation procedure used a SM-UF permeate as a raw material **(Table 1.2)**. In the dairy industry, SM-UF permeate or "ideal whey" is a co-product of the manufacturing of phosphocasein, or whey protein concentrate (WPC) (Mehra et al. 2014; Anand et al. 2013; Morison and She 2003) **(Fig. 1.6)**.

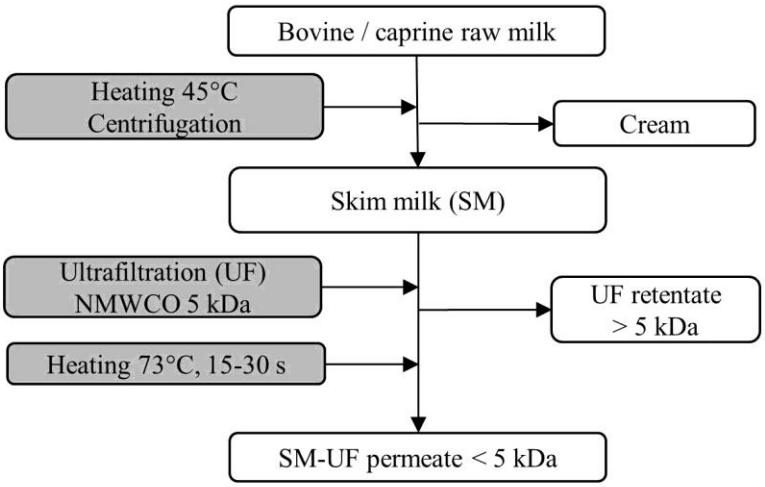



**Fig. 1.5:** Production of skimmed and ultrafiltered milk permeate as a raw material for the enrichment of MOS. NMWCO = nominal molecular weight cut-off

Moreover, cheese whey might be a valuable source for the enrichment of MOS (Barile et al. 2009). This aqueous fraction of milk is generated as a by-product of protein coagulation e.g. for cheesemaking **(Fig. 1.6)**. It is obtained by separation of the coagulum from milk, skim milk or cream in cheese manufacturing. The two basic types of whey are rennet or sweet whey and acid whey. While rennet whey is produced by application of rennet during cheesemaking (e.g. hard cheese, semi hard cheese), acid whey is generated as a co-product of milk coagulation by acid (e.g. quark or acid casein). Main solid components of both whey types are lactose (4.4 % to 5.0 %), minerals (0.52 % – 0.6 %) and whey proteins (Anand et al. 2013; Sienkiewicz and Riedel 1990). Moreover, it has been described that MOS are present in whey streams (Mehra et al. 2014). Whey is mainly processed by membrane filtration

procedures and spray drying, resulting in a variety of different products such as whey powder, whey protein concentrates or whey protein isolates. For instance, whey protein concentrates (WPC) containing different protein contents (e.g. WPC 34 = 34 % - 36 % protein content, WPC 80 = 80 % - 82 % protein content) are produced by ultrafiltration of whey (Anand et al. 2013) **(Fig. 1.6)**. A by-product of this UF process is whey-UF permeate containing all small molecules which can pass the membrane such as lactose, MOS and salts (Barile et al. 2009). While WPC is applied in various food products, whey-UF permeate is of low value (Barile et al. 2009). Whey-UF permeate is mainly used for the production of lactose by crystallization resulting in mother liquor **(Fig. 1.6)** (Mehra et al. 2014). Whey UF-permeate can be spray-dried and used as food ingredient in similar way as lactose, e.g. in confectionary, dairy and bakery products (Li et al. 2008). Due to the high lactose content, whey-UF permeate is used as fermentation medium, supplemented with 1-2 % WPC, for the propagation of lactic acid bacteria (Bury et al. 1998) or for the production of lactic acid by lactic acid bacteria such as *Lactobacillus helveticus* (Fitzpatrick and O'Keeffe 2001). Moreover, whey-UF permeate is fed to animals (Marwaha and Kennedy 1988). Another possibility to increase the economic value of whey-UF permeate might be the industrial enrichment of MOS by membrane filtration process. Whey-UF permeate is a suitable source for providing a MOS concentrate as functional food ingredient because it is a readily available dairy stream produced in high amounts. Because of the low concentration of MOS in bovine milk or in milk of other domestic animals, large quantities of raw material for the concentration of the sugars are mandatory. Mother liquor is the liquid separated from lactose after lactose crystallization **(Fig. 1.6)**, which contains low lactose amounts, MOS and milk salts. Mehra et al. (2014) demonstrated in their study **(Table 1.2)** that a concentration of the MOS from mother liquor by UF was possible. However, an additional MF step had to be performed for the clarification due to residual insoluble material and bacteria present in the liquor (Mehra et al. 2014).

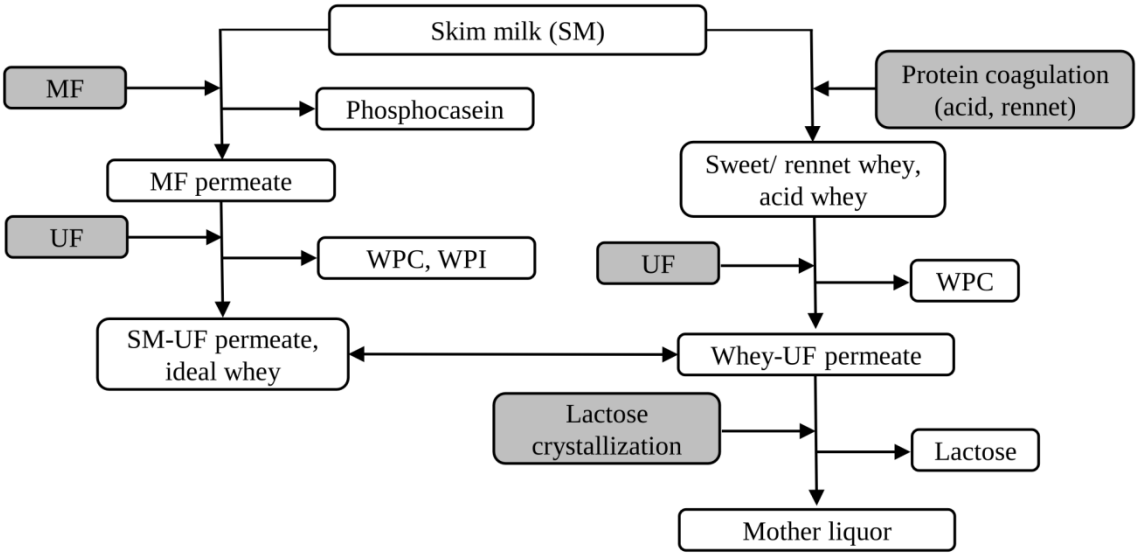



**Fig. 1.6** Whey streams for enrichment of MOS.

The dairy streams SM-UF and whey-UF permeate are far comparable regarding the lactose, sugar and protein content, however, SM-UF permeate contains no by-products of cheese processing. The composition of whey-permeate may be subject to daily variations associated with cheese manufacturing and contains compounds which might disturb the analytical identification and quantification of MOS by HPAEC. In our investigations, SM-UF permeate was applied as a raw material for the enrichment of MOS because its production is highly reproducible and no by-products of other manufacturing procedures are present.

### *I.1.2.6 Nanofiltration (NF) process for the enrichment of MOS*

For NF-enrichment of MOS, lactose hydrolyzed SM-UF permeate was applied as a raw material. As shown in our studies on laboratory scale *(Manuscript 1, chapter II)* and in other studies (Oliveira et al. 2014), the enrichment of MOS and the permeation of the residual sugars were higher when lactose in SM-UF permeate was hydrolyzed. The process of the NF performed on laboratory, pilot plant and industrial scale is described in *chapters II* and *III*. In brief, lactose hydrolyzed SM-UF permeate was circulated in the filtration device for 30 min to build a deposit layer for ensuring reproducible filtration procedures. In the first step of NF,

the initial sample was concentrated followed by diafiltration with demineralized water for a better permeation of the residual sugars (glucose, galactose) and milk salts. The MOS should be retained in the NF retentate.

As an example for a NF device, a flow chart of the membrane filtration device (MMS Membrane Systems, Urdorf, Switzerland), which was used for the enrichment of MOS on industrial scale, is shown in **Fig. 1.7**. The lactose hydrolyzed SM-UF permeate was transported by the feed and pressure pump to the membrane unit, which contains two membrane modules (DOW, NF-3840-30-FF®, the DOW chemical company, active membrane area = 7.5 $m^2$) with an active membrane area of 15 $m^2$. The double-walled membrane unit was cooled with ice water at a process temperature of 10°C. The retentate, which should contain the MOS, circulated back into the retentate vessel. The permeate was collected in a separate tank. Feed flow, permeate flow, pressure and conductivity were measured online during the NF process.

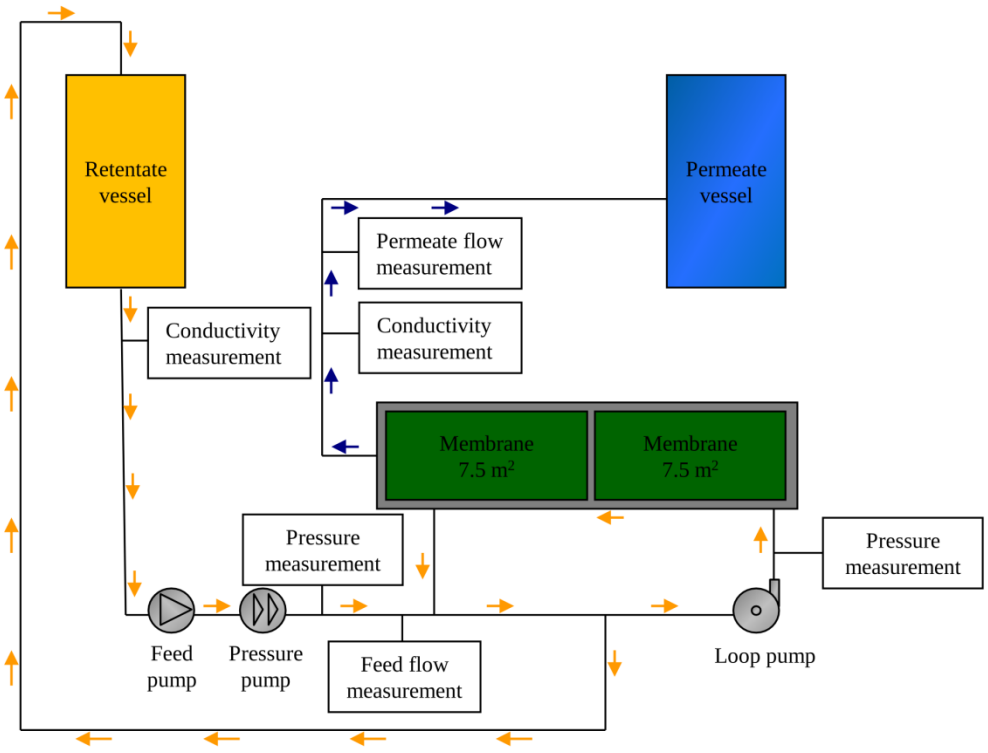



**Fig. 1.7:** Flow chart of the industrial scale NF applying the membrane module DOW NF-3840-30-FF at 10°C and 10-15 bar.

*I.1.2.7 Structure, production and biological activity of galactooligosaccharides (GOS)*

β-Galactooligosaccharides (GOS) are β-glycosidically linked non-digestible oligosaccharides composed of galactose with lactose at the reducing end and a degree of polymerization of 3 to 10 (mostly 3, 4 and 5) (Gänzle 2012; Bode 2012; Fox and McSweeney 2009). These oligosaccharides are synthesized from lactose by transgalactosylation activity of the enzyme β-galactosidase (EC 3.2.1.23) (Fox and McSweeney 2009). This glycosidase transfers the galactose moiety of a β-galactoside to an acceptor which contains a hydroxyl group. When the acceptor is water, galactose is synthesized. When the acceptor is lactose or galactose, galactooligosaccharides are formed (Nakamura et al. 1995; Fox and McSweeney 2009). Human and bovine milk contain only trace amounts of β-GOS (Gänzle 2012).

Structure and size of the GOS depend on the origin of the enzyme employed for the production of these sugars. For the production of GOS, β-galactosidases from yeast, fungi and bacteria are suitable. Thus, the choice of the enzyme is crucial for the GOS outcome and composition. By application of the β-galactosidases from *Kluyveromyces lactis*, *Aspergillus oryzae*, *Aspergillus acetulans* and *Streptococcus thermophilus*, primarily β(1-6) oligosaccharides (6'-galactosyllactose, 6'-GL) are produced (Gänzle et al. 2008; Fox and McSweeney 2009; Frenzel et al. 2015). The β-galactosidase from *Sterigmatomyces elviae* synthesizes mainly 4'-galactosyllactose (4'-GL). A wide range of different GOS with the glycosidic linkages β(1-2), β(1-3), β(1-6) and mainly β(1-4) are produced by the β-galactosidase from *Bacillus circulans* (Frenzel et al. 2015; Gänzle et al. 2008). Frenzel et al. (2015) determined a high affinity of the β-galactosidase from *Bacillus circulans* for transgalactosylation and a high GOS yield of 41 %, followed by *Aspergillus oryzae*, *Aspergillus acetulans* and *Kluyveromyces lactis* in a skimmed milk UF permeate with 40 % lactose concentration. Moreover, Lorenzen et al. (2013) developed a bi-enzymatic system for lactose conversion. A concentrated skim milk permeate with 40 % lactose content was incubated with a β-galactosidase and a glucose isomerase. 21 % galactooligosaccharides, such as 6-galactobiose, allolactose and 6'-GL, were produced after 4 h incubation time by almost complete hydrolysis of lactose (degree of hydrolysis of 95.3 %) as main reaction products of the β-galactosidase from *Kluyvmeromyces lactis*. Due to glucose isomerization (degree of isomerization up to 47.0 %), the fructose content increased, resulting in a lactulose concentration of 1.1 % after 4 h incubation (Lorenzen et al. 2013). Furthermore, an endless

variety of different GOS can be formed by β-galactosidases when different galactosyl acceptors are employed such as mannose, fructose, maltodextrins and N-acetylneuramic acid (Gänzle et al. 2008). For example, Black et al. (2014) produced heterooligosaccharides by conversion of lactose with a β-galactosidase from *Lactobacillus plantarum* and chitin-oligosaccharides or chitosan-oligosaccharides as galactosyl acceptors (Black et al. 2014).

Aside from the β-GOS also other lactose derivatives with a high degree of polymerization can be formed. Transglucosylation or transfructosylation of lactose as sugar acceptor and sucrose as glucosyl or fructosyl donor by application of glucansucrases or fructansucrases results in the formation of polymeric fructans and glucans, respectively. While glucansucrases produce linear or branched polymeric glucans with α-(1-2), α-(1-3), α-(1-4) or α-(1-6) linkages, fructansucrases produce linear or branched polymeric β-(1-2) fructans (inulin), or β-(1-6) fructans (levan) (Gänzle 2012).

GOS are widely known to exhibit prebiotic activity (Moreno et al. 2014; Fox and McSweeney 2009; Frenzel et al. 2015; Gosling et al. 2010; Gänzle 2012). The definition of prebiotics *(see chapter I.1.2.2)* implies that these compounds must resist the small intestinal digestion to reveal the positive effect in the large intestine (Bode 2012; Moreno et al. 2014). Various assays described that 90 % of GOS are stable to digestive enzymes and reach the colon to display their biological effects (Moreno et al. 2014). Moreover, GOS are able to modulate the immune system. During fermentation of GOS by intestinal microorganisms, butyrate and propionate are released, which are described to modulate the immune response e.g. by suppression of cytokines. The consumption of the commercially available GOS product Bimuno® resulted in a decrease in the secretion of inflammatory cytokines (IL-6, IL-1β, THF-α) and an increased synthesis of the anti-inflammatory cytokine IL-10 (Moreno et al. 2014). Furthermore, GOS are reported to inhibit the adhesion of pathogens at the gastrointestinal epithelial surface. These oligosaccharides are structurally similar to epithelial glycan receptors and prevent intestinal infections due to their capacity to act as “molecular decoys” by blocking the glycan receptors (Gänzle 2012; Moreno et al. 2014). This anti-adhesive or anti-infective effect was often reported for GOS, which inhibited the attachment of enterohepatic *E. coli*, *Salmonella enterica* or *Chronobacter sakazakii* (Goulas et al. 2007; Melnik et al. 2013; Karin and Lin 2002) in Caco-2 cells and HT29 cells. Some studies reported an improvement of Ca absorption due to GOS fermentation in the gut. One reason

might be that the short chain fatty acids, which are released during GOS fermentation in the large intestine, decrease the pH resulting in an increased salt solubility and therefore a better absorption across the epithelial cells of colon (Moreno et al. 2014).

Since the mid-1980s, GOS have been manufactured industrially. Food-grade oligosaccharide products are mixtures of mono-, di- and oligosaccharides rather than pure substances. The company Yakult Honsha Co. Ltd. produces three different GOS products (Oligomate 55, a syrup; Oligomate 55P, a powder; TOS-100, a purified powder). Furthermore, Nissin Sugar Manufacturing Co. Ltd. produces a syrup (Cup-Oligo H-70) and a powder (Cup-Oligo P) containing 70 % GOS in total solids. Friesland Foods Domo produces a syrup (Vivinal-GOS) composed of 60 % GOS in total solids. Due to their biologically activity, GOS are applied predominantly as functional ingredient to beverages, infant milk formula or infant foods (Fox and McSweeney 2009).

### *I.1.3 Bioactive peptides from milk*

#### *I.1.3.1 Anti-inflammatory peptides from milk and other sources*

It is widely known that milk provides a rich source of bioactive components (Mills et al. 2011; Nongonierma and FitzGerald 2015; Meisel et al. 1989). The milk protein fraction reveals a wide range of nutritional, technofunctional and biological activities (Korhonen and Pihlanto 2006; Meisel and Bockelmann 1999; Mills et al. 2011; Weimann et al. 2009). Increasing attention is paid to biologically active peptides from milk as most important source (Dziuba and Dziuba 2014; García-Tejedor et al. 2013; Korhonen and Pihlanto 2006; Picariello et al. 2013; Plaisancié et al. 2013). Within the sequence of a native protein precursor, milk protein-derived peptides are inactive. They can be released by enzymatic hydrolysis during food processing or gastrointestinal digestion (Hajirostamloo 2010; Korhonen and Pihlanto 2006; Meisel 1997b; Plaisancié et al. 2013; Weimann et al. 2009). Bioactive peptides were defined by Kitts and Weiler in 2003 as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler 2003). By oral consumption of active peptides or liberation of these peptides during gastrointestinal digestion, they may demonstrate various biological activities such as antioxidative, ACE inhibitory, antimicrobial, antihypertensive and opioid activities (Chakrabarti et al. 2014; Espeche Turbay et al. 2012; García-Tejedor et al. 2013; Hartmann and Meisel 2007; Korhonen and Pihlanto 2006; Mills et al. 2011; Plaisancié et al. 2013; Norris et al. 2015; Fitzgerald et al. 2004). Furthermore, some peptides are multifunctional by displaying more than one beneficial effect described (García-Tejedor et al. 2013; Hartmann and Meisel 2007; Korhonen and Pihlanto 2006; Mills et al. 2011). The field of bioactive peptides has been extensively reviewed (Dziuba and Dziuba 2014; Hartmann and Meisel 2007; Korhonen and Pihlanto 2006; Meisel 1998; Mills et al. 2011; Plaisancié et al. 2013; Wu 2013). Much less prevalent are studies dealing with anti-inflammatory food-derived peptides.

Recent studies exhibited that dietary peptides can modulate inflammatory response (Millán-Linares et al. 2014). Food proteins and peptides with anti-inflammatory activity are summarized in **Table 1.4**. Some investigations deal with the effect of plant-derived peptides on inflammatory markers (Millán-Linares et al. 2014; Vernaza et al. 2012; Young and Mine 2010) and anti-inflammatory peptides from fish proteins. Salmon protein hydrolysates

exhibited anti-inflammatory activity by concentration-dependent reduction of nitric oxide (NO) production and the pro-inflammatory cytokines TNF-α, IL-6, IL-β1 in lipopolysaccharide (LPS) stimulated macrophage cells (Ahn et al. 2012). Lysozyme and ovotransferrin from egg revealed anti-inflammatory activity by diminishing the expression of pro-inflammatory cytokines and inhibiting the proliferation of mouse spleen lymphocytes (Young and Mine 2010). The peptides IRW and IQW derived from ovotransferrin showed a reduction of the production of the inflammatory marker MCP-1, VACM-1 and ICAM-1 in TNF-α stimulated human umbilical vein endothelial cells (HUVEC) (Huang et al. 2010; Majumder et al. 2013).

Moreover, there exist some investigations about anti-inflammatory proteins and peptides from milk. Lactoferrin is reported to inhibit the production of pro-inflammatory cytokines *in vitro* and *in viv*o. This glycoprotein was described to inhibit the secretion of LPS induced synthesis of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 in human monocytic cells (Håversen et al. 2002). In addition, lactoferrin induced the production of anti-inflammatory cytokines IL-4 and IL-10 (Young and Mine 2010). κ-Casein is reported to inhibit the production of TNF-α, IL-10, IL-12, IL-6, and IL-1β in murine dendritic cells (Young and Mine 2010).

Only a few studies investigated the anti-inflammatory activity of milk-derived peptides **(Table 1.4)**. Glycomacropeptide (GMP), the C-terminal peptide fragment of κ-casein containing 64 amino acids, showed a reduction in pro-inflammatory cytokine production and a decrease in physiological colitis parameters of trinitrobenzene sulfonic acid (TBNS) stimulated colitis in rats (Young and Mine 2010). *In vitro* enzymatically digested whey protein hydrolysates exhibited anti-inflammatory activity in human colonic epithelial Caco-2 cells exposed to $H_2O_2$ by reducing the secretion of the pro-inflammatory cytokine IL-8. Whey proteins treated with hyperbaric pressure for unfolding of the proteins before enzymatic digestion resulted in an even higher reduction of IL-8 production (Piccolomini et al. 2012). Peptides generated from whey proteins by hydrolysis with proteases called cardosins from the plant *Cynara cardunculus* showed anti-inflammatory activity in mice by inhibition of prostaglandin synthesis (Tavares et al. 2013). Administration of β-casein hydrolyzed with a protease from *Lactobacillus delbrueckii* subspecies *lactis* exhibited anti-inflammatory effect in mice treated with the inflammatory agent TBNS by down-regulation of the production of

the inflammatory marker IFN-ɣ and increased secretion of the anti-inflammatory cytokine IL-10 (Espeche Turbay et al. 2012). The regular intake of nonsteroidal anti-inflammatory drugs (NSAID) such as ibuprofen, aspirin and indomethacin are known to cause gastrointestinal inflammation as adverse reaction. NSAID can disrupt the intercellular integrity of intestinal epithelial cells by increasing the gut permeability and inflammation. The bioactivity of casein hydrolysates (caseins were hydrolyzed with pepsin followed by corolase) on NSAID treated intestinal epithelial cells was evaluated. The casein hydrolysate showed anti-inflammatory activity by down-regulation of transcription of the inflammatory markers NFκB and COX-2 (Nielsen et al. 2012).

Even though a few studies investigated the anti-inflammatory activity of milk-derived peptides *in vitro* and *in vivo*, there exists almost no information about the primary structure of anti-inflammatory peptides from milk. Recently, anti-inflammatory activity of β-casein hydrolyzed with a porcine trypsin preparation TPCK (tosyl phenylalanyl chloromethyl ketone)-treated was tested. A group of large, hydrophobic peptides showed anti-inflammatory effects by TNF-α mediated activation of NFκB in $HEK^{nfκb\text{-}RE}$ cells. Furthermore, a casein-derived peptide that exhibited anti-inflammatory activity has been identified for the first time *in vitro* (Malinowski et al. 2014). Further research has to be performed for the generation and identification of anti-inflammatory peptides.

**Table 1.4** Food-derived anti-inflammatory proteins and peptides. (↓ = reduction of expression, secretion or activity, NO = nitric oxid, TNF-α = tumor necrosis factor-α, IL-6 = interleukin-6, IL-1β = interleukin-1β, iNOS = inducible nitric oxide synthase, COX-2 = cyclooxygenase, adlay = Chinese crop, TG = transglutaminase, $PLA_2$ = phospholipase A2, LPS = lipopolysaccharide, DDS = dextran sodium sulphate, PHA = phytohemagglutinin, HUVEC = human umbilical vein endothelial cells, ICAM-1 = intercellular adhesion molecule-1, MCP1 = monocyte chemoattractant protein-1, TBNS = trinitrobenzene sulfonic acid, ROS = reactive oxygen species, TGF-β1 = transforming growth factor-β1; NSAID = nonsteroidal anti-inflammatory drug, PG $E_2$ = prostaglandin $E_2$

| Sources | Proteins/ peptides | Test Conditions | Anti-inflammatory properties | References |
|---|---|---|---|---|
| **Chemical company** | VPP | Inhibition of THP-1 cell adhesion to HUVECs<br>ACE-inhibitory | | (Aihara et al. 2009) |
| **Fish** | Salmon by-product protein hydrolysates | LPS stimulated macrophage cells | ↓NO<br>↓TNF-α<br>↓IL-6<br>↓IL-1β | (Ahn et al. 2012) |
| **Plant** | Lupine protein hydrolysates | Enzyme activity assays *in vitro* | ↓thrombin<br>↓TG<br>↓$PLA_2$<br>↓COX-2 | (Millán-Linares et al. 2014) |
| | Hydrolyzed soybean flours | LPS stimulated macrophage cells | ↓NO<br>↓iNOS<br>↓PG $E_2$<br>↓COX-2<br>↓TNF-α | (Vernaza et al. 2012) |
| | Soy-derived Kunitz trypsin inhibitor | LPS stimulated fibroplasts,<br>LPS stimulated lethality in mice | ↓TNF-α<br>↓IL-6<br>↓IL-1β | (Young and Mine 2010) |

*Continued.*

| Sources | Proteins/ peptides | Test Conditions | Anti-inflammatory properties | References |
|---|---|---|---|---|
| **Plant** | Soy-derived Bowmann-Birk trypsin inhibitor | LPS stimulated macrophage cells,<br>DDS stimulated colitis in mice | ↓NO<br>↓prostaglandin $E_2$ | (Young and Mine 2010) |
| **Egg** | Lysozyme | DSS stimulated colitis in pigs | ↓TNF-α<br>↓IL-6<br>↓IFN-ɣ<br>↓IL-8<br>↓IL-17<br>↑TGF-β<br>↑Foxp3 | (Young and Mine 2010) |
| | Ovotransferrin | LPS and PHA stimulated mouse spleen lymphocytes | Inhibits lymphocyte proliferation | (Young and Mine 2010) |
| | Peptide: IRW | TNF-α stimulated HUVECs *in vitro* | ↓ICAM-1,<br>↓VACM-1,<br>↓MCP-1;<br>anti-oxidative and<br>ACE-inhibitory | (Huang et al. 2010) |
| | Peptide: IQW | TNF-α stimulated HUVECs *in vitro* | ↓ICAM-1;<br>anti-oxidative | (Majumder et al. 2013) |
| **Milk** | Lactoferrin | LPS stimulated THP-1 cells *in vitro* | ↓IL-6, ↓TNF-α, ↓IL-1β, ↓IL-8, ↓IL-10, ↓NFκB;<br>anti-microbial | (Håversen et al. 2002) |
| | α-Lactalbumin | Carrageenan induced paw exudates | ↓IL-6,<br>↓PG $E_2$,<br>↓COX, ↓phosphollpase $A_2$ | (Yamaguchi et al. 2009) |

*Continued.*

| Sources | Proteins/ peptides | Test Conditions | Anti-inflammatory properties | References |
|---|---|---|---|---|
| **Milk** | κ-Casein | LPS stimulated murine dendritic cells | ↓TNF-α<br>↓IL-6<br>↓IL-10<br>↓IL-12<br>↓IL-1ß | (Young and Mine 2010) |
| | Glycomacropeptide (GMP) | TBNS stimulated colitis in rats | ↓IL-1,<br>↓iNOS<br>↓IL-17<br>↓IL-1β | (Young and Mine 2010) |
| | Whey protein hydrolyzed with cardosins from *Cynara cardunculus* | Subcutaneous injection of carrageenan in the paw of mice resulted in odema | Inhibition of paw odema in mice by oral administration of whey protein hydrolysate | (Tavares et al. 2013) |
| | β-Casein hydrolysate generated by proteinase of *Lactobacillus delbrueckii ssp lactis* CRL 581. | TBNS induced colitis in mice | Decreased mortality rates, less microbial translocation to the liver, decreased β-glucuronidase and myeloperoxidase activites in the gut, decreased colonic damage; ↑IL-10, ↓IFN-ɣ | (Espeche Turbay et al. 2012) |
| | Pressurized and not pressurized whey protein isolate hydrolysates generated by enzymatic digestion *in vitro* | Caco-2 cells exposed to $H_2O_2$ | Inhibition of oxidative stress (ROS), ↓IL-8 | (Piccolomini et al. 2012) |
| | Casein hydrolysates generated by proteolysis with pepsin followed by hydrolysis with corolase | Rat intestinal epithelial cells tested with NSAID indomethacin; LPS induced macropahges | ↓TGF-β1, ↓NFκB, ↓COX-2 | (Nielsen et al. 2012) |
| | Pressurized and not pressurized whey protein was hydrolyzed by enzymatic digestion *in vitro* | Cystic fibrosis cell lines and non-cystic fibrosis respiratory cell lines were stimulated with LPS *in vitro* | ↓IL-8 (IL-8 production was higher diminished by pressurized whey protein hydrolysate) | (Iskandar et al. 2013) |

*I.1.3.2 β-Casein as a precursor of bioactive peptides*

Bovine milk contains 3.2 % proteins (Mills et al. 2011), which can be divided in caseins and whey proteins. Total milk protein consists of 80 % caseins ($\alpha_{S1}$-casein 34 %, $\alpha_{S2}$-casein 8 %, β-casein 25 %, κ-casein 9 %, γ-casein 4 %) and 20 % whey proteins (α-lactalbumin 4 %, β-lactoblunilin 9 %, serumalbumin 1 %, immunoglobulin 2 %, proteose-pepton 4 %) (Belitz 2009). β-Casein is the most hydrophobic of all caseins and contains a large amount of proline residues. 7 genetic variants of β-casein are known (A $^1$, A $^2$, A $^3$, B, C, D, E). The primary structure of β-casein $A^2$-5P is composed of 209 amino acids **(Fig. 1.8)** and has a molecular weight of 23 983 g/mol. 5 phosphoserine residues (5P) are located in the N-terminal domain within the position 1-35 forming the anionic section of β-casein. The other part (36-209) of the protein is composed mainly of hydrophobic amino acids residues building a hydrophobic C-terminal domain (Swaisgood 2003; Walstra et al. 1999; Belitz 2009). In this work, the one-letter notation for amino acid sequences according to IUPAC was used (see 5. Amino acid code) (IUPAC Commission, 1969).

β-Casein is described to be a precursor for the release of various biologically active peptides (Boutrou et al. 2013). **Table 1.5** summarizes the bioactive peptides released from β-casein by microorganisms and microbial enzymes. The location of the biologically active peptides in the primary structure of β-casein is shown in **Fig. 1.8**. One of the major risk factors for the cardiovascular system is hypertension (Hajirostamloo 2010). The angiotensin I-converting enzyme (ACE) is involved in the regulation of blood pressure by synthesizing the vasoconstrictor angiotensin-II. Antihypertensive peptides inhibit ACE as competitive inhibitors resulting in an antihypertensive effect (Mullally et al. 1997; Meisel 1997a) (Hajirostamloo 2010; Mills et al. 2011; Korhonen and Pihlanto 2006). β-Casein encodes various ACE inhibitory peptides **(Table 1.5)**. Masuda, Nakamura and Takano detected in 1996 the best known ACE inhibitory tripeptides VPP and IPP derived from β-casein in a Japanese sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae.* This product is marketed in Japan as a functional food named Calpis® (Korhonen and Pihlanto 2006; Hajirostamloo 2010; Mills et al. 2011) **(Table 1.5)**. Moreover, peptides with opioid activity were identified at position 60 to 70 in the primary structure of β-casein **(Fig. 1.8)**. Opioid peptides exhibit properties similar to opium or morphine by acting as receptor ligands interacting with opioid receptors, which are located in the nervous and in the immune

system and in the gastrointestinal tract. The major exogenous opioid peptides are fragments of β-casein called β-casomorphins such as β-Casomorphin-5 and β-Casomorphin-7 **(Table 1.5)** (Meisel 1998; Mills et al. 2011; Korhonen and Pihlanto 2006; Hajirostamloo 2010). The tryptic caseinophosphopeptide β-CN (f 1-25), which forms the anionic domain containing 5 phosphoseryl residues, can form soluble organophosphate salts and may function as a carrier for different minerals, especially calcium. Moreover, antioxidative peptides are encrypted in β-casein displaying free radical scavenging activity (Korhonen and Pihlanto 2006).

β-Casein was chosen as a substrate for the generation of anti-inflammatory peptides because this protein was described to be a precursor for the release of various biologically active peptides.

**Table 1.5** Bioactive peptides derived from β-casein.

| Peptide | Fragment of β-CN | Biological activity | Enzyme/ microorganism | Reference |
|---|---|---|---|---|
| VPP | β-CN(f 84-86) | ACE inhibitory | *Lactobacillus helveticus*<br>*Saccharomyces cerevisiae* | (Korhonen and Pihlanto 2006; Hajirostamloo 2010; Nakamura et al. 1995) |
| IPP | β-CN(f 74-76) | ACE inhibitory | *Lactobacillus helveticus*<br>*Saccharomyces cerevisiae* | (Hajirostamloo 2010; Korhonen and Pihlanto 2006; Nakamura et al. 1995) |
| SLVLPVPE | β-CN(f 57-64) | ACE inhibitory | *Lactobacillus helveticus* | (Fitzgerald and Meisel 2003) |
| YPFPGPI | β-CN(f 60-66) | ACE inhibitory | Pepsin | (Meisel 1998) |
| EMPFPK | β-CN(f 108-113) | ACE inhibitory | Tryptic activity of lactic acid bacteria | (Pihlanto-Leppälä et al. 1998) |
| KVLPVP | β-CN(f 169-174) | ACE inhibitory | *Lactobacillus helveticus* proteinase | (Maeno et al. 1996) |
| KVLPVPQ | β-CN(f 169-175) | ACE inhibitory | *Lactobacillus helveticus* proteinase | (Maeno et al. 1996) |
| AVP | β-CN(f 177-179) | ACE inhibitory | Synthesis | (Maruyama et al. 1987) |
| AVPYP | β-CN(f 177-181) | ACE inhibitory | Synthesis | (Maruyama et al. 1987) |
| AVPYPQR | β-CN(f 177-183)<br>β-Casokinin-7 | ACE inhibitory<br>antioxidative | Trypsin | (Maruyama et al. 1987; Rival et al. 2000) |
| PYP | β-CN(f 179-181) | ACE inhibitory | Synthesis | (Maruyama et al. 1987) |
| PQR | β-CN(f 181-183) | ACE inhibitory | Synthesis | (Maruyama et al. 1987) |
| YQQPVL | β-CN(f 193-198) | ACE inhibitory | Tryptic activity of lactic acid bacteria | (Pihlanto-Leppälä et al. 1998) |
| YQQPVLGPVR | β-CN(f 193-202)<br>β-Casokinin-10 | ACE inhibitory | Proteinase | (Meisel 1997a; Chiba et al. 1989) |

*Continued.*

| Peptide | Fragment of β-CN | Biological activity | Enzyme/ microorganism | Reference |
|---|---|---|---|---|
| FP | β-CN(f 62-63), β-CN(f 111-112) β-CN(f 157-158) β-CN(f 205-206) | ACE inhibitory | Proteinase K | (Saito 2008) |
| VYP | β-CN(f 59-61) | ACE inhibitory | Proteinase K | (Saito 2008) |
| VYPFPG | β-CN(f 59-64) | ACE inhibitory | Proteinase K | (Saito 2008) |
| RDMPIQAF | β-CN(f 183-190) | ACE inhibitory | Proteinase of *L. helveticus* | (Saito 2008) |
| TPVVVPPFLQP | β-CN(f 80-90) | ACE inhibitory | Proteinase K | (Saito 2008) |
| GPFPIIV | β-CN(f 203-209) | ACE inhibitory | | (Hayes et al. 2007) |
| YPFPGPIPNSL | β-CN(f 60-70) β-Casomorphin-11 | Opioid | - | (Fitzgerald and Meisel 2003) |
| YPFPGPI | β-CN(f 60-66) β-Casomorphin-7 | Opioid | Proteinase | (Fitzgerald and Meisel 2003) |
| YPFPGP | β-CN(f 60-65) β-Casomorphin-6 | Opioid | - | (Fitzgerald and Meisel 2003) |
| YPFPG | β-CN(f 60-64) β-Casomorphin-5 | Opioid | - | (Fitzgerald and Meisel 2003) |
| YPFP | β-CN(f 60-63) β-Casomorphin-4 | Opioid | *Lactobacillus GG* | (Fitzgerald and Meisel 2003; Chang et al. 1985) |
| RELEELNVPGEIVES$^{P}$LS$^{P}$S$^{P}$S$^{P}$EESITR | β-CN(f 1-25) Caseinophospho-peptide | Mineral-binding | Trypsin | (Meisel 1997b) |
| VKEAMAPK | β-CN(f 98-105) | Antioxidative | *Lactobacillus rhamnosus*+ digestion with pepsin and Colorase PP | (Hernández et al. 2009; Korhonen and Pihlanto 2007) |
| VLPVPQK | β-CN(f 170-176) | Antioxidative | Trypsin | (Rival et al. 2000) |
| KVLPVPQK | β-CN(f 169-176) | Antioxidative | Trypsin | (Rival et al. 2000) |

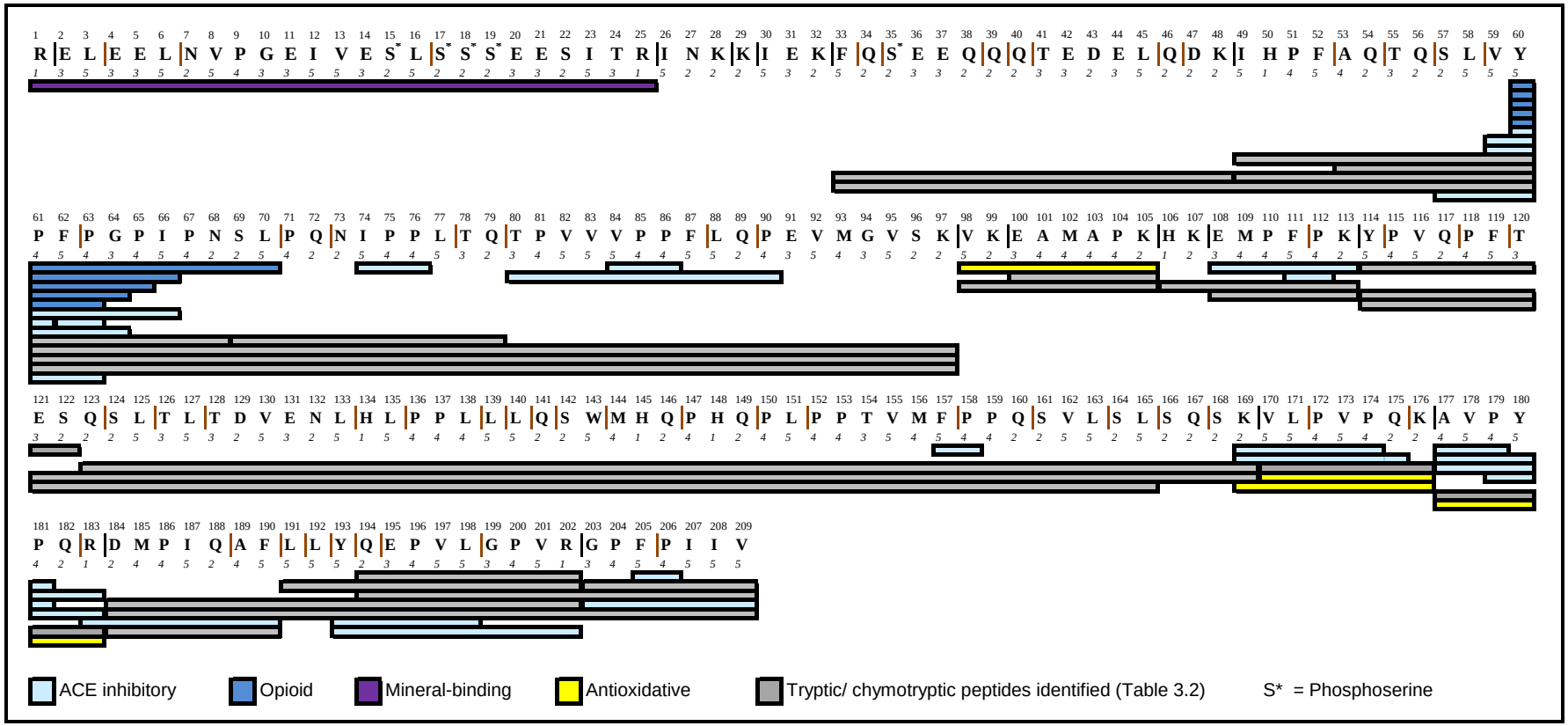



**Fig. 1.8** Primary structure of bovine β-casein $A^2$-5P (*Bos taurus*) with bioactive peptides encrypted within the sequence. Light blue = ACE inhibitory peptides, dark blue = opioid peptides, purple = mineral binding peptides, yellow = antioxidative peptides, grey = peptides generated by tryptic/ chymotryptic enzyme preparations (*chapter III*, **Table 3.2**), black line = tryptic cleavage site (lysine K, arginine R), brown line = chymotryptic cleavage site (tyrosine Y, tryptophan W, phenylalanine F, leucine L, glutamine Q, arginine R), italic numbers = degree of hydrophobicity according to Black and Mould et al. (1991).

*I.1.3.3 Isolation of β-casein and generation of bioactive peptides*

For the generation of anti-inflammatory peptides, bovine β-casein was applied as a substrate. Initially, β-casein was isolated from rennet casein according to a method by Le Magnen and Maugas with minor modifications *(Manuscript 3, chapter IV.3.1)*. Rennet casein is a product obtained after hydrolysis of the caseins with rennet containing $\alpha_{S1}$-, $\alpha_{S2}$- , β-casein and para-κ-casein, which is released from κ-casein during proteolysis (Le Magnen and Maugas 1995). The method for the isolation of β-casein from rennet casein is based on selective separation of β-casein from the other caseins by precipitation depending on pH and temperature. The procedure is composed of the main steps dissolving of rennet casein, precipitation and separation of $\alpha_S$- and para-κ-caseins and the precipitation and separation of β-casein **(Fig. 1.9)**.

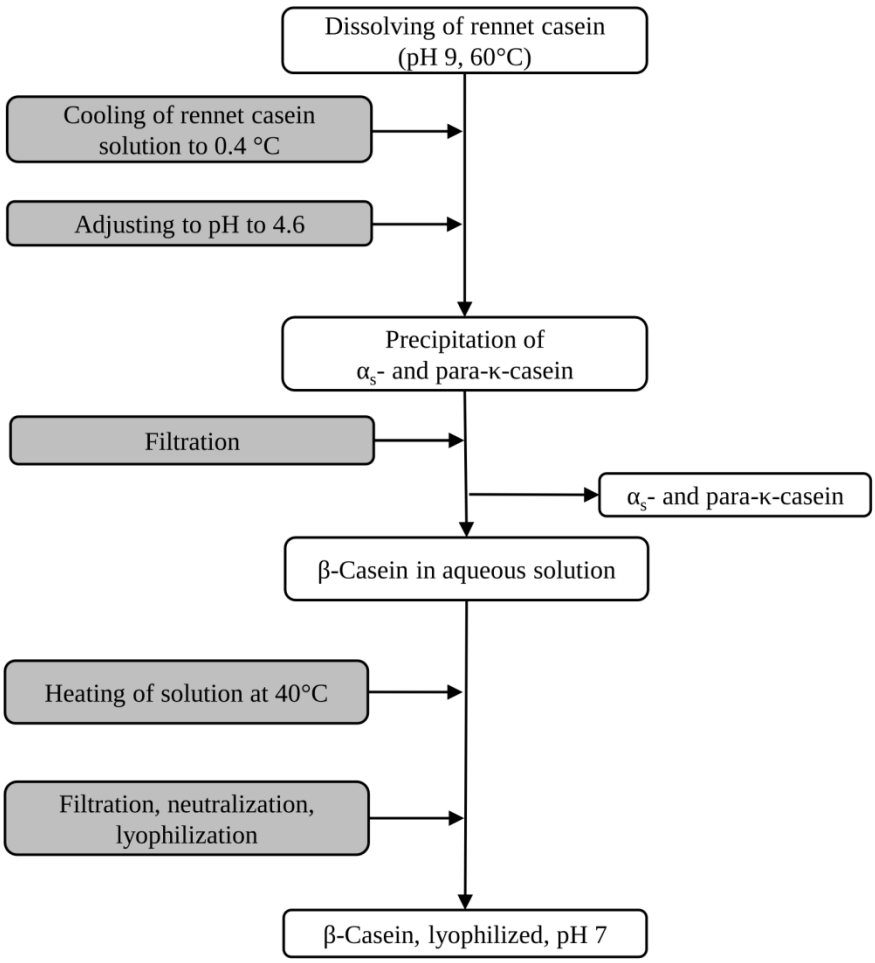



**Fig. 1.9:** Isolation of β-casein from rennet casein.

During cooling of the 5 % rennet casein solution and adjusting to pH 4.6, $\alpha_S$- and para-κ-caseins precipitated, while β-casein remained dissolved. After separation of the $\alpha_S$- and para-κ-caseins, the β-casein precipitated by warming the aqueous phase to 40°C. The precipitated β-casein fraction was separated from the aqueous solution, dissolved in demineralized water at pH 7 and lyophilized.

For the generation of anti-inflammatory peptides, proteolysis of a 5 % β-casein solution was performed at pH-stat conditions of 7.8 for 3 to 4 h at 37 °C *(Manuscript 3, chapter IV)* by application of different tryptic/chymotryptic enzyme preparations from cod and hog **(Table 4.1) (Fig. 1.10)**. The enzymatic reaction was stopped by heating the hydrolysate at 90°C for 10 min. The hydrolysates were fractionated by UF with a 5 kDa membrane according to the molecular size. The retentate 5 kDa was mainly composed of peptides > 5 kDa and the permeate contained peptides < 5 kDa.

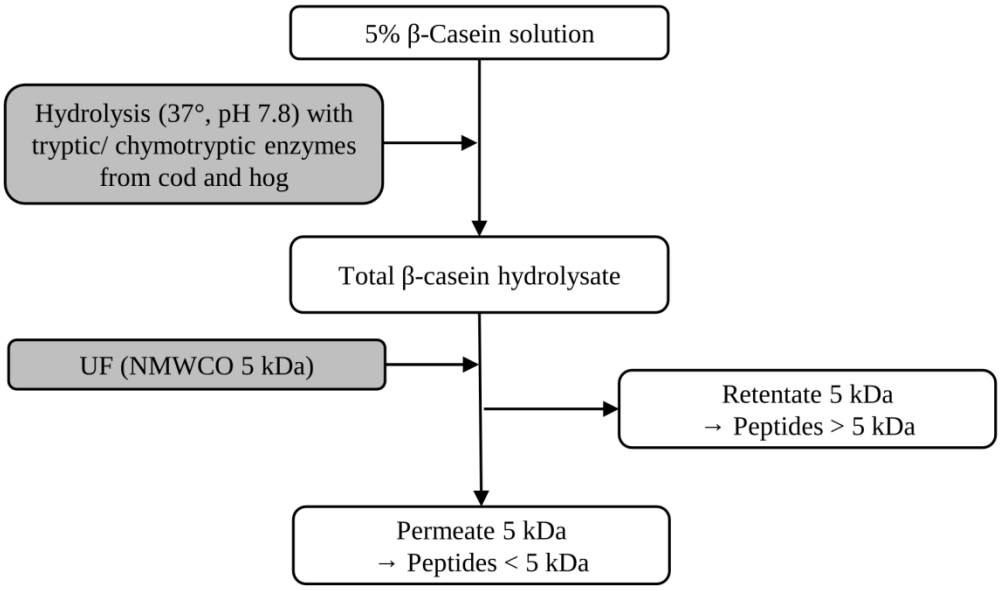



**Fig. 1.10:** Hydrolysis of β-casein with tryptic/ chymotryptic enzyme preparations.

Proteases derived from animals, plants and microorganisms have extensive industrial applications in food processing (Klomklao et al. 2010; Ktari et al. 2012). As part of the viscera, the pyloric caeca is a by-product of the fish manufacturing industry and rich in

digestive enzymes. Proteolytic enzymes isolated from viscera of fish are adapted to cold environment like the serine protease trypsin and chymotrypsin. These fish enzymes display high stability and activity under harsh conditions, such as high pH and low temperature (Arvizu-Flores et al. 2012; Freitas-Júnior et al. 2012; Ktari et al. 2012). The serine endoprotease trypsin (EC 3.4.21.4) from Atlantic cod and hog hydrolyzes peptide bonds at the carboxylic end of the amino acid residue arginine (R) and lysine (K) (Raae et al. 1995; Bunkenborg et al. 2013; Gudmundsdottir et al. 2013). Cod trypsin has shown higher extent of degradation of the proteins lactoferrin, lysozyme and bovine serum albumin at temperatures of 4, 25 and 37°C compared to bovine trypsin (Gudmundsdottir et al., 2013). Chymotrypsin (EC 3.4.21.1) from Atlantic cod and hog cleaves peptide chains at the carboxyl side of the amino acids phenylalanine (F), leucine (L) and tyrosine (Y) (Raae et al. 1995; Belitz 2009). In addition, chymotrypsin derived from hog cleaves peptides at the carboxylic end of glutamine (Q) and chymotrypsin derived from cod liberates peptides at the carboxylic end of arginine (R) (Belitz 2009; Raae et al. 1995). The theoretical cleavage sites of the tryptic and chymotryptic enzyme preparations form cod and hog in the primary structure of β-casein $A^2$-5P are shown in **Fig. 1.8**. Even though fish proteases have already been applied in food processing such as in dairy industry for substitution of rennet in cheese manufacturing, for the removal of oxidized flavour from milk or for shortening of cheese ripening time (Rossano et al. 2011), their use is still limited. The non-food grade TPCK-treated porcine trypsin preparation may be replaced by food-grade enzyme preparations, e.g. from cod, for the generation of anti-inflammatory peptides, which may add value to by-products from fish processing industry.

Enzymatic hydrolysates of β-casein are often described to have a bitter taste. Even very limited hydrolysis produces bitter peptides. The bitter off-flavor is a known problem in cheese ripening. Ney et al. (1971) postulated a correlation between the hydrophobicity of the peptides and their bitter taste. The tendency of a protein to form bitter peptides can be predicted from its primary structure. The hydrophobic amino acid side chains are responsible for the bitter taste. In the intact globular protein, the majority of the hydrophobic amino acid side chains are concealed in the interior. By degradation of the protein, the hydrophobic side chains are exposed to the solvent and can interact with the taste buds. The higher the degree of hydrolysis, the stronger the bitter taste. A very high degree of hydrolysis up to free amino acids results in a decrease in bitterness (Walstra et al. 1999). The tryptic and chymotryptic β-

casein hydrolysates produced in our investigation *(Manuscript 3, chapter IV)* exhibited strong bitter taste. The peptides identified in the total β-casein hydrolysate, in the retentate > 5 kDa and the permeate < 5 kDa via LC-MS are listed in **Table 4.2** and their location in the primary structure of the parent protein are shown as grey bars in **Fig. 1.8**. Black and Mould (1991) determined hydrophobicity parameters for the side chains of amino acids. According to these parameters the degree of hydrophobicity was classified from 1 (lowest hydrophobicity) to 5 (highest hydrophobicity) **(Table 1.6)**. In **Fig. 1.8**, the degree of hydrophobicity of the amino acid residues is shown as italic number below the primary structure of β-casein.

**Table 1.6** Classification of amino acids according to their degree of hydrophobicity (Black and Mould 1991). 1= lowest hydrophobicity, 5= highest hydrophobicity

| **Amino acid** | **Hydrophobicity parameter according to Black and Mould (1991)** | **Degree of hydrophobicity** |
|---|---|---|
| His (H), Arg (R) | 0.0-0.2 | 1 |
| Asp (D), Asn (N), Gln (Q), Ser (S), Lys (K) | 0.2-0.4 | 2 |
| Glu (E), Gly (G), Thr (T) | 0.4-0.6 | 3 |
| Ala (A), Cys (C), Met (M), Pro (P) | 0.6-0.8 | 4 |
| Phe (F), Ile (I), Leu (L), Val (V), Trp (W), Tyr (Y) | 0.8-1.0 | 5 |

### *I.1.4 Determination of NFκB activation in HEK$^{nfκb\text{-}RE}$ cells*

Inflammation is generated by the body as a response to infection, irritation and injury. Generally, it is involved in the development of various chronic diseases such as diabetes, cancer, asthma and obesity (Huang et al. 2014; Millán-Linares et al. 2014). Inflammatory process is triggered by the synthesis of pro-inflammatory cytokines such as TNF-α mainly in macrophages but also in mast cells, lymphoid cells, endothelial cells and fibroplasts (Li 2007; Li et al. 2010; Malinowski et al. 2014; Rahman and McFadden 2006; Xu and Chen 2011). Moreover, inflammation resulting in a limited extent of cytokine expression can result in immunostimulation by activating the innate immune responses (Pasparakis 2009; Kurakevich et al. 2013).

Nuclear factor κ B (NFκB) is a homo- and heterodimeric transcription factor, which plays a key role in inflammatory process and is responsible for expression and regulation of genes

involved in inflammation as well as immunity and apoptosis (Xu and Chen 2011). This transcription factor is a central regulator of cellular responses and exhibits a dual function. On the one hand, NFκB promotes the expression of pro-inflammatory genes that are important for the activation of immune responses such as cytokines and chemokines. On the other hand, NFκB protects cells from apoptosis by inducing the expression of anti-apoptotic proteins (BCL-$X_L$= B-cell lymphoma-extra large) and from necrosis by inhibiting the accumulation of reactive oxygen species (ROS) through transcriptional up-regulation of proteins with antioxidant functions (e.g MnSOD = manganese superoxide dismutase) (Pasparakis 2009; Karin and Lin 2002). NFκB has a dual function by helping cells to survive and at the same time inducing an immunostimulatory response triggered by pro-inflammatory cytokines to protect the organism from infection and injury (Pasparakis 2009).

**Fig. 1.11** shows the predominant canonical signaling pathway of NFκB. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) plays an important role in the regulation of cell proliferation, differentiation and apoptosis (Li 2007; Zelová and Hošek 2013). Binding of TNF-α to cell surface receptors such as tumor necrosis factor receptor (TNFR) initiate the NFκB signaling pathway (Kamada et al. 2002; Zelová and Hošek 2013). TNF-α stimulation most often triggers the canonical signaling pathway (Zelová and Hošek 2013) by activation of IKK complex (IκB-kinase complex). The transcription factor NFκB is kept inactive, because the RHD (rel-homology domain), which contain sequences responsible for DNA binding and nuclear translocation, is blocked by binding of IkB (inhibitor of κ B). The activated IKK complex phosphorylates N-terminal serine residues on IκB. IκB gets degraded by proteasomes and releases NFκB. The liberated transcription factor NFκB translocates into the nucleus to induce expression of genes responsible for immune regulation, which could trigger inflammatory as well as immunostimulatory processes (Li 2007; Rahman and McFadden 2006; Xu and Chen 2011; Zelová and Hošek 2013).

The reduction of the NFκB activity leads to a reduced expression of pro-inflammatory genes. Therefore, a decreased NFκB activity is often related with anti-inflammatory activity (Pasparakis 2009). Substances which down-regulate the activity of NFκB can be described as anti-inflammatory. In our investigation, the anti-inflammatory effect of the tryptic and chymotryptic β-casein hydrolysates was evaluated by the reduction of the NFκB activity in $HEK^{nfkb\text{-}RE}$ cells *in vitro*.

A high NFκB activity is often related with inflammatory processes. Activation of NFκB in a limited extent could also result in immunostimulation (Pasparakis 2009). Components such as MOS could act as a signal to prime innate immune system. By stimulation of pro-inflammatory cytokine production, the oligosaccharides might educate the immune system and prepare the organism for an encounter with pathogenic microorganisms (Kurakevich et al. 2013). In our study, the immunostimulatory activity of the MOS concentrates produced by NF was determined by the activation of NFκB activity in HEK$^{nfκb\text{-}RE}$ cells *in vitro*.

HEK cells were transfected with an NFκB response element. The reporter gene was luciferase (Malinowski et al. 2014). After cultivation, the HEK$^{nfκb\text{-}RE}$ cells were incubated with the pro-inflammatory cytokine TNF-α and the sample (β-casein hydrolysates or MOS enriched NF-retentates) for 24 h. In a subsequent luciferase assay, the NFκB activity was measured as luciferase activity. The extent of NFκB activation correlated with the luciferase activity. The method is described in *chapter II (Manuscript I)* and *chapter III (Manuscript 2)*.

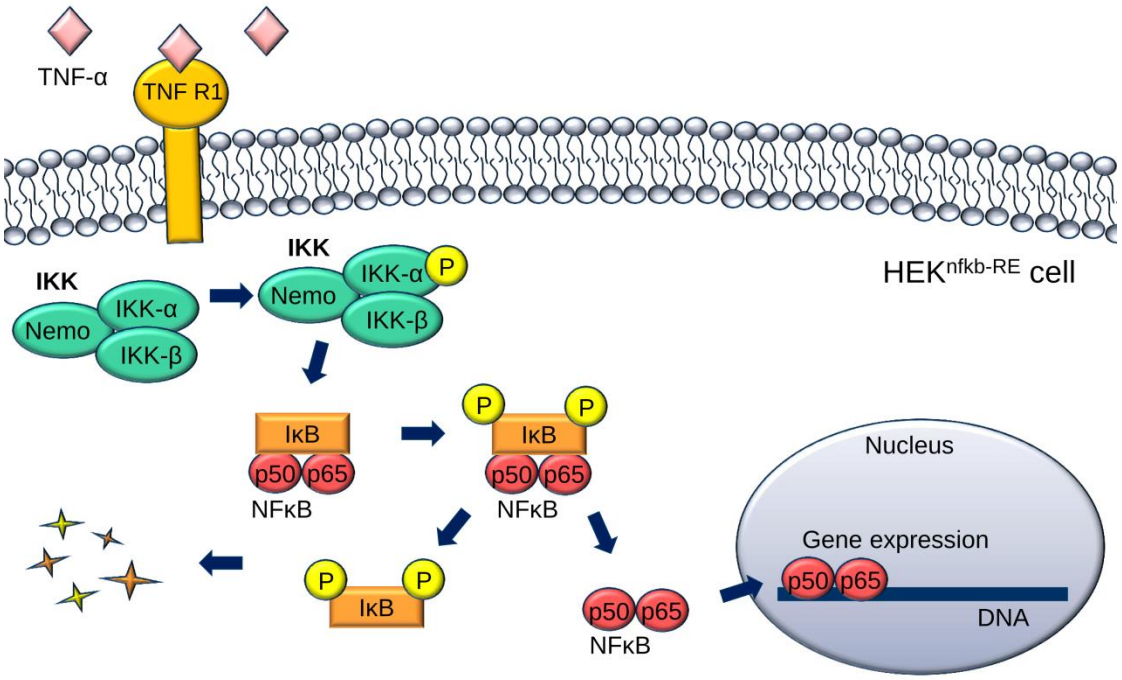



**Fig. 1.11:** Canonical NFκB signaling pathway. (NFκB = Nuclear factor κ B, IKK = IκB-kinase, IκB = inhibitor of κ B, TNF R1 = tumor necrosis factor receptor 1)

## I.2 Motivation

### *I.2.1 Development of procedures for the enrichment of milk oligosaccharides (MOS) by nanofiltration (NF)*

The application of concentrates enriched in MOS as functional food ingredient is of high value due to the various biological activities of these natural abundant oligosaccharides in milk *(chapter I.1.2.2)*. A few investigations for the enrichment of MOS by membrane filtration technology were performed on laboratory and pilot plant scale (Sarney et al. 2000; Martinez-Ferez et al. 2006a; Oliveira et al. 2014) and only one study was conducted on industrial scale (Mehra et al. 2014) for the enrichment of MOS by membrane separation technology *(chapter I.1.2.3)*. Due to the low concentration of MOS in milk of domestic animals (e.g. bovine and caprine milk), low productivity and operational complexity, no efficient process for the enrichment of MOS has been implemented at industrial scale so far. For the development of an efficient method for MOS enrichment, a readily available raw material, which can be provided in high amounts should be applied. Especially for the performance on industrial scale, the developed NF process should be feasible with short processing times and low fouling of the membranes. Until now, there exist no study about a screening of different nanofiltration membranes for an enrichment of MOS and a subsequent feasibility study for transferring this membrane process into large scale. It depends on the priorities whether a high recovery of MOS and/ or a high purity of the concentrate wants to be achieved. For obtaining a higher purity of the MOS in NF retentate, the number of diafiltration steps can be increased and conditions for facilitating the permeation of milk salts have to be chosen. The pH has a strong impact on the dissociation degree of salts. By acidification to pH 5 the dissociation of milk salts is increased improving the permeation of the salts due to its lower molecular size (Töpel 2004; Fox and McSweeney 2009). The influence of the pH on the degree of enrichment of MOS during NF has not been evaluated yet.

For a successful establishment of a membrane filtration method for the enrichment of MOS, the first step should be a screening of different membrane modules on laboratory scale using a developed NF procedure *(Manuscript 1, chapter II, V.1)*. In consideration of the results, the NF process should be transferred into pilot plant and industrial scale by applying the same or

similar suitable membranes (NMWCO, membrane material and properties) from laboratory scale trials *(Manuscript 1, chapter II, V.1)*. For further optimization of the NF process, e.g. by achieving a better permeation of salts and residual sugars, the influence of the pH during NF at acidic (pH 5) or neutral (pH 7) milieu should be determined *(Manuscript 2, chapter III, V.1)*. Moreover, the application of the developed and optimized process on caprine milk could be valuable to benefit of the different MOS profile which is described to be closer to human milk *(chapter Manuscript 2, III, V.1)*.

### *I.2.2 Development of procedures for the generation of anti-inflammatory peptides*

Inflammation is a contributing factor for the development of various chronic diseases e.g. diabetes, cancer, asthma and obesity (Millán-Linares et al. 2014). Some studies exhibited that food-derived peptides from fish plant and egg can modulate inflammatory response by exhibiting anti-inflammatory activity *(chapter I.1.3.1)*. Only a few studies investigated the anti-inflammatory activity of milk-derived peptides. In a recent study from Malinowski et al. (2014) a β-casein-derived peptide (β-CN A (f 184-202)), produced by application of a non-food grade porcine TPCK (tosyl phenylalanyl chloromethyl ketone)-treated tryptic enzyme preparation, was identified as anti-inflammatory by reducing the NFκB activity in $HEK^{nfκb\text{-}RE}$ cells *in vitro*. With exception of this investigation, there exists no further information about the primary structure of anti-inflammatory peptides from milk proteins. Extensive research has to be performed for the development of a procedure for generation of anti-inflammatory peptides and the identification of their primary structure. The non-food grade TPCK-treated porcine trypsin preparation may be replaced by food-grade tryptic enzyme preparations from cod, as by-product of the fish processing industry, for the generation of anti-inflammatory peptides.

Since β-casein encodes various bioactivities *(chapter I.1.3.2)* **(Table 1.5)**, this protein could be a valuable substrate for the production of anti-inflammatory peptides (Boutrou et al. 2013). The first step should be the isolation of bovine β-casein from rennet casein in sufficient amounts and purity. The generation and identification of anti-inflammatory peptides from β-casein with different tryptic and chymotryptic enzyme preparations from cod and hog should be performed *(Manuscript 3, chapter IV, V.2)*. A fractionation of the β-casein hydrolysate by

molecular size could support the identification of the bioactive peptides. Moreover, the non-food grade TPCK-treated porcine trypsin preparation should be replaced by food-grade enzyme preparations, e.g. from cod as by-products of the fish processing industry.

### *I.2.3 Characterization of the biological activity of enriched MOS concentrates and generated β-casein hydrolysates*

The transcription factor NFκB is a regulator of cell response with dual function. On the one hand, NFκB induces the expression of pro-inflammatory genes which activate the immune response. On the other hand, NFκB protects cells from apoptosis and necrosis *(chapter I.1.4)* (Pasparakis 2009; Karin and Lin 2002).

The reduction of the NFκB activity results in diminished expression of pro-inflammatory genes. Thus, substances which reduce the NFκB activity are described to exhibit anti-inflammatory activity (Pasparakis 2009). The anti-inflammatory activity of the whole β-casein hydrolysates and peptide fractions should be evaluated in human embryonic kidney cells ($HEK^{nfκb\text{-}RE}$ cells) *in vitro* by measuring the activity of the immune regulating transcription factor NFκB *(chapter I.1.4, Manuscript 3, chapter IV, V.3).*

High NFκB activity is often associated with inflammatory processes. However, the activation of NFκB in a limited extent can result in immunostimulation by activating the immune response (Pasparakis 2009). By stimulation of the pro-inflammatory cytokine production, MOS could prime and educate the immune system for an encounter with pathogens (Kurakevich et al. 2013). The immunostimulatory activity of the NF retentates enriched in MOS content should be evaluated by the activation of NFκB in $HEK^{nfκb\text{-}RE}$ cells *in vitro (chapter I.1.4, Manuscript 1, chapter II, V.3).*

## I.3 References

Ahn C-B, Je J-Y & Cho Y-S (2012) Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis. *Food Research International* 49(1), 92-98.

Aihara K, Ishii H & Yoshida M (2009) Casein-derived tripeptide, Val-Pro-Pro (VPP), modulates monocyte adhesion to vascular endothelium. *Journal of Atherosclerosis and Thrombosis* 16(5), 594-603.

Albrecht S, Lane AL, Mariño K, Al Busadah KA, Carrington SD, Hickey RM & Rudd PM (2014) A comparative study of free oligosaccharides in the milk of domestic animals. *British Journal of Nutrition* 111(7), 1313-1328.

Anand S, Khanal SN & Marella C (2013) Whey and whey products. In: Park YW & Haenlein GFW (eds) *Milk and dairy products in human nutrition*. Wiley-Blackwell, Chichester, UK.

Arvizu-Flores AA, Quintero-Reyes IE, Felix-Lopez M, Islas-Osuna MA, Yepiz-Plascencia G, Pacheco-Aguilar R, Navare A, Fernández FM, Velazquez-Contreras EF, Sotelo-Mundo RR & Castillo-Yañez FJ (2012) Thermodynamic activation and structural analysis of trypsin I from Monterey sardine (*Sardinops sagax caerulea*). *Food Chemistry* 133(3), 898-904.

Barile D, Tao N, Lebrilla CB, Coisson J-D, Arlorio M & German JB (2009) Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *International Dairy Journal* 19(9), 524-530.

Belitz H-D, Grosch, W., Schieberle, P. (2009) *Food Chemistry*. Springer Berlin Heidelberg

Black BA, Yan Y, Galle S, Hu Y, Curtis JM & Gänzle MG (2014) Characterization of novel galactosylated chitin-oligosaccharides and chitosan-oligosaccharides. *International Dairy Journal* 39(2), 330-335.

Black SD & Mould DR (1991) Development of hydrophobicity parameters to analyze proteins which bear post- or cotranslational modifications. *Analytical Biochemistry* 193(1), 72-82.

Bode L (2006) Recent advances on structure, metabolism, and function of human milk oligosaccharides. *Journal of Nutrition* 136(8), 2127-2130.

Bode L (2009) Human milk oligosaccharides: prebiotics and beyond. *Nutrition Reviews* 67(2), 183-191.

Bode L (2012) Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* 22(9), 1147-1162.

Bode L & Rudloff S (2002) Human milk oligosaccharides (HMO) inhibit monocyte adhesion to endothelial cells in a concentration dependent manner. *Faseb Journal* 16(5), 983-983.

Boutrou R, Gaudichon C, Dupont D, Jardin J, Airinei G, Marsset-Baglieri A, Benamouzig R, Tome D & Leonil J (2013) Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition* 97(6), 1314-1323.

Bunkenborg J, Espadas G & Molina H (2013) Cutting edge proteomics: benchmarking of six commercial trypsins. *Journal of Proteome Research* 12(8), 3631-3641.

Bury D, Jelen P & Kimura K (1998) Whey protein concentrate as a nutrient supplement for lactic acid bacteria. *International Dairy Journal* 8(2), 149-151.

Capitán-Cañadas F, Ortega-González M, Guadix E, Zarzuelo A, Suárez MD, de Medina FS & Martínez-Augustin O (2014) Prebiotic oligosaccharides directly modulate proinflammatory cytokine production in monocytes via activation of TLR4. *Molecular Nutrition & Food Research* 58(5), 1098-1110.

Chakrabarti S, Jahandideh F & Wu JP (2014) Food-derived bioactive peptides on inflammation and oxidative stress. *Biomed Research International*.

Chang KJ, Su YF, Brent DA & Chang JK (1985) Isolation of a specific mu-opiate receptor peptide, morphiceptin, from an enzymatic digest of milk proteins. *Journal of Biological Chemistry* 260(17), 9706-9712.

Chiba H, Tani F & Yoshikawa M (1989) Opioid antagonist peptides derived from kappa-casein. *Journal of Dairy Research* 56(3), 363-366.

Choi YH, Kim JH, Park JH, Lee N, Kim DH, Jang KS, Park IH & Kim BG (2014) Protein engineering of alpha 2,3/2,6-sialyltransferase to improve the yield and productivity of in vitro sialyllactose synthesis. *Glycobiology* 24(2), 159-169.

Commission I-I (1969) IUPAC-IUB Commission on biochemical nomenclature. A one-letter notation for amino acid sequences. Tentative rules. *Biochemical Journal* 113(1).

Dziuba B & Dziuba M (2014) Milk proteins-derived bioactive peptides in dairy products: molecular, biological and methodological aspects. *Acta Sci Pol, Technol Aliment* 13(1), 5-25.

Ebringer L, Ferenčík M & Krajčovič J (2008) Beneficial health effects of milk and fermented dairy products — Review. *Folia Microbiologica* 53(5), 378-394.

Eiwegger T, Stahl B, Schmitt J, Boehm G, Gerstmayr M, Pichler J, Dehlink E, Loibichler C, Urbanek R & Szepfalusi Z (2004) Human milk-derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. *Pediatric Research* 56(4), 536-540.

Espeche Turbay MB, de Moreno de LeBlanc A, Perdigón G, Savoy de Giori G & Hebert EM (2012) β-Casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. *Journal of Dairy Science* 95(3), 1108-1118.

Fierfort N & Samain E (2008) Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides. *Journal of Biotechnology* 134(3–4), 261-265.

Fitzgerald RJ & Meisel H (2003) Milk protein hydrolysates and bioactive peptides. In: Fox PF & McSweeney PLH (eds) *Advanced Dairy Chemistry—1 Proteins*. 675-698. Springer US.

Fitzgerald RJ, Murray BA & Walsh DJ (2004) Hypotensive peptides from milk proteins. *Journal of Nutrition* 134(4), 980-988.

Fitzpatrick JJ & O'Keeffe U (2001) Influence of whey protein hydrolysate addition to whey permeate batch fermentations for producing lactic acid. *Process Biochemistry* 37(2), 183-186.

Fox PF & McSweeney P (2009) *Advanced Dairy Chemistry- Volume 3: Lactose, water, salts and minor constituents*. 3. edn. Springer, Ireland.

Freitas-Júnior ACV, Costa HMS, Icimoto MY, Hirata IY, Marcondes M, Carvalho LB, Oliveira V & Bezerra RS (2012) Giant Amazonian fish pirarucu *(Arapaima gigas)*: Its viscera as a source of thermostable trypsin. *Food Chemistry* 133(4), 1596-1602.

Frenzel M, Zerge K, Clawin-Rädecker I & Lorenzen PC (2015) Comparison of the galacto-oligosaccharide forming activity of different β-galactosidases. *LWT - Food Science and Technology* 60(2), 1068-1071.

Gänzle M (2012) Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *International Dairy Journal* 22(2), 116-122.

Gänzle M, Haase G & Jelen P (2008) Lactose: Crystallization, hydrolysis and value-added derivatives. *International Dairy Journal* 18(7), 685-694.

García-Tejedor A, Padilla B, Salom JB, Belloch C & Manzanares P (2013) Dairy yeasts produce milk protein-derived antihypertensive hydrolysates. *Food Research International* 53(1), 203-208.

Gibson GR & Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition* 125(6), 1401-1412.

Gnoth MJ, Rudloff S, Kunz C & Kinne RKH (2001) Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. *Journal of Biological Chemistry* 276(37), 34363-34370.

Gopal PK & Gill HS (2000) Oligosaccharides and glycoconjugates in bovine milk and colostrum. *British Journal of Nutrition* 84(supplement 1), 69-74.

Gosling A, Stevens GW, Barber AR, Kentish SE & Gras SL (2010) Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry* 121(2), 307-318.

Goulas A, Tzortzis G & Gibson GR (2007) Development of a process for the production and purification of alpha- and beta-galactooligosaccharides from *Bifobacterium bifidum* NCIMB 41171. *International Dairy Journal* 17(6), 648-656.

Gudmundsdottir A, Hilmarsson H & Stefansson B (2013) Potential use of Atlantic cod trypsin in biomedicine. *Biomed Research International*.

Guo Y, Jers C, Meyer AS, Arnous A, Li H, Kirpekar F & Mikkelsen JD (2014) A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3′-sialyl and 6′-sialyl glycans. *Journal of Biotechnology* 170, 60-67.

Hajirostamloo B (2010) Bioactive Component in Milk and Dairy Product. In World Academy of Science, Engineering and Technology 72.

Han NS, Kim T-J, Park Y-C, Kim J & Seo J-H (2012) Biotechnological production of human milk oligosaccharides. *Biotechnology Advances* 30(6), 1268-1278.

Hartmann R & Meisel H (2002) Cytochemical assessment of phosphopeptides derived from casein as potential ingredients for functional food. *Food / Nahrung* 46(6), 427-431.

Hartmann R & Meisel H (2007) Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology* 18(2), 163-169.

Håversen L, Ohlsson BG, Hahn-Zoric M, Hanson LÅ & Mattsby-Baltzer I (2002) Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB. *Cellular Immunology* 220(2), 83-95.

Hayes M, Ross RP, Fitzgerald GF & Stanton C (2007) Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part I: Overview. *Biotechnology Journal* 2(4), 426-434.

Hernández O, Ruiz-Matute AI, Olano A, Moreno FJ & Sanz ML (2009) Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. *International Dairy Journal* 19(9), 531-536.

Huang DW, Wu CH, Shih CK, Liu CY, Shih PH, Shieh TM, Lin CI, Chiang WC & Hsia SM (2014) Application of the solvent extraction technique to investigation of the anti-inflammatory activity of adlay bran. *Food Chemistry* 145, 445-453.

Huang W, Chakrabarti S, Majumder K, Jiang Y, Davidge ST & Wu J (2010) Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *Journal of Agricultural and Food Cemistry* 58(20), 10840-10846.

Iskandar MM, Dauletbaev N, Kubow S, Mawji N & Lands LC (2013) Whey protein hydrolysates decrease IL-8 secretion in lipopolysaccharide (LPS)-stimulated respiratory epithelial cells by affecting LPS binding to Toll-like receptor 4. *British Journal of Nutrition* 110(1), 58-68.

IUPAC Commission on Biochemical Nomenclature (1969) A one-Letter notation for amino acid sequences. Tentative rules. *Biochemical Journal*, 113 (1).

Kamada T, Nakajima M, Nabetani H, Saglam N & Iwamoto S (2002) Availability of membrane technology for purifying and concentrating oligosaccharides. *European Food Research and Technology* 214(5), 435-440.

Karin M & Lin A (2002) NF-kB at the crossroads of life and death. *Nature Immunology* 3(3), 221-227.

Kitts DD & Weiler K (2003) Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design* 9(16), 1309-1323.

Klomklao S, Kishimura H, Benjakul S, Simpson BK & Visessanguan W (2010) Cationic trypsin: A predominant proteinase in pacific saury *(Cololabis Saira)* pyloric ceca. *Journal of Food Biochemistry* 34(5), 1105-1123.

Korhonen H & Pihlanto A (2006) Bioactive peptides: Production and functionality. *International Dairy Journal* 16(9), 945-960.

Korhonen H & Pihlanto A (2007) Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Current Pharmaceutical Design* 13(8), 829-843.

Korhonen HJ (2009) Bioactive Components in Bovine Milk. In: *Bioactive Components in Milk and Dairy Products*. 13-42. Wiley-Blackwell.

Ktari N, Ben Khaled H, Nasri R, Jellouli K, Ghorbel S & Nasri M (2012) Trypsin from zebra blenny *(Salaria basilisca*) viscera: Purification, characterisation and potential application as a detergent additive. *Food Chemistry* 130(3), 467-474.

Kunz C & Rudloff S (2006) Health promoting aspects of milk oligosaccharides. *International Dairy Journal* 16(11), 1341-1346.

Kunz C & Rudloff S (2008) Potential anti-inflammatory and anti-infectious effects of human milk oligosaccharides. In: Bösze Z (ed) *Bioactive Components of Milk*, vol 606. Advances in Experimental Medicine and Biology. 455-466. Springer New York.

Kunz C, Rudloff S, Baier W, Klein N & Strobel S (2000) Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Annual Review of Nutrition* 20, 699-722.

Kurakevich E, Hennet T, Hausmann M, Rogler G & Borsig L (2013) Milk oligosaccharide sialyl(alpha2,3)lactose activates intestinal CD11c+ cells through TLR4. *Proceedings of the National Academy of Sciences of the United States of America* 110(43), 17444-17449.

Le Magnen C & Maugas J-J (1995) Method for obtaining beta casein. France Patent 5,397,577.

Li H (2007) The role of RIP ubiquitination in the TNF-α signaling pathway. Doctor Thesis, Department of Biochemistry, School of Medicine and Biomedical Sciences, University at Buffalo, Proquest, Ann Arbor.

Li W, Li H, Bocking AD & Challis JR (2010) Tumor necrosis factor stimulates matrix metalloproteinase 9 secretion from cultured human chorionic trophoblast cells through TNF receptor 1 signaling to IKBKB-NFKB and MAPK1/3 pathway. *Biology of Reproduction* 83(3), 481-487.

Li Y, Shahbazi A, Williams K & Wan C (2008) Separate and concentrate lactic acid using combination of nanofiltration and reverse osmosis membranes. *Applied Biochemistry and Biotechnology* 147(1-3), 1-9.

Lorenzen PC, Breiter J, Clawin-Rädecker I & Dau A (2013) A novel bi-enzymatic system for lactose conversion. *International Journal of Food Science & Technology* 48(7), 1396-1403.

Luo J, Nordvang RT, Morthensen ST, Zeuner B, Meyer AS, Mikkelsen JD & Pinelo M (2014) An integrated membrane system for the biocatalytic production of 3′-sialyllactose from dairy by-products. *Bioresource Technology* 166(0), 9-16.

Maeno M, Yamamoto N & Takano T (1996) Identification of an antihypertensive peptide from casein hydrolysate produced by a poteinase from *Lactobacillus helveticus CP790*. *Journal of Dairy Science* 79(8), 1316-1321.

Majumder K, Chakrabarti S, Davidge ST & Wu J (2013) Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of Agricultural and Food Cemistry* 61(9), 2120-2129.

Malinowski J, Klempt M, Clawin-Rädecker I, Lorenzen PC & Meisel H (2014) Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. *Food Chemistry* 165(0), 129-133.

Martinez-Ferez A, Guadix A & Guadix EM (2006a) Recovery of caprine milk oligosaccharides with ceramic membranes. *Journal of Membrane Science* 276(1-2), 23-30.

Martinez-Ferez A, Rudloff S, Guadix A, Henkel CA, Pohlentz G, Boza JJ, Guadix EM & Kunz C (2006b) Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal* 16(2), 173-181.

Maruyama S, Mitachi H, Tanaka H, Tomizuka N & Suzuki H (1987) Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agricultural and Biological Chemistry* 51(6), 1581-1586.

Marwaha SS & Kennedy JF (1988) Whey—pollution problem and potential utilization. *International Journal of Food Science & Technology* 23(4), 323-336.

Mehra R, Barile D, Marotta M, Lebrilla CB, Chu C & German JB (2014) Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. *PLoS One* 9(5).

Mehra R & Kelly P (2006) Milk oligosaccharides: Structural and technological aspects. *International Dairy Journal* 16(11), 1334-1340.

Mehra R, Marnila P & Korhonen H (2006) Milk immunoglobulins for health promotion. *International Dairy Journal* 16(11), 1262-1271.

Meisel H (1997a) Biochemical properties of bioactive peptides derived from milk proteins: Potential nutraceuticals for food and pharmaceutical applications. *Livestock Production Science* 50(1–2), 125-138.

Meisel H (1997b) Biochemical properties of regulatory peptides derived from milk proteins. *Peptide Science* 43(2), 119-128.

Meisel H (1998) Overview on Milk Protein-derived Peptides. *International Dairy Journal* 8(5–6), 363-373.

Meisel H & Bockelmann W (1999) Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* 76(1-4), 207-215.

Meisel H, Frister H & Schlimme E (1989) Biologically active peptides in milk proteins. *Zeitschrift für Ernährungswissenschaft* 28(4), 267-278.

Melnik BC, John SM & Schmitz G (2013) Milk is not just food but most likely a genetic transfection system activating mTORC1 signaling for postnatal growth. *Nutrition Journal* 12, 103.

Michalak M, Larsen DM, Jers C, Almeida JRM, Willer M, Li H, Kirpekar F, Kjærulff L, Gotfredsen CH, Nordvang RT, Meyer AS & Mikkelsen JD (2014) Biocatalytic production of 3′-sialyllactose by use of a modified sialidase with superior *trans*-sialidase activity. *Process Biochemistry* 49(2), 265-270.

Millán-Linares MdC, Yust MdM, Alcaide-Hidalgo JM, Millán F & Pedroche J (2014) Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway. *Food Chem* 151(0), 141-147.

Mills S, Ross RP, Hill C, Fitzgerald GF & Stanton C (2011) Milk intelligence: Mining milk for bioactive substances associated with human health. *International Dairy Journal* 21(6), 377-401.

Moreno FJ, Montilla A, Villamiel M, Corzo N & Olano A (2014) Analysis, structural characterization, and bioactivity of oligosaccharides derived from lactose. *Electrophoresis* 35(11), 1519-1534.

Morison KR & She X (2003) Optimisation and graphical representation of multi-stage membrane plants. *Journal of Membrane Science* 211(1), 59-70.

Mulder M (1992) *Basic principles of membrane technology*. Kulwer Academic Publishers, Dordrecht, The Netherlands.

Mullally MM, Meisel H & FitzGerald RJ (1997) Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Letters* 402(2-3), 99-101.

Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S & Takano T (1995) Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science* 78(4), 777-783.

Nielsen DS, Theil PK, Larsen LB & Purup S (2012) Effect of milk hydrolysates on inflammation markers and drug-induced transcriptional alterations in cell-based models. *Journal of Animal Sience* 90 Suppl 4, 403-405.

Nielsen WK (2000) *Membrane filtration and related molecular separation technologies*. APV Systems, Silkeborg, Denmark.

Nongonierma AB & FitzGerald RJ (2015) The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A Review. *Journal of Functional Foods* 17, 640-656.

Norris R, O'Keeffe MB, Poyarkov A & FitzGerald RJ (2015) Peptide identification and angiotensin converting enzyme (ACE) inhibitory activity in prolyl endoproteinase digests of bovine alpha(s)-casein. *Food Chemistry* 188, 210-217.

Oliveira D, Wilbey RA, Grandison A & Roseiro L (2014) Natural caprine whey oligosaccharides separated by membrane technology and profile evaluation by capillary electrophoresis. *Food and Bioprocess Technology* 7(3), 915-920.

Oliveira DL, Wilbey RA, Grandison AS & Roseiro LB (2015) Milk oligosaccharides: A review. *International Journal of Dairy Technology* 68(3), 305-321.

Ortega-González M, Ocón B, Romero-Calvo I, Anzola A, Guadix E, Zarzuelo A, Suárez MD, Sánchez de Medina F & Martínez-Augustin O (2014) Nondigestible oligosaccharides exert nonprebiotic effects on intestinal epithelial cells enhancing the immune response via activation of TLR4-NFκB. *Molecular Nutrition & Food Research* 58(2), 384-393.

Park YW (2009) Overview of Bioactive Components in Milk and Dairy Products. In: *Bioactive Components in Milk and Dairy Products*. 1-12. Wiley-Blackwell.

Pasparakis M (2009) Regulation of tissue homeostasis by NF-kB signalling: implications for inflammatory diseases. *Nature Reviews Immunology* 9(11), 778-788.

Picariello G, Iacomino G, Mamone G, Ferranti P, Fierro O, Gianfrani C, Di Luccia A & Addeo F (2013) Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food chemistry* 139(1-4), 203-212.

Piccolomini AF, Iskandar MM, Lands LC & Kubow S (2012) High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food & Nutrition Research* 56, 1-10.

Pihlanto-Leppälä A, Rokka T & Korhonen H (1998) Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal* 8(P4), 325-331.

Plaisancié P, Claustre J, Estienne M, Henry G, Boutrou R, Paquet A & Léonil J (2013) A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. *Journal of Nutritional Biochemistry* 24(1), 213-221.

Priem B, Gilbert M, Wakarchuk WW, Heyraud A & Samain E (2002) A new fermentation process allows large-scale production of human milk oligosaccharides by metabolically engineered bacteria. *Glycobiology* 12(4), 235-240.

Raae AJ, Flengsrud R & Sletten K (1995) Chymotrypsin isoenzymes in Atlantic cod; differences in kinetics and substrate specificity. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 112(2), 393-398.

Rahman MM & McFadden G (2006) Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathogens* 2(2), 66-77.

Ramaswamy H & Marcotte (2005) *Food processing- Principles and application*. CRC Press, Boca Raton, USA.

Rival SG, Fornaroli S, Boeriu CG & Wichers HJ (2000) Caseins and casein hydrolysates. 1. lipoxygenase inhibitory properties. *Journal of Agricultural and Food Chemistry* 49(1), 287-294.

Rossano R, Larocca M & Riccio P (2011) Digestive enzymes of the crustaceans *Munida* and their application in cheese manufacturing: A review. *Marine Drugs* 9(7), 1220-1231.

Saito T (2008) Antihypertensive peptides derived from bovine casein and whey proteins. In: Bösze Z (ed) *Bioactive Components of Milk*, vol 606. Advances in Experimental Medicine and Biology. 295-317. Springer New York.

Sarney DB, Hale C, Frankel G & Vulfson EN (2000) A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. *Biotechnology and Bioengineering* 69(4), 461-467.

Sienkiewicz T & Riedel C-L (1990) *Whey and whey utilization*. Th. Mann, Gelsenkrichen, Germany.

Swaisgood HE (2003) Chemistry of the caseins. In: Fox PF & McSweeney PLH (eds) *Advanced dairy chemistry- Volume 1: Proteins part A*. Springer, New York.

Tamime AY (2009) *Dairy powders and concentrated products*. vol 1. Wiley-Blackwell, West Sussex, United Kingdom.

Tao N, Depeters EJ, Freeman S, German JB, Grimm R & Lebrilla CB (2008) Bovine milk glycome. *Journal of Dairy Science* 91(10), 3768-3778.

Tao N, Ochonicky KL, German JB, Donovan SM & Lebrilla CB (2010) Structural determination and daily variations of porcine milk oligosaccharides. *Journal of Agricultural and Food Chemistry* 58(8), 4653-4659.

Tavares TG, Spindola H, Longato G, Pintado ME, Carvalho JE & Malcata FX (2013) Antinociceptive and anti-inflammatory effects of novel dietary protein hydrolysate produced from whey by proteases of *Cynara cardunculus*. *International Dairy Journal* 32(2), 156-162.

ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Feitsma AL, van Hoffen E & Schoterman MHC (2014) Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutrition Reviews* 72(6), 377-389.

Töpel A (2004) *Chemie und Physik der Milch: Naturstoff-Rohstoff-Lebensmittel*. Behr's Verlag, Hamburg.

Urashima T, Saito T, Nakamura T & Messer M (2001) Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal* 18(5), 357-371.

Vernaza MG, Dia VP, Gonzalez de Mejia E & Chang YK (2012) Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry* 134(4), 2217-2225.

Walstra P, Geurts TJ, Noomen A, Jellema A & Boekel MAJSv (1999) *Dairy Technology-Principles of Milk Properties and Processes*. Marcel, Dekker, Inc., Basel.

Weimann C, Meisel H & Erhardt G (2009) Short communication: bovine kappa-casein variants result in different angiotensin I converting enzyme (ACE) inhibitory peptides. *Journal of Dairy Science* 92(5), 1885-1888.

Wu G (2013) Arginine and immune function. In: Calder PC & Yaqoob P (eds) *Diet, immunity and inflammation*. 523-543. Woodhead Publishing, Cambridge.

Wu S, Tao N, German JB, Grimm R & Lebrilla CB (2010) Development of an annotated library of neutral human milk oligosaccharides. *Journal of Proteome Research* 9(8), 4138-4151.

Xu M & Chen ZJ (2011) Ubiquitin mediated regulation of protein kinases in NFkB signaling. In: Bradshaw RA & Dennis EA (eds) *Regulation of organelle and cell compartment signaling*. 71-82. Academic Press, China.

Yamaguchi M, Yoshida K & Uchida M (2009) Novel functions of bovine milk-derived alpha-lactalbumin: anti-nociceptive and anti-inflammatory activity caused by inhibiting cyclooxygenase-2 and phospholipase A(2). *Biological & Pharmaceutical Bulletin* 32(3), 366-371.

Young D & Mine Y (2010) Anti-inflammatory/Oxidative Stress Proteins and Peptides. In: *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals*. 13-27. Wiley-Blackwell.

Zelová H & Hošek J (2013) TNF-α signalling and inflammation: interactions between old acquaintances. *Inflammation Research* 62(7), 641-651.

Zenhom M, Hyder A, de Vrese M, Heller KJ, Roeder T & Schrezenmeir J (2011) Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinal Caco-2 cells via

activation of PPARgamma and peptidoglycan recognition protein 3. *Journal of Nutrition* 141(5), 971-977.

Zeuner B, Luo J, Nyffenegger C, Aumala V, Mikkelsen JD & Meyer AS (2014) Optimizing the biocatalytic productivity of an engineered sialidase from *Trypanosoma rangeli* for 3′-sialyllactose production. *Enzyme and Microbial Technology* 55(0), 85-93.

Zivkovic AM & Barile D (2011) Bovine milk as a source of functional oligosaccharides for improving human health. *Advances in Nutrition* 2(3), 284-289.

## 1.4 Objectives

The aim of the thesis was the development of procedures for the generation, enrichment and characterization of bioactive oligosaccharides and peptides from milk. This work focused on the production of functional food ingredients of the carbohydrate and protein fraction of milk. The developed processes should be suitable and feasible for the implementation on industrial scale.

Therefore, the objectives of the present study were:

- Carbohydrates: To develop a procedure for the **enrichment** of milk oligosaccharides (MOS) by nanofiltration (NF) *(chapter II, III, V.1)*
  - The comparison of the efficiency of different NF membranes for the enrichment of MOS from bovine milk and the transfer of the process from laboratory to pilot plant and industrial scale *(Manuscript 1, chapter II, V.1).*
  - The influence of the pH on the NF process for a better separation of milk salts during NF for the enrichment of MOS from bovine milk *(Manuscript 2, chapter III, V.1).*
  - The establishment of the process for the enrichment of MOS to caprine milk *(Manuscript 2, chapter III, V.1).*
  - The **characterization** of the immunostimulatory activity of the MOS concentrates *(Manuscript 1, chapter II, V.3).*

- Proteins: To develop a procedure for the **generation** of anti-inflammatory peptides *(chapter IV, V.2)*
  - The synthesis and identification of anti-inflammatory peptides from bovine β-casein using enzyme preparations from cod and hog *(Manuscript 3, chapter IV, V.2).*
  - The potential of a cod tryptic enzyme preparation to generate anti-inflammatory peptides for the replacement of non-food grade TPCK treated porcine tryptic enzyme preparation *(Manuscript 3, chapter IV, V.2).*
  - The fractionation of the β-casein hydrolysates by molecular size *(Manuscript 3, chapter IV, V.2).*
  - The **characterization** of the anti-inflammatory activity of the β-casein hydrolysates and fractions *(Manuscript 3, chapter IV, 5.3).*

# Chapter II

## Comparison of the efficiency of different NF membranes for the enrichment of milk oligosaccharides from bovine milk

Karina Altmann[1]*, Adam Wutkowski[1], Sebastian Kämpfer[2], Martin Klempt[1], Peter Chr. Lorenzen[1], Ingrid Clawin-Rädecker[1]

*Accepted in European Food Research and Technology*

*December 2015*

[1] Max Rubner-Institut Kiel, Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Straße 1, 24103 Kiel, Germany

[2] Central Production and Engineering, Müller Service GmbH, An den Breiten, 01454 Leppersdorf, Germany

*corresponding author

## II.1 Abstract

Oligosaccharides in milk (MOS) have been reported in literature to exert various bioactivities and to modulate the immune system. However, processes to obtain milk oligosaccharides on industrial scale as food ingredients are currently not available. Therefore, the aim of the study on hand was the evaluation of different nanofiltration (NF) membranes for the enrichment of MOS from bovine milk. Moreover, a transfer of the NF process from laboratory to pilot plant and industrial scale was performed. The immunostimulatory effect of the MOS concentrates was studied by the activity of NFκB in human embryonic kidney cells ($HEK^{nfκb\text{-}RE}$ cells).

NF was carried out with lactose hydrolyzed skimmed and ultrafiltered milk permeate by application of different membranes. The quantification of MOS was determined by high pH-anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and parallel online electrospray ion-trap mass spectrometry (IT-MS).

The enrichment of MOS (3-sialyl-lactose, 6-sialyl-lactose, N-acetylgalactosaminyl-lactose) on laboratory, pilot plant and industrial scale was achieved by a retention of these oligosaccharides of at least 50 % in NF retentate. The content of MOS in relation to total sugar content in the retentate from NF on industrial scale was 100 fold higher than in the initial sample. The MOS retentates and the standards (3-sialyl-lactose, 6-sialyl-lactose) exhibited increased NFκB activity in $HEK^{nfκb\text{-}RE}$ cells. Although there exist a few studies about the enrichment of MOS by NF, this is the first report about a screening of the efficiency of different NF membranes for the enrichment of MOS on different technological scales of production.

**Keywords**

enrichment of bovine milk oligosaccharides (MOS) by nanofiltration (NF), retention of MOS, content of MOS in relation to total sugar content, transfer of NF process from laboratory to industrial scale, NFκB activity, immunostimulatory effect

## II.2 Introduction

Oligosaccharides in milk (MOS) exert biologically active effects locally in the gut and systemically after absorption [1]. In the gut, MOS can show prebiotic activity by contribution to the growth of beneficial intestinal flora in the colon. As competitive inhibitors for binding sites on the epithelial surface, they show anti-infective and anti-adhesive properties by reducing or preventing the adhesion of pathogens [1-7]. It was described that human MOS resist digestion, partly get absorbed and remain in the circulation in concentrations high enough to exert systemic effects [3]. As shown in Caco-2 intestinal epithelial cells *in vitro*, MOS can be absorbed via the paracellular or transcellular route [1,8]. After absorption, MOS may exhibit systemically bioactive effects [9], by interacting with immune cells [10-14]. An expamle for the immune regulatory effect is that sialic acid containing oligosaccharides reduce the adhesion of leukocytes to endothelial cells [3]. Moreover, MOS show immunostimulatory activity by up-regulation of cytokine production (IFN-γ, IL-13, TNF-α, IL-6 and IL-10) in human cord blood- derived T cells [9], in primary mouse splenocytes [15] and in colonic epithelial cells (HT-29) [10] *in vitro*. In a study from Ortega-González et al. an activation of NFκB by MOS from goat milk in intestinal epithelial cells from rat (IEC18 cells) was described. Incubation of this cell line with 0.05 to 5 mg/ml MOS resulted in a concentration dependent increase of cytokine production (GROα, MCP1, MIP2, IL6, IP10).

Research has demonstrated that bovine milk contains MOS, which are identical with oligosaccharides to human milk such as the acidic oliogosaccharides 3-sialyl-lactose (3'-SL) and 6-sialyl-lactose (6'-SL) [16,17]. Therefore, it can be suggested that the biological effects, which MOS provide for infants could also be available to humans of all ages [17,7]. The MOS content in human milk is between with 5 to 8 g/L and presents the third largest solid component after lactose and lipids [2,18,5,7]. Compared with human milk, the levels of oligosaccharides in milk of domestic mammalian animals are much lower. The concentration of MOS in bovine milk is between 0.03 to 0.06 g/L. Because of the low concentration of MOS in bovine milk, large amounts of MOS for the application as functional ingredient in food are not yet available [2]. The structural and health promoting aspects of MOS are intensively reported. However, there exist only few studies about the enrichment of MOS by membrane filtration on laboratory and pilot plant scale [19,18,20] and only one study deals with the concentration of MOS from bovine milk permeate on industrial scale [7]. Until now,

there exist no study about a screening of different nanofiltration (NF) membranes for an enrichment of MOS followed by a feasibility study for transferring this membrane process into large scale. For the concentration, an important parameter is on the one hand the retention of MOS in NF retentate. On the other hand, the content of MOS in relation to total sugar content gives information about the purity of the product. It depends on the priorities, whether a high recovery of MOS and/ or a high purity of the concentrate wants to be achieved. An evaluation of the bioactive properties of MOS concentrates gained by application of NF has not been performed yet.

Therefore, the aim of the present study is the evaluation of the efficiency of different NF membranes for the enrichment of MOS. The efficiency of the membranes for this application was characterized by the recovery of MOS and the content of MOS in relation to total sugar content. Subsequently, the process of the enrichment of MOS should be transferred from laboratory scale into pilot plant and industrial scale. Furthermore, the immunostimulatory effect of bovine MOS should be determined analyzing their effect on the activity of the transcription factor NFκB in human embryonic kidney cells ($HEK^{nfκb\text{-}RE}$ cells).

## II.3 Materials and methods

### *II.3.1 Nanofiltration assays*

Bovine milk (from the experimental farm of the MRI in Schädtbek) was skimmed using a disc centrifuge (LWA 205-1, Westfalia Separator AG, 4740 Oelde, Germany) and ultrafiltered on a 5 kDa hollow fiber membrane (CTG.1" HF 1.0-43-PM5-P3, Koch Membrane Systems, Wilmington, USA) in order to get a permeate of skimmed milk. For a better separation of the MOS from the residual sugar, the lactose was hydrolyzed (150 NL U/g lactose) by the action of β-galactosidase (HA-Lactase 2100, Chr. Hansen, DK-2970 Horsholm, Denmark, 150 NL U/g lactose) at 40°C for 3 h in a stirred tank reactor. For the enrichment of MOS, cross-flow NF of the lactose hydrolyzed skimmed and ultrafiltered milk permeates (lactose hydrolyzed SM-UF-permeates) were performed by application of flat-sheet and spiral-wound membranes with different nominal molecular weight cut-off (NMWCO) and different material at varying parameters **(Table 2.1)**.

For characterization of the NF process, the following factors were applied:

In the first part of NF, ultrafiltered skimmed milk permeate was concentrated to enrich the content of MOS in NF retentate. The concentration factor *CF* is the ratio of the initial feed volume $V_f$ [L] to the retentate volume $V_r$ [L].

(1)

$$CF = \frac{V_f}{V_r}$$

In the second part the retentate was diafiltered with demineralized water to decrease the contents of glucose, galactose, residues of lactose, and milk salts in the MOS retentate. The number of diavolumes *DV* is defined as the ratio of the total volume of liquid permeated $V_p$ [L] and the initial volume, which is in our experiments the retentate volume $V_r$ [L] from the first step of the NF.

(2)

$$DV = \frac{V_p}{V_r}$$

The volumetric flux of permeate F [L m $^{-2}$ h $^{-1}$] is the ratio of liquid $V_p$ [L] permeated in one hour $t$ [h] and the active membrane area $A$ [m$^2$] of the corresponding module.

(3)

$$F = \frac{V_p}{A\ t}$$

Another important parameter is the percental retention $R_i$ of a component i (e.g. 3'-SL, 6'-SL, GalNAc-Gal-Gluc) in NF retentate. $C_{r,i}$ is the concentration of the component i in retentate and $C_{f,i}$ is the concentration of the component i in the initial feed sample.

(4)

$$R_i = \frac{C_{r,i}\ V_r}{C_{f,i}\ V_f}\ 100$$

Furthermore, the percental content of MOS (3'-SL, 6'-SL, GalNAc-Gal-Glc) in relation to total sugar content (MOS, glucose, galactose, lactose) $M_{MOS/total\ sugar}$ was calculated as ratio of the concentration of MOS in retentate $C_{r,MOS}$ [mg/L] to the concentration of total sugar in retentate $C_{r,total\ sugar}$ [mg/L].

(5)

$$M_{MOS/total\ sugar} = \frac{C_{r,MOS}}{C_{r,total\ sugar}}\ 100$$

A transfer of the NF process from laboratory scale to pilot plant scale and to industrial scale was performed **(Table 2.1)**.

**Table 2.1** Properties of NF membranes applied for the enrichment of MOS on laboratory, pilot plant and industrial scale and corresponding experimental set-ups (a= flux at 5 bar; b= flux at 10 bar; c= module SR 50 on technical scale; d= module DL on technical scale; NMWCO = nominal molecular weight cut-off; concentration factor *CF*= the ratio of the initial feed volume $V_f$ [L] to the retentate volume $V_r$ [L]; diavolume *DV* = the ratio of the total volume of liquid permeated $V_p$ [L] and the initial volume).

| **Dimensions of experiments** | **Membranes** | **Manufacturer** | **Material** | **NMWCO [Da]** | **Flux *(F)* [L/m$^2$h$^1$]** |
|---|---|---|---|---|---|
| | **Flat-sheet** | | | | |
| laboratory scale | DL ® | Desalogics | composite | 150-300 | 6.7 |
| | CK ® | Desalogics | cellulose acetate | 200 | 2.9 |
| | SR 100 ® | Koch Membrane Systems | thin film composite | 200-300 | 3.5 |
| | GE ® | Desalogics | composite | 500-1000 | 7.8 |
| | MPF 36 ® | Koch Membrane Systems | composite | 700-1000 | 10.9 |
| | **Spiral-wound** | | | | |
| pilot plant scale | SR 50 (2540 SR2-N1)® | Koch Membrane Systems | thin film composite | 300-400 | 300.0 [a]<br>531.6 [b] |
| | DL (2540C1077)® | Desalogics | thin film | 150-300 | 34.94 [a]<br>71.18 [b] |
| | **Spiral-wound** | | | | |
| industrial scale | DOW (NF-3840-30-FF)® | The DOW chemical company | not specified | 300 | 9.36 |

| | **Experimental set-up** | | | | | |
|---|---|---|---|---|---|---|
| **Dimensions of experiments** | **Temperature [°C]** | **Pressure [bar]** | **Initial feed volume *($V_f$)* [L]** | **Concentration factor *(CF)*** | **Diavolume *(DV)*** | **Active membrane area** |
| laboratory scale | 40 | 4 | 0.2 | 2 | 10 | 160 cm$^2$ |
| pilot plant scale | 40 | 5<br>10 | 6 | 2 | 10 | 2.5 m$^2$ [c]<br>1.7 m$^2$ [d] |
| industrial scale | 10 | 10-15 | 1000 | 10 | 4.6 | 15 m$^2$ |

NFs on laboratory scale were performed with the ÄKTAcrossflow™ system (Ge Healthcare Bio-Sciences AB, 75184 Uppsala, Sweden) by application of different flat-sheet membranes with a membrane area of 160 $cm^2$ at 4 bar and 40°C **(Table 2.1)**. To get a deposition layer, retentate and permeate were circulated into the retentate tank for 30 min. 200 ml ultrafiltered skimmed milk permeate were concentrated to 100 mL (concentration factor = 2). After that a continuous diafiltration step of 100 mL NF retentate was conducted with 10 diavolumes. Therefore, 1 L demineralized water was added to 100 mL NF-retentate at the same rate as the permeate flux, thus keeping retentate volume constant during operation.

NFs on pilot plant scale were performed with the modules SR 50 (active membrane area of 2.5 $m^2$) and DL (active membrane area of 1.7 $m^2$) at 40°C and the pressures 5 bar and 10 bar **(Table 2.1)**. The membrane filtration was performed with the Alfa Laval Lab M20 device (Alfa Lava Corporate AB, SE-22655, Lund, Sweden). Retentate and permeate were circulated into the retentate tank for 30 min to get a deposition layer. 6 L ultrafiltered skimmed milk permeate were concentrated in the first step to 3 L (concentration factor = 2). The NF retentate was diafiltered with 10 diavolumes by stepwise addition of 6 L demineralized water to 3 L retentate for 5 times. Water was added when retentate was concentrated to 3 L. After discontinuous diafiltration with 30 L demineralized water, the NF retentate was further concentrated up to the residual volume of the membrane filtration device of 2.2 to 2.4 L.

The industrial scale NF process was conducted with the MMS nanofiltration device (MMS Membrane Systems, 8902 Urdorf, Switzerland) in cooperation with the Theo Müller Group at Sachsenmilch in Leppersdorf. For the membrane separation process two modules of the spiral wound membrane DOW with an active membrane area of 7.5 $m^2$ were used at 10-15 bar and 10 °C **(Table 2.1)**. At the beginning, retentate and permeate were circulated into the retentate tank for 30 min for forming a deposition layer. 1000 L of ultrafiltered skimmed milk permeate were concentrated to 100 L (concentration factor = 10). The NF retentate was diafiltered with 4.6 diavolumes by adding stepwise 100, 200 and 160 L of demineralized water when the retentate was concentrated to 100 L. After discontinuous diafiltration with 460 L water, the NF retentate was concentrated to 58 - 60 L in the first experiment and to 45 L in the second experiment.

### *II.3.2 Analytical methods*

#### *II.3.2.1 Quantification of MOS, mono- and disaccharides*

##### *II.3.2.1.1 Sample pretreatment*

Milk oligosaccharides were purified by graphitized carbon solid-phase extraction according to Packer et. al. 1998 [21]. The cartridges (Extract-clean SPE Carbo 150mg, Alltech Grom GmbH, Worms) were washed with 80 % acetonitrile/0.1 % trifluoroacetic acid (v/v) in water, followed by conditioning with pure water. 100 µl of skim milk permeate or enriched NF-retentate were loaded onto the cartridge and washed with 4ml water and 4 ml 4 % acetonitril to remove the high amounts of mono- and disaccharides. Milk oligosaccharides were eluted by consecutive washing with 4 ml 20 % acetonitril and 4 ml of a solution of 40 % acetonitrile and 0.1 % trifluoroacetic acid (v/v). Samples were dried in a vacuum centrifuge and reconstituted in water and diluted with solvent A (10mmol/L NaOH) prior to HPAEC-PAD/MS analysis.

For the determination of galactose, glucose and lactose-content the skim milk permeate or enriched NF-retentate samples were clarified using Carrez solutions I and II (Carrez I = 3.60g/100ml potassium hexacyanoferrate (II), Carrez II = 7.20g/100ml zinc sulphate) and were diluted to required concentration with 10 mmol/L NaOH.

##### *II.3.2.1.2 Determination of milk oligosaccharides by HPAEC-PAD/MS*

The analyses of carbohydrates were performed by a high performance anion exchange chromatography with pulsed amperometric detection and parallel online mass spectrometry [22-24]. The analytical system consisted of a Dionex ICS-3000 chromatographic system (Idstein, Germany) with an SP gradient pump, an SP autosampler and an ED 40 electrochemical detector with a gold working electrode and Ag/AgCl as a reference electrode. Separations were performed on a CarboPac PA-100 analytical column (2mm x 250mm, 8.6µm), with a CarboPac PA-100 guard column (2mm x 50mm, 8.6µm, Dionex, Idstein, Germany) with a flow rate of 0.2 ml/min at 25°C. Elution was carried out with a linear

gradient with increasing sodium hydroxide and sodium acetate concentrations. For identification of the oligosaccharides the flow was split in a 1:1 ratio for ED and MS detection after elution from the analytical column. MS data were acquired on an ion trap mass spectrometer LTQ XL (Thermo Fischer Scientific, Dreieich, Germany) with an electrospray interface (ESI). For coupling to the mass spectrometer, an online desalinisation with Suppressor ASRS 300, 2 mm (Dionex, Idstein, Germany) and post-column addition of 30 µl/min 5 mM LiCl were used. Mass spectra were generated in the positive ionisation mode in the full scan range 150 up to 2000 m/z and a data-dependent scan with fragmentation of the three most intense ions (activation type = CID, normalised collision energy = 35.0 eV, isolation width 2 m/z). The ESI spray voltage was set to 4.0 kV, the heated capillary temperature was 300°C.

Sample pretreatment and analysis of the samples was performed in duplicate. Glucose, galactose, lactose, GalNAc-Gal-Glc, 6-sialyl-lactose and 3-sialyl-lactose were used as external standards. Glucose, galactose and lactose were purchased from VWR (Darmstadt, Germany). The milk oligosaccharides 6-sialyllactose and 3-sialyllactose were obtained from Carbosynth (Berkshire, U.K.) and N-acetyl-galactosaminyl-lactose (GalNAc-Gal-Glc) was obtained from McBiTec (Strasbourg, France). For evaluation of the sample pretreatment the recovery of the individual oligosaccharides spiked into ultrafiltered SM-UF-permeate and NF retentate samples were determined. The recovery of the oligosaccharides was 73.9 ± 7.7 % for 3'-SL, 87.4 ± 4.1 % for 6'-SL and 95.6 ±7.1 % for GalNAc-Gal-Glc.

*II.3.2.1.3 Quantification of other components*

Dry mass was measured by the VDLUFA method C 35.5. An aluminium foil and filter paper was weighted before and after addition of 2 g sample. After drying for 2 h at 102 ± 2°C the foil and filter paper with sample were weighted again. The dry mass was calculated as percentage by weight of the dried sample to the initial sample before drying.

Total protein and non-protein nitrogen (NPN) contents in MOS concentrates from industrial scale NF were measured by the Kjeldahl method (VDLUFA VI C 30.2). For determination of the NPN content (VDLUFA VI C 30.3) the samples were first acidified to a concentration of

12 % with trichloroacetic acid. The nitrogen in the NPN fraction was determined by the Kjeldahl method (VDLUFA VI C 30.2). The ash content was measured according to the method VDLUFA IV C 10.2. Determination of the salts Na, K, Mg, Ca and P were performed with slight modifications according to the Method VDLUFA III 10.8.1.2 and 10.8.2. Chloride content was measured with HPLC and ion-exchange chromatography according to a modified DIN method (DIN EN 12014 Part 2). The Citric acid content was measured with a citric acid test kit (r-biopharm AG, Darmstadt, Germany).

### *II.3.3 Effect of NF MOS concentrates on NFκB activation in HEK$^{nfκb\text{-}RE}$ cells and followed Luciferase assay*

HEK$^{nfkb\text{-}RE}$ cells [25] were cultivated at 5 % $CO_2$ in DMEM medium (PAA, Cölbe, Germany) supplemented with 10 % fetal calf serum (FCS) (PAA, Cölbe, Germany). Lyophilized MOS concentrates from NF (50 mg/ml) were dissolved in culture medium and sterile filtered. HEK$^{nfkb\text{-}RE}$ cells were seeded in white (clear bottom) 96-well plates at a density of 10 000 cells/well. After overnight incubation, cells were incubated with MOS concentrates (final concentration 25 mg/ml). For control cells were treated only with DMEM medium without sample. After 24 hours, medium was removed, cells were washed twice with PBS and lysed by freezing and defrosting two times. Luciferase assay was started by incubation of cells with 45 µl of Beetle Lysis-Juice (PJK, Kleinblittersdorf, Germany) for 10 min. The light product developed by luciferin degradation was measured over a period of 1 s in the Chameleon plate reader (Hidex, Turku, Finland). Quadruplicated incubation and measurements were considered to be one experiment. The data are expressed as arbitrary units of luciferase activity of HEK$^{nfkb\text{-}RE}$ cells compared with the control (cells with TNF-α without sample are stated as 1).

*Statistical analysis*

Statistical significance values of relative luciferase activity between NF MOS concentrates and control without sample were determined by using Kruskal-Wallis one-way analysis followed by the Dunns multiple comparison test with the software SigmaPlot 11.0 (SigmaPlot

Software, Systat Software Inc., USA). The data are expressed as the arithmetic means ± standard deviation (SD) of at least three independent experiments. Values are reported in relation to the luciferase activity of the control.

## II.4 Results and discussion

### *II.4.1 Efficiency of different NF membranes for the enrichment of MOS*

#### *II.4.1.1 Laboratory scale NF*

For the enrichment of MOS, NF of lactose hydrolyzed SM-UF-permeate was performed on laboratory scale by application of different flat-sheet membranes **(Table 2.1)**. The MOS content was quantified by determination of the main components of the MOS fraction (6'-SL, 3'-SL, GalNAc-Gal-Glc). The concentration of the MOS, monosaccharides (glucose, galactose), and residual lactose in the initial sample and the NF-retentates determined as average of two different NFs are listed in **Table 2.2**. The total content of MOS ( 6'-SL, 3'-SL, GalNAc-Gal-Glc) in the lactose hydrolyzed SM-UF-permeate was about 67.1 mg/L. Fong et al. (2011) [26] quantified six different MOS in bovine mature milk, colostrum and infant formula using a hydrophilic interaction chromatography – mass spectroscopy method. The total content of the main oligosaccharides 3'-SL, 6'-SL and GalNAc-Gal-Glc in mature milk vary from 50.6 to 67.4 mg/L and is consistent with our results. Due to lactose hydrolysis the glucose and galactose content was about 20 g/L and the residual lactose concentration was 0.30 g/L. Because of the concentration factor of 2 on laboratory scale NF, the highest possible MOS content, which could be achieved after NF, is the double of the concentration in the initial sample. As a result of NF, MOS content in the NF retentates after filtration with the membranes DL, CK, SR 100 and GE are slightly higher than in the initial sample. Only by application of the composite membrane MPF 36 the retention of MOS was markedly lower than in the original sample. Moreover, the concentration of mono- and disaccharides and the dry mass content in all retentates decreased distinctly in comparison to the lactose hydrolyzed SM-UF-permeate.

**Table 2.2** Concentration of MOS (3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose), mono- and disaccharides (glucose, galactose, lactose) and dry mass in lactose hydrolyzed SM-UF-permeate and retentates of laboratory scale NF (average of two trials, n=2). The recovery of the standards was 73.9 ± 7.7 % for 3'-SL, 87.35 ± 4.05 % for 6'-SL and 95.6 ±7.1 % for GalNAc-Gal-Glc. (n=2)

| sample | lactose hydrolyzed SM-UF-permeate | NF retentate DL | NF retentate CK | NF retentate SR 100 | NF retentate GE | NF retentate MPF 36 |
|---|---|---|---|---|---|---|
| **6'-SL [mg/L]** | 6.8 +/- 0.3 | 7.9 +/- 0.4 | 8.5 +/- 1.7 | 11.5 +/- 1.6 | 9.0 +/- 0.1 | 3.6 +/- 0.2 |
| **3'-SL[mg/L]** | 35.7 +/- 1.7 | 36.6 +/- 1.8 | 37.1 +/- 4.1 | 35.5 +/- 4.3 | 36.1 +/- 4.7 | 16.3 +/- 1.3 |
| **GalNAc-Gal-Glc [mg/L]** | 24.7 +/- 2.3 | 33.2 +/- 2.4 | 26.9 +/- 2.0 | 29.0 +/- 3.9 | 8.5 +/- 5.3 | 3.0 +/- 0.2 |
| **∑ MOS [mg/L]** | 67.1 | 77.7 | 72.3 | 76.0 | 53.6 | 22.8 |
| **lactose [g/L]** | 0.3 +/- 0.0 | 0.1 +/- 0.0 | 0.1 +/- 0.0 | 0.1 +/- 0.0 | 0.0 +/- 0.0 | 0.2 +/- 0.0 |
| **glucose [g/L]** | 22.6 +/- 0.2 | 0.0 +/- 0.0 | 0.1 +/- 0.1 | 0.0 +/- 0.0 | 0.0 +/- 0.0 | 0.2 +/- 0.0 |
| **galactose [g/L]** | 20.3 +/- 0.2 | 0.1 +/- 0.0 | 0.3 +/- 0.1 | 1.0 +/- 0.0 | 0.1 +/- 0.0 | 0.1 +/- 0.0 |
| **dry mass [g/L]** | 61.0 +/- 0.1 | 6.6 +/- 0.5 | 5.9 +/- 0.3 | 7.6 +/- 0.1 | 3.8 +/- 0.0 | 1.0 +/- 0.2 |

**Figure 2.1** indicates the retention of MOS (6‘-SL, 3‘-SL, GalNAc-Gal-Glc) in NF retentates after filtrations with the membranes DL, CK, SR 100, GE, and MPF 36 (white, grey, and black bars; left ordinate). Furthermore, the MOS content in relation to the total sugar content is shown for the original sample and the NF retentates (striped bars, right ordinate).

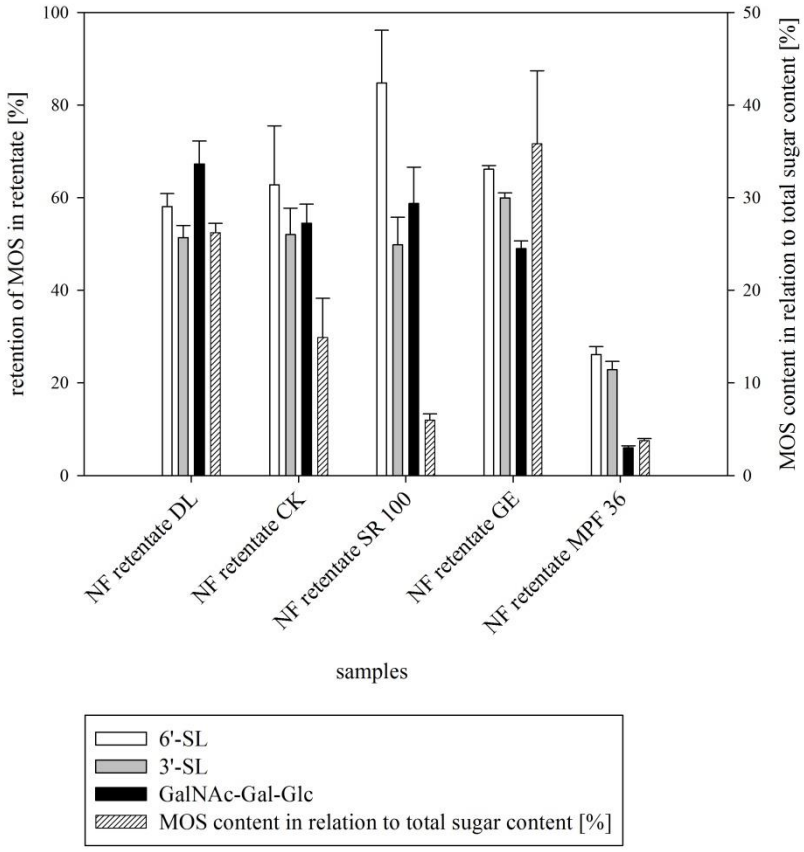



**Fig. 2.1** Retention of MOS by NF on laboratory scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate), (average of two trials, n=2, 3’-SL = 3-sialyl-lactose, 6’-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose). MOS content in relation to total sugar content in the initial SM-UF-permeate is 0.1 - 0.2 %.

The content of MOS in relation to the total sugar content in the lactose hydrolyzed SM-UF-permeate was 0.2 ± 0.0 %. The final retention of the three MOS was highest in the NF retentate of the thin film composite membrane SR 100 (MWCO of 200-300 Da). From the initial sample 84.8 ± 11.4 % of 6‘-SL, 49.8 ± 6.0 % of 3‘-SL and 58.7 ± 7.9 % of GalNAc-Gal-Glc were retained in the NF retentate. However, the MOS content in relation to total

sugar content (6.0 ± 0.7 %) was low, when this membrane was used. This is mainly due to the higher retention of mono- and disaccharides **(Table 2.2)**. Application of the cellulose acetate membrane CK (NMWCO 200 Da), resulted in a retention of MOS in the same order of magnitude. In addition, the MOS content in relation to total sugar content (14.9 ± 4.2 %) was much higher with the CK than with the membrane SR 100. The retention of MOS by application of the composite membrane DL (NMWCO 150-300 Da) was at the same level as by using the CK membrane. Due to the high permeation of mono- and disaccharides, an increased content of MOS in relation to total sugar content (26.2 ± 1.0 %) was obtained. By application of the composite membrane GE (NMWCO 500-1000 Da) the retention of MOS in the retentate was in the same order as with the DL and CK. Furthermore, the highest MOS content in relation to total sugar content (35.8 ± 7.9 %) was detected. However, the application of the GE membrane resulted in high fouling so that the permeate flux decreased rapidly during diafiltration. As a result, the processing time at constant pressure (4 bar) increased up to 13 h. NF with the composite membrane MPF 36 did not exhibit a high retention of MOS resulting in a low MOS content in relation to total sugar content. It can be assumed that a NMWCO of 700-1000 Da was too large for an effective retention of MOS. As a result of the studies on laboratory scale, the membranes DL and SR 100 were chosen for a transfer of the NF to pilot plant scale. The application of this membranes resulted in a high retention of MOS in the retentate. Using these membranes led to low fouling and increasing permeate fluxes during diafiltration. It can be concluded that both the membrane NMWCO of the membrane and the composition have to be considered for the enrichment of MOS. A direct effect of the permeate flux on the retention of MOS during NF could not be observed. The flux could not be directly correlated with pore size probably due to the differences in membrane materials, which was also reported by Luo et al. 2014 [27]. However, this process parameter gives important information about the fouling of the membrane. In laboratory scale the permeate flux is between 2.93 to 10.90 L $m^{-2}$ $h^{-1}$ during the concentration of the initial sample in the first step of NF **(Table 2.1)**.

In addition to the studies described above, the same range of flat-sheet membranes were applied in the NF on laboratory scale of SM-UF-permeate without lactose hydrolysis. The retention of the three MOS in NF retentate using the modules DL, CK, SR 100 and GE was between 25.4 ± 0.7 to 65.2 ± 10.3 % and was therefore distinctly lower than applying lactose hydrolyzed SM-UF-permeate as substrate. Especially, the MOS content in relation to total

sugar content was substantially lower (0.2 ± 0.0 % to 1.6 ± 0.1 %) and almost on the same level as in the original sample (0.1 ± 0.0%). This results reveal that the enrichment of MOS and the permeation of residual sugars is more effective, when lactose hydrolyzed SM-UF-permeate is used. This result is comparable with the work of Oliveira et al. (2014), who reported that NF by the DL membrane did not result in a sufficient separation of MOS from lactose in lactose unhydrolyzed ultrafiltered caprine whey [20].

*II.4.1.2 Pilot plant scale NF*

The enrichment of MOS on pilot plant scale was performed by NF using the spiral-wound membranes DL and SR 50 at 5 and 10 bar. The DL is similar to the membrane used on laboratory scale. The SR 50 membrane is comparable to the flat-sheet membrane SR 100 **(Table 2.1)**, but conducted as a spiral-wound membrane. **Table 2.3** reveals the contents of MOS, as well as mono- and disaccharides in the lactose hydrolyzed SM-UF-permeate and retentates after NF at different pressures. The sum of the three MOS determined in the initial lactose hydrolyzed SM-UF-permeate was slightly higher (83.31 mg/L) than in the initial sample for NF on laboratory scale. This is maybe due to the biological variation of bovine milk composition and to a possible small variability in the UF processes performed. The highest MOS content, which could be achieved after NF in the retentate was twice of the concentration in the initial sample (concentration factor = 2). The amounts of monosaccharides in the MOS retentates after NF on pilot plant scale were higher than in retentates on laboratory scale. The retentates produced by the application of the membranes DL and SR 50 at 5 and 10 bar exhibited MOS contents of 14.9 ± 3.3 mg/L to 18.8 ± 4.6 mg/L 6'-SL, 96.8 ± 10.1 mg/L to 112.2 ± 16.8 mg/L 3'-SL and 36.0 ± 16.0 mg/L to 46.9 ± 12.6 mg/L GalNAc-Gal-Glc. No significant differences in the concentration of single MOS by using the different membranes and pressures were observed. The dry mass contents in the retentates of pilot plant scale NF were slightly higher than in the concentrates of NF performed on laboratory scale. This is might be due to the higher content of monosaccharides **(Table 2.3)**.

**Table 2.3** Concentration of MOS (3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose), mono- and disaccharides (glucose, galactose, lactose) and dry mass in lactose hydrolyzed SM-UF-permeate and retentates of pilot plant scale NF (average of two trials, n=2), (n.d.= below limit of determination). The recovery of the standards was 73.9 ± 7.7 % for 3'-SL, 87.35 ± 4.05 % for 6'-SL and 95.6 ±7.1 % for GalNAc-Gal-Glc. (n=2)

| **sample** | **lactose hydrolyzed SM-UF-permeate** | | | **NF retentate SR 50** | | | | | | **NF retentate DL** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 bar | | | 10 bar | | | 5 bar | | | 10 bar | | |
| **6'-SL [mg/L]** | 8.6 | +/- | 0.5 | 18.8 | +/- | 4.6 | 15.7 | +/- | 0.2 | 15.5 | +/- | 3.6 | 14.9 | +/- | 3.3 |
| **3'-SL[mg/L]** | 47.5 | +/- | 1.8 | 112.2 | +/- | 16.8 | 96.8 | +/- | 10.1 | 97.2 | +/- | 19.5 | 97.3 | +/- | 22.3 |
| **GalNAc-Gal-Glc [mg/L]** | 27.2 | +/- | 2.9 | 45.8 | +/- | 5.8 | 36.0 | +/- | 16.0 | 46.9 | +/- | 12.6 | 44.7 | +/- | 10.1 |
| **∑ MOS [mg/L]** | | 83.3 | | | 176.8 | | | 148.5 | | | 159.6 | | | 156.9 | |
| **lactose [g/L]** | | n.d. | | | n.d. | | | n.d. | | | n.d. | | | n.d. | |
| **glucose [g/L]** | 21.7 | +/- | 0.9 | 1.4 | +/- | 0.7 | 1.2 | +/- | 0.3 | 2.0 | +/- | 0.2 | 2.2 | +/- | 0.3 |
| **galactose [g/L]** | 19.3 | +/- | 0.6 | 1.4 | +/- | 0.6 | 1.1 | +/- | 0.4 | 1.8 | +/- | 0.2 | 2.1 | +/- | 0.2 |
| **dry mass [g/L]** | 61.8 | +/- | 0.8 | 11.0 | +/- | 2.2 | 8.8 | +/- | 2.3 | 13.4 | +/- | 0.4 | 14.0 | +/- | 0.4 |

**Figure 2.2** reveals the retention of MOS in NF retentate applying the DL and SR 50 membranes at 5 bar and 10 bar (white, grey, and black bars; left ordinate). In addition, the MOS contents in relation to total sugar content are shown (striped bars, right ordinate). The retention of MOS in the retentates of pilot plant scale NF was at the same order of magnitude than on laboratory scale NF. During NF on pilot plant scale applying the membranes DL and SR 50 at 5 and 10 bar a sufficient enrichment of the MOS was achieved with a retention between 51.6 ± 18.7 % and 77.5 ± 9.3 % for all experiments. No significant differences in the retention of MOS in the NF retentates between the different membranes and pressures applied could be observed. The content of MOS in relation to total sugar content was between 5.0 ± 2.0 % and 7.2 ± 3.7 % in the retentates using the membrane SR 50 and 3.6 ± 1.1 % to 4.0 ± 0.5 % in the retentate applying the module DL. The contents are lower, than those achieved on laboratory scale. The reason therefore seems to be the lower permeation of the mono- and disaccharides **(Table 2.3)**. The total yield of MOS could be increased by the transfer from laboratory to pilot plant scale NF due to higher input volumes of lactose hydrolyzed SM-UF-permeate and higher amounts of retentates achieved after NF on pilot plant scale.

The permeate flux of the filtration processes on pilot plant scale NF are with 300.0 L $m^{-2}$ $h^{-1}$ to 531.6 L $m^{-2}$ $h^{-1}$ for the module SR 50 and with 34.9 L $m^{-2}$ $h^{-1}$ to 71.2 L $m^{-2}$ $h^{-1}$ for the membrane DL at 5 and 10 bar much higher than by filtration on laboratory scale. Although, the same types of membranes on laboratory and pilot plant scale NF were applied, large differences in flux were detected. The main reason could be that with flat sheet membranes and spiral wound membranes different constructional types of membranes were employed. In addition, the pressure varied between 4 bar on laboratory scale and 5 to 10 bar on pilot plant scale NF. Furthermore, different devices for NF on the scales of production were used, which could also influence the permeate flux. Nevertheless, comparable retention of MOS in retentate was achieved with NF on laboratory and pilot plant scale.

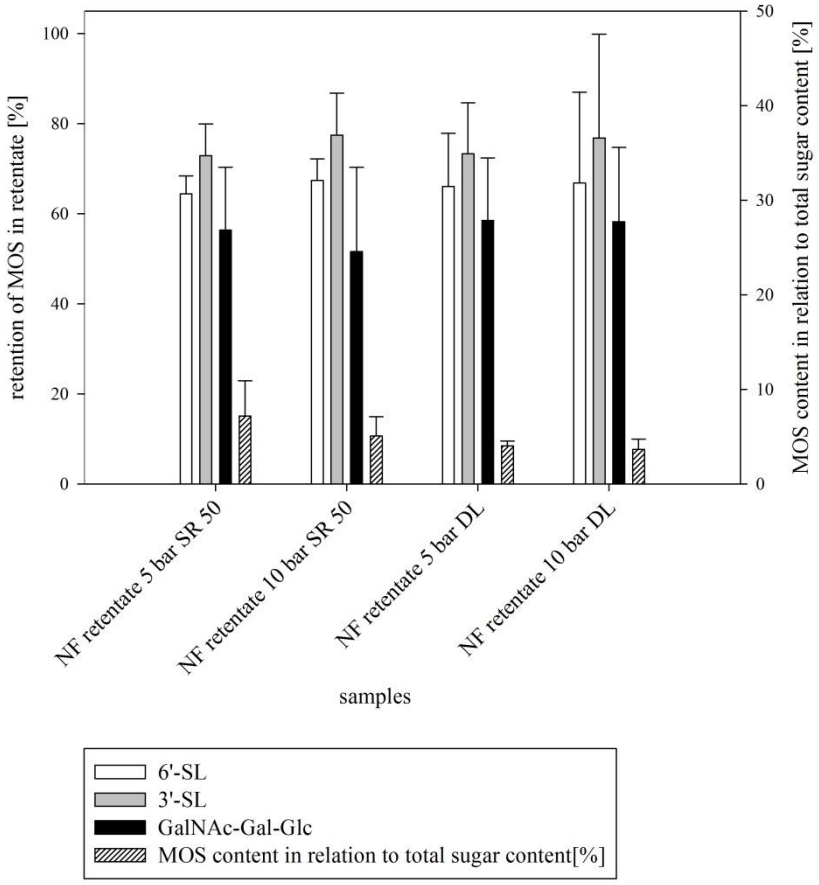



**Fig. 2.2** Retention of MOS by NF on pilot plant scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate), (average of two trials, n=2, 3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose). MOS content in relation to total sugar content in the initial SM-UF-permeate is 0.1 - 0.2 %.

*II.4.1.3 Industrial scale NF*

NFs on industrial scale were performed in cooperation with the Theo Müller Group at Sachsenmilch, Leppersdorf, Germany. 1000 L lactose hydrolyzed SM-UF-permeate were concentrated by NF and diafiltered with the 300 Da spiral-wound membrane DOW **(Table 2.1)**. NFs were performed in duplicate. The average concentration of the MOS, mono- and disaccharides in the lactose hydrolyzed SM-UF-permeate, the retentate after concentration and the retentate after diafiltration are listed in **Table 2.4 (a)**.

**Table 2.4 (a)** Concentration of MOS (3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose), mono- and disaccharides (glucose, galactose, lactose) and dry

mass in lactose hydrolyzed SM-UF-permeate and retentates of industrial scale NF (average of two trials, n=2). Variation of concentration in NF retentate after diafiltration relates to differing trial volumes (trial 1: 60 L, trial 2: 45 L), (n.d.= below limit of determination). The recovery of the standards was 73.9 ± 7.7 % for 3'-SL, 87.35 ± 4.05 % for 6'-SL and 95.6 ±7.1 % for GalNAc-Gal-Glc. (n=2)

| **sample** | **lactose hydrolyzed SM-UF-permeate** | **NF retentate after concentration** | **NF retentate after diafiltration** |
|---|---|---|---|
| | | **DOW** | |
| **6'-SL [mg/L]** | 9.5 +/- 1.4 | 90.9 +/- 1.7 | 105.1 +/- 32.0 |
| **3'-SL[mg/L]** | 46.6 +/- 6.3 | 456.3 +/- 6.9 | 515.0 +/- 132.2 |
| **GalNAc-Gal-Glc [mg/L]** | 41.4 +/- 4.3 | 287.4 +/- 49.5 | 253.2 +/- 32.6 |
| **∑ MOS [mg/L]** | 97.5 | 834.5 | 873.3 |
| **lactose [g/L]** | n.d. | n.d. | n.d. |
| **glucose [g/L]** | 22.3 +/- 0.9 | 33.8 +/- 5.2 | 4.6 +/- 0.1 |
| **galactose [g/L]** | 19.0 +/- 0.7 | 27.8 +/- 4.6 | 3.6 +/- 0.1 |
| **dry mass [g/L]** | 58.8 +/- 2.0 | 105.1 +/- 18.9 | 29.4 +/- 0.0 |

The total MOS content in the lactose hydrolyzed SM-UF permeate (97.5 mg/L) differ again slightly to the initial samples for laboratory and pilot plant scale NF, probably due to the natural variation of the composition of bovine milk and a possible small variability in the UF process. The dry mass content in the original sample was at the same level as on laboratory and pilot plant scale. The dry mass is composed mainly of sugars, milk salts and organic acids. Because of the high concentration factor of 10, the MOS content in the retentates of industrial scale NF was much higher than in retentates from laboratory and pilot plant scale experiments. In the NF retentate after concentration, but before diafiltration, the total MOS content in 100 L was about 834.5 mg/L. The concentration of the acidic MOS 6'-SL (90.9 ± 1.7 mg/L) and 3'-SL (456.3 ± 6.9 mg/L) were almost 10 fold higher and the content of the neutral MOS GalNAc-Gal-Glc (287.4 ± 49.5 mg/L) was about 7 fold higher than in the initial lactose hydrolyzed SM-UF-permeate. Furthermore, high amounts of the monosaccharides permeated during filtration so that only 14 to 15 % of these sugars were retained in the retentate. As expected, the dry mass content in the NF retentate after concentration (105.1 ± 18.9 g/L) was about 2 fold higher than in the initial sample. This is probably due to the concentration of salts, monosaccharides, organic acids and other minor components. The contents of the monosaccharides in NF retentate after concentration were higher than in the

initial sample. In NF retentate after diafiltration (trial 1: 60 L, trial 2: 45 L) the total MOS content was 873.23 mg/L. Moreover, a higher permeation of monosaccharides, salts and other components could be achieved by the diafiltration steps. The concentration of monosaccharides and the dry mass content in the retentate after diafiltration could be reduced markedly.

**Table 2.4 (b)** Protein, non-protein nitrogen, ash and salt content of NF retentate after diafiltration generated on industrial scale NF by using the membrane DOW (average of two trials, n=2).

| **sample** | **NF retentate after diafiltration** | | |
|---|---|---|---|
| | **DOW** | | |
| **protein [g/L]** | 0.6 | ± | 0.5 |
| **non-protein nitrogen [g/L]** | 0.2 | ± | 0.1 |
| **ash [g/L]** | 7.3 | ± | 0.1 |
| **sodium [g/L]** | 0.3 | ± | 0.1 |
| **potassium [g/L]** | 1.6 | ± | 0.2 |
| **magnesium [g/L]** | 0.3 | ± | 0.1 |
| **calcium [g/L]** | 1.4 | ± | 0.1 |
| **phosphor [g/L]** | 0.6 | ± | 0.1 |
| **chloride [g/L]** | 0.1 | ± | 0.0 |
| **citric acid [g/L]** | 6.9 | ± | 0.6 |

Protein content (0.6 ± 0.5 g/L) and non-protein nitrogen content (0.2 ± 0.1 g/L) were low in the NF retentate after diafiltration from industrial scale experiments **(Table 2.4 (b))**. The citric acid content was surprisingly high (6.9 ± 0.6 g/L) and contributes to 23.5 % of the dry mass of the NF retentate. The concentration of ash was 7.3 ± 0.1 g/L and therefore at the same order of magnitude as in bovine milk (7 g/L) [28]. Main components of ash were salts. The concentrations of potassium (1.6 ± 0.2 g/L) and calcium (1.4 ± 0.1) were as high as in bovine milk (potassium 1.5 g/L, calcium 1.2 g/L) [28]. The concentrations of sodium (0.3 ± 0.1) and chloride (0.1 ± 0.0) in the MOS retentate after diafiltration were lower than in bovine milk (sodium 0.5 g/L, chloride 1.0 g/L) [28]. Only magnesium was concentrated (0.3 ± 0.1 g/L) to the 3 fold in comparison the concentration in to bovine milk (magnesium 0.1 g/L) [28]. Other components of ash could be sulfate and micronutrients.

**Figure 2.3** shows the retention of MOS in the retentate after concentration and in the retentate after diafiltration (white, grey, and black bars; left ordinate) on industrial scale NF. In addition, the MOS content in relation to total sugar content for the lactose hydrolyzed SM-UF-permeate, the retentate after concentration and the retentate after diafiltration is shown (striped bars, right ordinate).

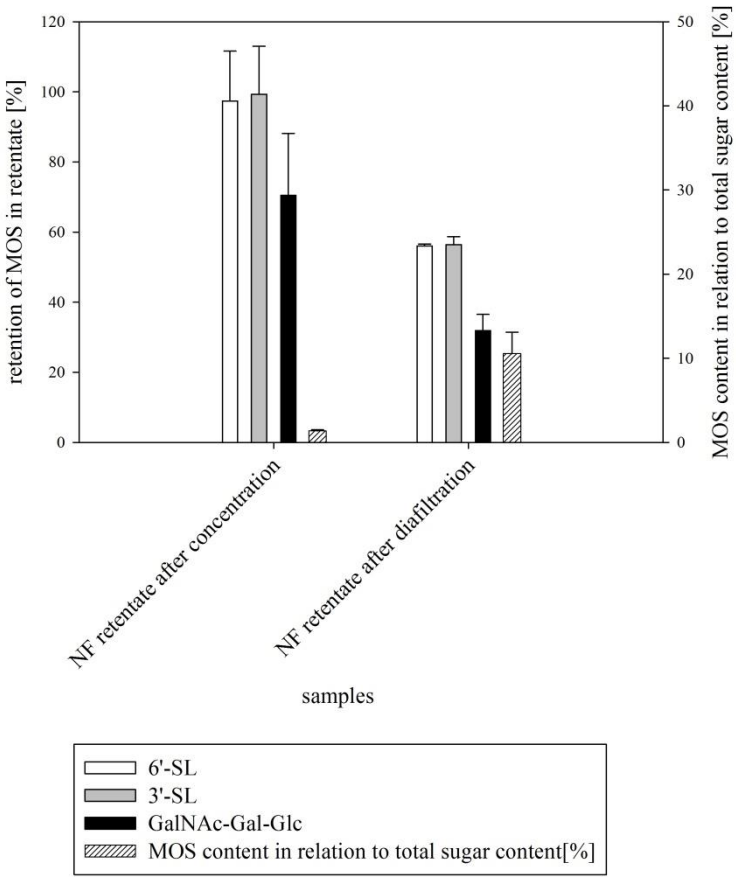



**Fig. 2.3** Retention of MOS by NF on industrial scale [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate), (average of two trials, n=2, 3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose). MOS content in relation to total sugar content in the initial SM-UF-permeate is 0.1 - 0.2 %.

A substantially high retention of MOS was achieved in NF retentate after concentration (97.4 ± 14.2 % 6'-SL, 99.3 ± 13.7 % 3'-SL, 70.4 ± 17.7 % GalNAc-Gal-Glc). The MOS content in relation to total sugar content (1.4 ± 0.1 % ) was only slightly higher than in the initial sample,

which can be explained by a high retention of monosaccharides **(Table 2.4 (a))**. In the NF retentate after diafiltration the retention of MOS was about 45 to 57 % lower than in the retentate after the concentration. 56.0 ± 0.6 % 6'-SL, 56.4 ± 2.3 % 3'-SL and 31.9 ± 4.6 % GalNAc-Gal-Glc were retained in the retentate after diafiltration. The MOS content in relation to total sugar content was 10.6 ± 2.5 % and thereby 10 fold higher than in the retentate after concentration and 100 fold higher than in the initial sample of the lactose hydrolyzed SM-UF-permeate. The diafiltration resulted in a lower retention of MOS in retentate but also in a much higher content of MOS in relation to total sugar content because of the higher permeation of monosaccharides. However, the content of MOS in relation to total sugar content in the retentate from laboratory scale NF is 3 fold higher than in retentate after diafiltration from industrial scale NF. A reason for the lower separation of the residual sugars from the MOS on industrial scale could be the diafiltration with 4.6 diavolumes. Diafiltration on laboratory scale was performed with 10 diavolumes. For a better separation of the residual sugar from the MOS in retentate produced on industrial scale, a further NF and diafiltration with the spiral-wound membranes SR 50 and DL might be useful.

The dry mass of the NF retentate after diafiltration is composed of 2.3 % protein, 0.6 % non-protein nitrogen, 2.6 % MOS (3'-SL, 6'-SL, GalNAC-Gal-Glc), 28.3 % mono- and disaccharides (glucose, galactose, lactose), 23.5 % citric acid and 24.7 % ash. These results are comparable to the findings of Mehra et al. (2014) [7]. In this study MOS were enriched by ultrafiltration in previously clarified mother liquor. The concentration of sialyl-lactoses (3'-SL, 6'-SL), ash and disaccharides (lactose) in the concentrate are at the same level as in our experiments on industrial scale. However, the protein content (7.4 %) was 5-fold higher and the non-protein nitrogen content (2.0 %) was about 3-fold higher than in the NF-retentate produced on industrial scale in our study. It can be concluded that our experiment on industrial scale resulted in a MOS concentrate with comparable composition than the concentrate of Mehra et al. (2014) [7] but lower protein and non-protein nitrogen content. It has to be considered that apart from diafiltration also other parameters could influence the permeation of mono-and disaccharides. Even though the NF on pilot plant scale was also performed in the same manner as on laboratory scale with 10 diavolumes, the content of MOS in relation to total sugar content on pilot plant scale was lower than on laboratory and industrial scale. The permeate flux of the NF on industrial scale was with 9.4 L $m^{-2}$ $h^{-1}$ at the

same level as by application of the membranes MPF 36 (10.9 L $m^{-2}$ $h^{-1}$) and GE (7.8 L $m^{-2}$ $h^{-1}$) on laboratory scale.

### *II.4.2 Composition of sugars in MOS concentrates*

**Figure 2.4** shows the HPAEC chromatograms of the initial SM-UF-permeate (I), the lactose hydrolyzed SM-UF-permeate (II), and the NF retentate after diafiltration by the DOW membrane at 10-15 bar (III, industrial scale). Due to the separation of interfering high amounts of mono- and disaccharides by sample pretreatment with graphitized carbon solid-phase extraction, only low residual amounts of galactose (peak 1), glucose (peak 2) and lactose (peak 3) were detected in the samples. In bovine colostrum more than fifty different milk oligosaccharides have been detected and 37 oligosaccharides have been structural characterized [29-31]. Thirteen of the complex oligosaccharides in bovine milk have been identified previously in human milk. In mature bovine milk the concentration of the oligosaccharides is low compared to colostrum [10]. In the SM-UF-permeate (I) the most abundant oligosaccharides GalNAc(α1-3)-Gal(β1-4)-Glc (peak 5, retention time 23.9 min), 6'-SL (peak 6, retention time 39.3 min), 3'-SL (peak 7, retention time 39.8 min) and a hexosetrisaccharide (Peak *, retention time 34.3 min) could be identified by their molecular masses and fragmentation patterns in the $ms^2$ mass spectra. Four different galactotrioses have been so far characterized in bovine milk [31] : Gal(α1-3)-Gal(β1-4)-Glc, Gal(β1-3)-Gal(β1-4)-Glc, Gal(β1-4)-Gal(β1-4)-Glc (4-GL), and Gal(β1-6)-Gal(β1-4)-Glc (6-GL). According to the retention time this galactotriose could be related to Gal(α1-3)-Gal(β1-4)-Glc or Gal(β1-3)-Gal(β1-4)-Glc, but Gal(α1-3)-Gal(β1-4)-Glc has been described as most abundant galactotriose in bovine milk [30].

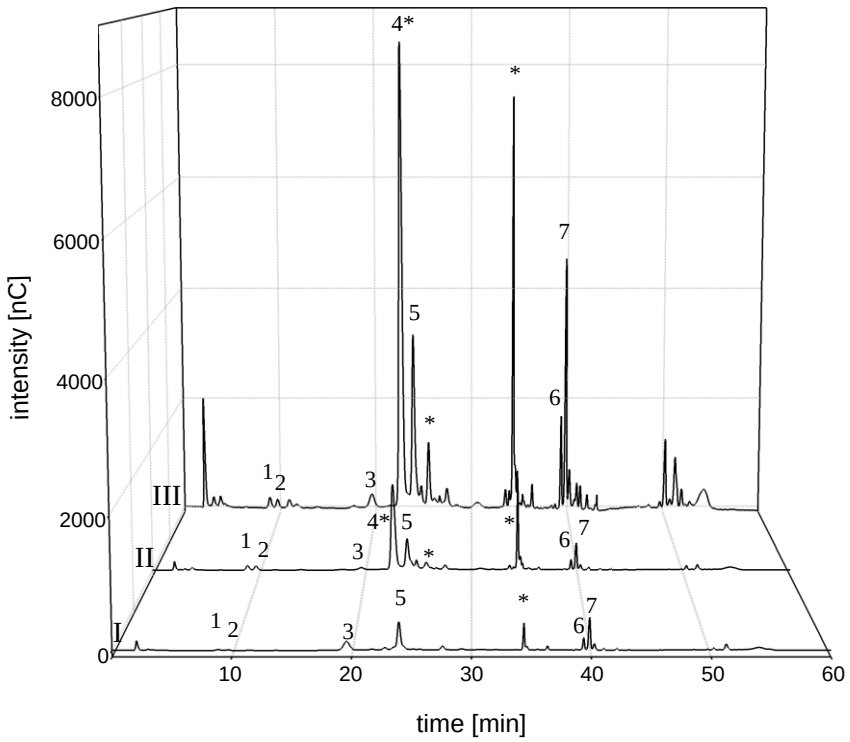



**Fig. 2.4** HPAEC chromatograms of the SM-UF-permeate before lactose hydrolysis (I) lactose hydrolyzed SM-UF-permeate (II), and NF retentate after diafiltration of the membrane DOW at 10-15 bar (III, industrial scale). For separation of mono- and disaccharides samples were purified by graphitized carbon solid-phase extraction. (1= galactose, 2= glucose, 3= lactose, 4= 6'-glalctosyl-lactose, 5= GalNAc-Gal-Glc, 6= 6'-SL, 7= 3'-SL, * = GOS).

The comparison of the SM-UF-permeate before lactose hydrolysis (I) and after lactose hydrolysis (II) reveals comparable peak areas of the MOS (Peaks 5-7). Obviously, the process of enzymatic lactose hydrolysis did not led to a degradation of the MOS. However, as a result of the lactose hydrolysis by the β-galactosidase from *Kluyveromyces lactis* considerably amounts of galacto-oligosaccharides (GOS, indicated with an asterisk) were synthesized. The GOS 6'-galactosyllactose is depicted in peak 4 at a retention time of 22.6 min in the lactose hydrolyzed SM-UF-permeate (II) and in the NF retentate after diafiltration applying the DOW membrane. As described in a study by Frenzel et al. 2015, 6'-glalctosyllactose belongs to the main GOS produced by the transgalactosylation activity of this β-galactosidase [23]. Moreover, other GOS were detected in the lactose hydrolyzed SM-UF-permeate (II) (peaks

indicated with asterisk). In comparison to the chromatogram of the SM-UF-permeate (I) a significant increase of the galactotriose (Peak *, retention time 34.3 min) could also be detected in the lactose hydrolyzed SM-UF-permeate (II) indicating also the synthesis of Gal(β-13)-Gal(β1-4)-Glc by the transgalactosylation activity of this β-galactosidase. In the retentate produced on industrial scale NF using the membrane DOW (III), a markedly increase of the peak areas of the MOS (peaks 5-7) and GOS (peak 4 and peaks with asterisks) in comparison to the SM-UF-permeate before lactose hydrolysis (I) and after lactose hydrolysis (II) can be seen.

### *II.4.3 The effect of MOS concentrates on the NFκB activity in TNF-α induced HEK$^{nfkb\text{-}RE}$ cells*

Applying the NF on pilot plant and industrial scale provided a sufficient amount of MOS concentrates for the evaluation of the immune stimulatory effects. Therefore, the effect of the NF retentates on NFκB activity in HEK$^{nfkb\text{-}RE}$ cells *in vitro* was evaluated. Luciferase activity of cells treated with DMEM medium without sample was standardized to 1 **(Figure 2.5)**. The lactose hydrolyzed SM-UF-permeate and the retentates produced by NF on pilot plant and industrial scale revealed increased luciferase activity, and therefore, increased NFκB activity in HEK$^{nfkb\text{-}RE}$ cells. Addition of the lactose hydrolyzed SM-UF-permeate to the cells resulted in an over 1.5 fold increase of luciferase activity from 1 to 1.8 ± 0.6. The highest luciferase activity (2.8 ± 0.3) was measured by incubation of the cells with the NF retentate produced by the membrane DL on pilot plant scale filtration. The retentate generated on pilot plant scale NF with the membrane SR50 showed also an increased luciferase activity (1.7 ± 0.2) in comparison to the control. An effect in the same order of magnitude was achieved by incubation of the cells with the retentate generated on industrial scale NF with the membrane DOW. Moreover, treatment of cells with the standard 6'-SL resulted in a significant increase in luciferase activity (1.5 ± 0.1). Also the standard 3'-SL showed an increased luciferase activity at the same level (1.6 ± 0.3).

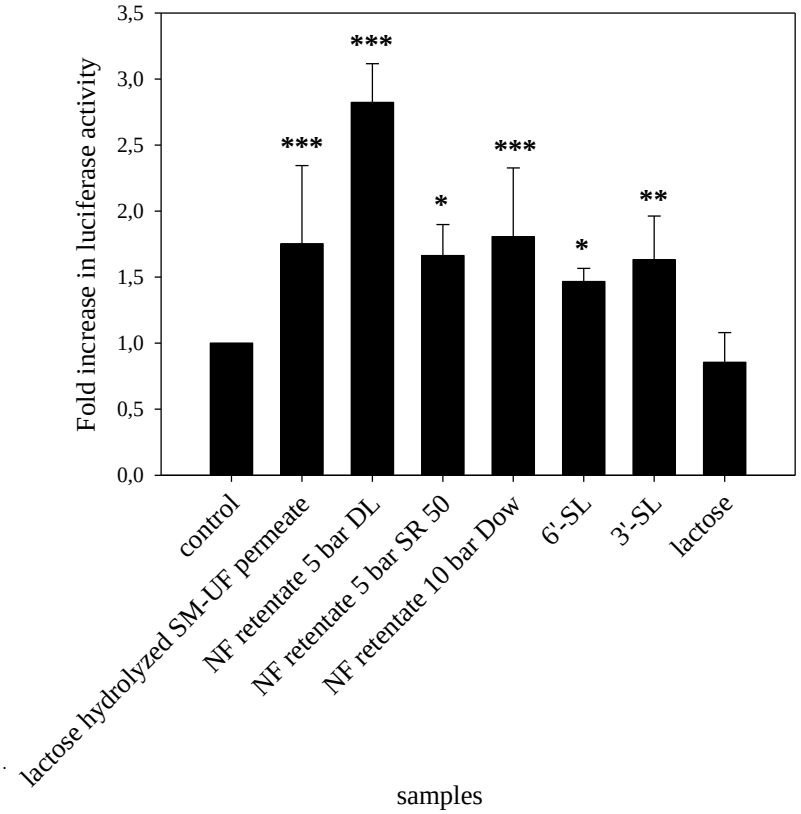



**Fig. 2.5** Potentially immunostimulatory activity of NF retentates in HEK$^{nfκB\text{-}RE}$ cells measured as luciferase activity. Lactose hydrolyzed SM-UF permeate, NF retentates produced by the membranes DL and SR 50 on pilot plant scale, NF retentates generated by the module DOW on industrial scale, and the standards 6'-SL, 3'-SL, and lactose were tested. Luminescence of treated cells is shown as fold increase compared with the control (=1). Values are means ± standard deviations (SD) of two NF retentates with at least two independent replicates of cell incubation and luciferase activity determination. Significance values were calculated using Kruskal Wallis one-way analysis followed by the Dunn's multiple comparison test. Significance values were determined in comparison to the control without sample, ***: $p < 0.001$ versus control, **: $p < 0.01$ versus control, *: $p < 0.05$ versus control.

The incubation of the TNF-α stimulated cells with lactose exhibited no significant effect on luciferase activity in comparison to the control. The increase of the NFκB-activity in TNF-α stimulated HEK$^{nfkb\text{-}RE}$ cells by incubation with NF-retentates and the standards 6'-SL and 3'-SL can be an incident for an immunostimulatory activity of MOS. As reported in the introduction, the immunostimulatory activity of MOS by the increase of cytokine production

was measured in different cells. Moreover, a regulating effect of MOS on the cytokine production was observed. While the incubation of IEC18 cells with MOS from goat milk exhibited an increased cytokine production in comparison to control without MOS, the treatment of these cells with MOS and the endotoxin LPS resulted in a reduction of cytokine production compared with cells which were incubated only with LPS. There is no significant difference in the concentration of cytokines produced by incubation of cells with MOS or MOS and LPS [32].

It has to be considered that the MOS concentrates can also contain further substances such as peptides, nucleotides or GOS, which could influence the effect of NFκB activity the stimulated cells. As shown in **Figure 4** some GOS are present in the NF-retentates. Ortega-González et al. reported also about an increase of cytokine production (GROα, MIP2, MCP1, GRO) in intestinal epithelial cells from rat (IEC18 cells) by activation of NFκB during incubation with GOS. However, the cytokine production was not as high as by treatment with MOS from goat milk and this effect could not be observed in human HT29 cells [32].

## II.5 Conclusion

The paper presents studies about the enrichment of MOS in lactose hydrolyzed skimmed and ultrafiltered milk permeate (SM-UF-permeate) by nanofiltration (NF) on laboratory, pilot plant and industrial scale. Most efficient results were achieved by application of membranes with a NMWCO of 150-400 Da. On laboratory scale, NF with the flat-sheet membranes DL and SR 100 resulted in a retention of MOS of 49.0 % to 84.8 %. The same level of retention could be achieved by application of the spiral-wound modules DL and SR 50 on pilot plant scale and by the membrane DOW on industrial scale NF. As a result of the feasibility study to transfer the NF process to large scale, markedly higher yields of MOS could be achieved. After NF on industrial scale using the module DOW a content of MOS in relation to total sugar content of 10.6 ± 2.5 % were reached (initial sample 0.1 – 0.2 %). This is equivalent to an enrichment of MOS by the factor 100. Therefore, the process developed, enables the successful enrichment of MOS and permeation of mono- and disaccharides during NF on industrial scale. The process parameters permeate flux and pressure could not be correlated to the retention of MOS in retentate or on the MOS content in relation to total sugar content. In consideration of the immune regulating role of NFκB, the evaluation of the influence of the NF retentate on this transcription factor in $HEK^{nf\kappa B\text{-}RE}$ cells is an incidence for immunostimulatory properties of the MOS concentrates.

The immunostimulatory effect of the MOS concentrates has to be further investigated *in vitro* e.g. by the measurement of the secretion and expression of cytokines in immune cells. In addition, it is important to investigate the immunostimulatory activity of the NF retentates in humans by a clinical trial *in vivo*. Moreover, a study about the filtration characteristics during the NF for the enrichment of MOS would help to understand the building and composition of the deposition of the layer and give additional information about the retention properties. Depending on the further application and preferences, it could be advantageous to conduct a further NF of the MOS concentrate for the reduction of the salt content.

Skimmed and ultrafiltered milk permeate is a product stream in the dairy industry and a high valuable source of MOS. This study shows process parameters for a method for an enrichment of MOS by NF on industrial scale. The feasibility of the concentration of MOS on large scale devices can result in the development of a bioactive food ingredient for the beverage, food and infant formula industry. Although there exist a few studies about the

enrichment of MOS by NF, this is the first report about a screening of the efficiency of different NF membranes for the enrichment of MOS on different technological scales of production. Furthermore, the immunostimulatory effect of the MOS concentrates produced by membrane separation process has not been evaluated before.

**Acknowledgement**

This research project is funded by the Federal Ministry for Education and Research (BMBF) as part of the competence network "FoCus- Food Chain Plus". NF on industrial scale was performed at the Theo Müller Group, Sachsenmilch, Leppersdorf, Germany.

## II.6 References

1. ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Feitsma AL, van Hoffen E, Schoterman MHC (2014) Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr Rev 72: 377-389

2. Mehra R, Kelly P (2006) Milk oligosaccharides: structural and technological aspects. Int Dairy J 16: 1334-1340

3. Kunz C, Rudloff S (2006) Health promoting aspects of milk oligosaccharides. Int Dairy J 16: 1341-1346

4. Oliveira DL, Wilbey RA, Grandison AS, Duarte LC, Roseiro LB (2012) Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. Int Dairy J 24: 102-106

5. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL, Killeen K, Grimm R, Lebrilla CB (2006) A strategy for annotating the human milk glycome. J Agric Food Chem 54: 7471-7480

6. Boehm G, Stahl B (2007) Oligosaccharides from milk. J of Nutr 137: 847S-849

7. Mehra R, Barile D, Marotta M, Lebrilla CB, Chu C, German JB (2014) Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. Plos One. doi:10.1371/journal.pone.0096040

8. Gnoth MJ, Rudloff S, Kunz C, Kinne RKH (2001) Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. J Biol Chem 276: 34363-34370

9. Eiwegger T, Stahl B, Schmitt J, Boehm G, Gerstmayr M, Pichler J, Dehlink E, Loibichler C, Urbanek R, Szepfalusi Z (2004) Human milk-derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. Pediatr Res 56: 536-540

10. Lane JA, O'Callaghan J, Carrington SD, Hickey RM (2013) Transcriptional response of HT-29 intestinal epithelial cells to human and bovine milk oligosaccharides. Br J Nutr 110: 2127-2137

11. Kurakevich E, Hennet T, Hausmann M, Rogler G, Borsig L (2013) Milk oligosaccharide sialyl(alpha2,3)lactose activates intestinal CD11c+ cells through TLR4. Proc Natl Acad Sci USA 110: 17444-17449

12. Rijnierse A, Jeurink PV, van Esch BCAM, Garssen J, Knippels LMJ (2011) Food-derived oligosaccharides exhibit pharmaceutical properties. European Journal of Pharmacology 668, (Supplement 1): 117-S123

13. Comstock SS, Wang M, Hester SN, Li M, Donovan SM (2014) Select human milk oligosaccharides directly modulate peripheral blood mononuclear cells isolated from 10-d-old pigs. Br J Nutr 111: 819-828

14. Mills S, Ross RP, Hill C, Fitzgerald GF, Stanton C (2011) Milk intelligence: mining milk for bioactive substances associated with human health. Int Dairy J 21: 377-401

15. Capitán-Cañadas F, Ortega-González M, Guadix E, Zarzuelo A, Suárez MD, de Medina FS, Martínez-Augustin O (2014) Prebiotic oligosaccharides directly modulate proinflammatory cytokine production in monocytes via activation of TLR4. Mol Nutr Food Res 58: 1098-1110

16. Gopal PK, Gill HS (2000) Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br J Nutr 84: 69-74

17. Zivkovic AM, Barile D (2011) Bovine milk as a source of functional oligosaccharides for improving human health. Adv Nutr 2: 284-289

18. Martinez-Ferez A, Guadix A, Guadix EM (2006) Recovery of caprine milk oligosaccharides with ceramic membranes. J Membr Sci 276: 23-30

19. Sarney DB, Hale C, Frankel G, Vulfson EN (2000) A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. Biotechnol Bioeng 69: 461-467

20. Oliveira D, Wilbey RA, Grandison A, Roseiro L (2014) Natural caprine whey oligosaccharides separated by membrane technology and profile evaluation by capillary electrophoresis. Food Bioprocess Technol 7: 915-920

21. Packer N, Lawson M, Jardine D, Redmond J (1998) A general approach to desalting oligosaccharides released from glycoproteins. Glycoconj J 15: 737-747

22. Bruggink C, Maurer R, Herrmann H, Cavalli S, Hoefler F (2005) Analysis of carbohydrates by anion exchange chromatography and mass spectrometry. J of Chromatogr A 1085: 104-109

23. Frenzel M, Zerge K, Clawin-Rädecker I, Lorenzen PC (2015) Comparison of the galacto-oligosaccharide forming activity of different β-galactosidases. LWT - Food Sci Technol 60: 1068-1071

24. Zerge K (2014) Charakterisierung und Gewinnung von Oligosacchariden als potenielle funktionelle Lebensmittelinhaltsstoffe. Doctoral Thesis,Technical University Dresden, Dresden, Germany

25. Malinowski J, Klempt M, Clawin-Rädecker I, Lorenzen PC, Meisel H (2014) Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. Food Chem 165: 129-133

26. Fong B, Ma K, McJarrow P (2011) Quantification of bovine milk oligosaccharides using liquid chromatography–selected reaction monitoring–mass spectrometry. J Agric Food Chem 59: 9788-9795

27. Luo J, Nordvang RT, Morthensen ST, Zeuner B, Meyer AS, Mikkelsen JD, Pinelo M (2014) An integrated membrane system for the biocatalytic production of 3′-sialyllactose from dairy by-products. Bioresour Technol 166: 9-16

28. Schlimme E, Buchheim W (1999) Milch und ihre Inhaltstoffe. Chemische und Physikalische Eigenschaften. Th. Mann, Gelsenkirchen, Germany

29. Mariño K, Lane JA, Abrahams JL, Struwe WB, Harvey DJ, Marotta M, Hickey RM, Rudd PM (2011) Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography. Glycobiology 21: 1317-1330

30. Albrecht S, Lane AL, Mariño K, Al Busadah KA, Carrington SD, Hickey RM, Rudd PM (2014) A comparative study of free oligosaccharides in the milk of domestic animals. Br J Nutr 111: 1313-1328

31. Urashima T, Fujita S, Fukuda K, Nakamura T, Saito T, Cowan P, Messer M (2014) Chemical characterization of milk oligosaccharides of the common brushtail possum *(Trichosurus vulpecula)*. Glycoconj J 31: 387-399

32. Ortega-González M, Ocón B, Romero-Calvo I, Anzola A, Guadix E, Zarzuelo A, Suárez MD, Sánchez de Medina F, Martínez-Augustin O (2014) Nondigestible oligosaccharides exert nonprebiotic effects on intestinal epithelial cells enhancing the immune response via activation of TLR4-NFκB. Mol Nutr Food Res 58: 384-393

# Chapter III

## NF enrichment of milk oligosaccharides (MOS) in relation to process parameters

Karina Altmann*, Ingrid Clawin-Rädecker, Wolfgang Hoffmann, Peter Chr. Lorenzen

*Submitted in Food and Bioprocess Technology*

*12 October 2015*

*Currently under review.*

Max Rubner-Institut Kiel, Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Straße 1, 24103 Kiel, Germany

*corresponding author

## III.1 Abstract

Concentrates containing bioactive milk oligosaccharides (MOS) are not yet available as functional ingredients for food, because of the lack of suitable procedures for their enrichment.

The aim of the present study was to develop an optimized nanofiltration (NF) process for the enrichment of MOS on pilot plant scale by achieving a better permeation of milk salts and residual sugars during NF at acidic (pH 5) and neutral (pH 7) milieu. A bovine retentate produced by NF with a 100-fold increase of MOS content in relation to total sugar content was applied. In addition, the process should be suitable for the enrichment of MOS in caprine milk.

MOS were identified by high performance anion exchange chromatography with pulsed amperometric detection. Generally, a higher enrichment of MOS in the final NF retentate (14 % MOS in dry mass, 5-fold higher than in first NF retentate, 140-fold higher than in initial sample) and a better separation of salts and residual sugars were achieved in bovine milk by NF at pH 5. The high MOS content in relation to total sugar content in the final NF retentate (92 %, 9-fold than in first NF retentate, 900-fold higher than in initial sample) indicated a nearly complete permeation of mono- and disaccharides. NF of caprine milk resulted in a 23-fold to 31-fold higher MOS content in relation to total sugar content than in the initial sample. With the present work the influence of the pH on the degree of enrichment of MOS by NF was evaluated for the first time.

**Keywords**

Enrichment of milk oligosaccharides (MOS), nanofiltration (NF), bovine milk, caprine milk, retention of MOS, MOS content in relation to total sugar content

## III.2 Introduction

Milk oligosaccharides (MOS) stimulate the immune system, inhibit the adhesion of pathogens on the epithelial surface and exhibit prebiotic activity (ten Bruggencate et al. 2014; Kunz and Rudloff 2006; Bode 2009; Gopal and Gill 2000; A Martinez-Ferez et al. 2006; R. Mehra and Kelly 2006). Especially in human milk, the concentration of MOS (5-8 g/L) is very high consisting of 200 different molecular structures, which are composed to 50-80 % neutral mainly fucosylated sugars and to 10-30 % acidic sialylated sugars (D. L. Oliveira et al. 2015; Bode 2012). Milk from domestic animals has a various oligosaccharide in common with human milk, even though their structure is less complex (Albrecht et al. 2014; Thum et al. 2015; Urashima et al. 2001; Boehm and Stahl 2007). Therefore, the scientists make efforts to develop procedures for the preparation of MOS concentrates as an alternative of MOS from human milk (D. L. Oliveira et al. 2015). Unfortunately, the MOS content in animal milk is markedly lower than in human milk. Bovine milk contains only 0.03-0.06 g/L MOS consisting of 91 % acidic acidic sugars (mainly 3-sialyl-lactose and 6-sialyl-lactose) (Albrecht et al. 2014). Oliveira et al. 2015 and Kiskini et al. 2013 reported that the amount of MOS in caprine milk is about 0.25-0.30 g/L and thereby 5-fold higher than in bovine milk, but still significantly lower than in human milk (Kiskini and Difilippo 2013; D. L. Oliveira et al. 2015). Caprine milk has a high level of sialylated (95 %) oligosaccharides and a neutral oligosaccharide fraction (5 %) with a profile closer to that of human milk than bovine or ovine milk profiles (Albrecht et al. 2014) (D. L. Oliveira et al. 2015).

MOS retentates are not yet available as functional ingredients for the food industry, because of the lack of established processes for their enrichment. Because of the low MOS concentration in domestic animal milk, and high capital cost and operational complexity no processes for the enrichment of MOS have been efficiently implemented on industrial scale so far. Only a few studies reported about the enrichment of MOS by membrane separation on laboratory scale (A. Martinez-Ferez et al. 2006; Sarney et al. 2000; D. Oliveira et al. 2014; Martinez-Ferez et al. 2008; A Martinez-Ferez et al. 2006; D. L. Oliveira et al. 2012). In a study from Mehra et al. (Raj Mehra et al. 2014) the concentration of bovine MOS by ultrafiltration (UF) on industrial scale was performed. Moreover, in our previous work (Altmann et al. 2015), a screening of the efficiency of different nanofiltration (NF) membranes for the enrichment of MOS on laboratory, pilot plant and industrial scale has been

performed for the first time. The NF retentate produced on industrial scale contained 10.6 % MOS (N-acetylgalactosaminyl-lactose, 3-sialyl-lactose, 6-sialyl-lactose) in total sugar, and 28.3 % monosaccharides (glucose, galactose), 24.7 % ash and 23.5 % citric acid in dry mass. These results are comparable to the achievement of Mehra et al. (2014), even though the filtration processes were conducted differently.

Depending on the envisage targets, a high recovery of MOS and/or a high purity of the retentate can be reached. For getting a higher purity of the MOS retentate, lactose has to be hydrolyzed to achieve a higher permeation of the saccharides and conditions for facilitating the permeation of milk salts have to be chosen. The pH has a strong impact on the dissociation degree of salts. For example, acidification to pH 5 increases dissociation of milk salts, especially calcium, and improves the permeation of the minerals due to its lower molecular masses. An improved separation of MOS from residual monosaccharides can be achieved by increasing the number of diafiltration steps with water.

Therefore, the aim of the present study was a further optimization of the process on pilot plant scale by achieving a better permeation of milk salts and residual sugars by nanofiltration (NF) at acidic (pH 5) and neutral milieu (pH 7). In addition, the process should be suitable for the enrichment of MOS in caprine milk.

## III.3 Materials and Methods

### *III.3.1 Nanofiltration assays*

In this work a pre-manufactured NF retentate of SM-UF permeate from bovine milk was used as a basic raw material for further NF. The method of the enrichment of MOS for production of the raw material is described in a study before (Altmann et al. 2015). In brief, bovine milk (from the Theo Müller Group, Sachsenmilch, Leppersdorf, Germany) was skimmed and ultrafiltered in order to get a skimmed milk permeate (bovine SM-UF permeate). For a better separation of the MOS from the residual sugar, the lactose in bovine SM-UF permeate was hydrolyzed (150 NL U/g lactose) by β-galactosidase (HA-Lactase 2100, Chr. Hansen, DK-2970 Horsholm, Denmark, 150 NL U/g lactose) at 40°C for 3 h in a tank reactor while stirring **(Fig. 3.1 a)**.

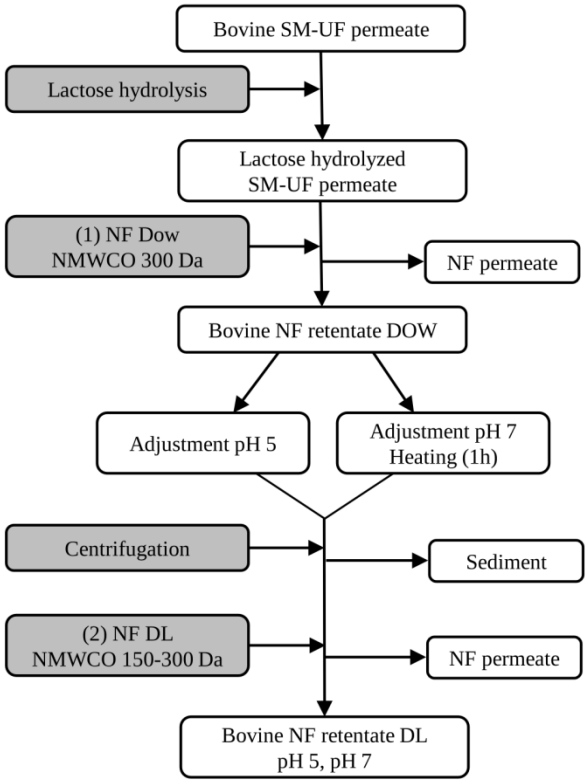



**Fig. 3.1 a)** Process for the enrichment of MOS by NF from bovine milk.

For the enrichment of MOS a first NF was conducted by using the spiral wound membrane module DOW® (NF-380-30-FF, the DOW chemical company, Midland, USA) on industrial scale in cooperation with the Theo Müller Group. 1000 L of lactose hydrolyzed SM-UF permeate were concentrated to 100 L (concentration factor = 10). The NF retentate was diafiltered with 460 L demineralized water (4.6 diavolumes). This bovine NF retentate **(Fig. 3.1a)** was the source for the experiments in this study.

Initially, one half of the bovine NF retentate was adjusted to pH 5 and the other half of the concentrate was adjusted to pH 7 and heated for 1 h at 75°C. The precipitates in the NF retentates were separated by centrifugation. For a further enrichment of MOS in the NF retentates and a better separation of residual sugars and salts a second NF was conducted. These cross-flow NFs were performed with the bovine NF retentate at pH 5 and 7 using the spiral wound membrane module DL (150-300 Da nominal molecular weight cut-off (NMWCO), 1.7 $m^2$ active membrane area) **(Table 3.1)**. The membrane filtration was performed with the Alfa Laval Lab M20 device (Alfa Laval Corporate AB, SE-22655, Lund, Sweden) at 5 bar and 40 °C.

**Table 3.1** Properties of NF membranes applied for the enrichment of MOS on pilot plant scale and corresponding experimental set-ups (a= module DL; b= module SR 50; NMWCO = nominal molecular weight cut-off; concentration factor *CF*= the ratio of the initial feed volume $V_f$ [L] to the retentate volume $V_r$ [L]; diavolume *DV* = the ratio of the total volume of liquid permeated $V_p$ [L] and the initial volume).

| **Dimensions of experiments** | **Spiral-wound membranes** | **Manufacturer** | **Material** | **NMWCO [Da]** | **Active membrane area** |
|---|---|---|---|---|---|
| Pilot plant scale | SR 50 (2540 SR2-N1)® | Koch Membrane Systems | thin film composite | 300-400 | 2.5 $m^2$ |
| | DL (2540C1077)® | Desalogics | thin film | 150-300 | 1.7 $m^2$ |

| **Experimental set-up** | | | | | | |
|---|---|---|---|---|---|---|
| **Origin of milk** | **Temperature [°C]** | **Pressure [bar]** | **Initial feed volume *($V_f$)* [L]** | **Concentration factor *(CF)*** | **Diavolume *(DV)*** | **Flux *(F)* [L/$m^2$h]** |
| Caprine milk | 40 | 5 | 6 | 2 | 10 | 37.6 ± 1.0 [a]<br>28.7 ± 1.6 [b] |
| Bovine milk | 40 | 5 | 10 | 4 | 20 | 31.6 ± 2.6 [a,c]<br>27.8 ± 0.2 [a,d] |

For characterization of the NF process, the following parameters were considered:

In the first part of NF, the initial sample was concentrated to enrich the content of MOS in NF retentate. The concentration factor *CF* is the ratio of the initial feed volume $V_f$ [L] to the retentate volume $V_r$ [L].

(1)

$$CF = \frac{V_f}{V_r}$$

In the second part the retentate was diafiltered with demineralized water to decrease the contents of glucose, galactose, residues of lactose, and milk salts in the MOS retentate. The number of diavolumes *DV* is defined as the ratio of the total volume of liquid permeated $V_p$ [L] and the initial volume, which corresponds to the retentate volume $V_r$ [L] from the first step of the NF in our ecperiments.

(2)

$$DV = \frac{V_p}{V_r}$$

The volumetric flux of permeate *F* [$L/m^2h^1$] is the ratio of liquid $V_p$ [L] permeated in one hour *t* [h] and the active membrane area *A* [$m^2$] of the corresponding module.

(3)

$$F = \frac{V_p}{A\,t}$$

Another important parameter is the percental retention $R_i$ of a component i (e.g. 3'-SL, 6'-SL, GalNAc-Gal-Gluc) in NF retentate. $C_{r,i}$ is the concentration of the component i in retentate and $C_{f,i}$ is the concentration of the component i in the initial feed sample.

(4)

$$R_i = \frac{C_{r,i}\,V_r}{C_{f,i}\,V_f}\ 100$$

Furthermore, the percental content of MOS (3'-SL, 6'-SL, GalNAc-Gal-Glc) in relation to total sugar content (quantified major MOS, glucose components, galactose, lactose) $M_{MOS/total\ sugar}$ was calculated as ratio of the concentration of MOS in retentate $C_{r,MOS}$ [mg/L] to the concentration of total sugar in retentate $C_{r,total\ sugar}$ [mg/L].

(5)

$$M_{MOS/total\ sugar} = \frac{C_{r,MOS}}{C_{r,total\ sugar}}\ 100$$

At the beginning of nanofiltering the bovine concentrate, retentate and permeate were circulating in the filtration device for 30 min to firm a deposit layer. 10 L of the NF retentate at pH 5 and 7 were concentrated to 2.5 L (concentration factor= 4). The NF retentate was diafiltered with 20 diavolumes by stepwise addition of 10 L demineralized water to 2.5 L retentate for five times. Water was added when retentate was concentrated to 2.5 L. After discontinuous diafiltration with 50 L demineralized water, the NF retentate was further concentrated up to the residual volume of the filtration device of 2.2-2.4 L.

Caprine milk (from the caprine farm Rehder, Boksee, Germany) was skimmed using a disc centrifuge (LWA 205-1, Westfalia Separator AG, 4740 Oelde, Germany) and ultrafiltered on a 5 kDa hollow fiber membrane (CTG.1" HF 1.0-43-PM5-P3, Koch Membrane Systems, Wilmington, USA). The lactose in the caprine skimmed and ultrafiltered milk permeate (caprine SM-UF permeate) was hydrolyzed (150 NL U/g lactose) (HA-Lactase 2100, Chr. Hansen, DK-2970 Horsholm, Denmark, 150 NL U/g lactose) at 40°C for 3 h in a tank reactor while stirring **(Fig. 3.1 b)**. For the enrichment of MOS, cross-flow NFs of the lactose hydrolyzed SM-UF-permeate were performed at 5 bar and 40°C **(Table 3.1)** by using the spiral wound membrane modules DL (150-300 Da NMWCO, 1.7 $m^2$ active membrane area) and SR 50 (300-400 Da NMWCO, 2.5 $m^2$ active membrane area). The membrane filtration was performed with the Alfa Laval Lab M20 device (Alfa Laval Corporate AB, SE-22655, Lund, Sweden). To get a deposit layer, retentate and permeate were circulating in the unit for 30 min. Then 6 L of lactose hydrolyzed caprine SM-UF permeate were concentrated to 3 L (concentration factor= 2). The NF retentate was diafiltered with 10 diavolumes by adding stepwise 6 L of demineralized water for 5 times when the retentate was concentrated to 3 L.

After discontinuous diafiltration with 30 L water, the NF retentate was concentrated to the residual volume of the membrane device of 2.2-2.4 L.

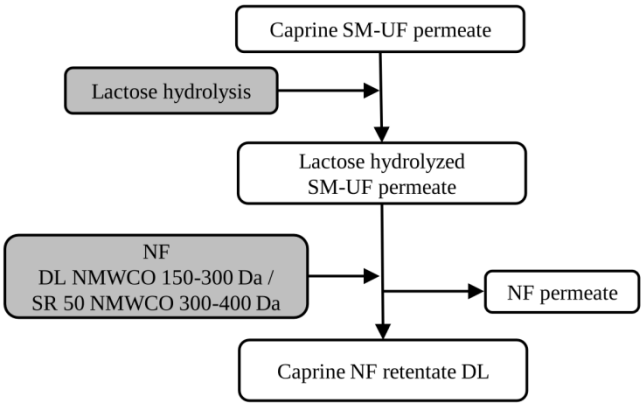



**Fig. 3.1 b)** Process for the enrichment of MOS by NF from caprine milk.

### *III.3.2 Analytical methods*

#### *III.3.2.1 Quantification of MOS, mono- and disaccharides*

##### *III.3.2.1.1 Sample pretreatment*

Milk oligosaccharides were purified by graphitized carbon solid-phase extraction according to Altmann et al. 2015 (Altmann et al. 2015). For the determination of galactose, glucose and lactose content the samples were clarified using Carrez solutions I and II.

##### *III.3.2.1.2 Determination of milk oligosaccharides by HPAEC-PAD/MS*

The analyses of carbohydrates were performed by a high performance anion exchange chromatography with pulsed amperometric detection and parallel online mass spectrometry (Bruggink et al. 2005; Frenzel et al. 2015; Zerge 2014; Altmann et al. 2015).

Pretreatment and analysis of the samples were performed in duplicate. Glucose, galactose, lactose (from VWR, Darmstadt, Germany), N-acetyl-galactosaminyl-lactose (GalNAc-Gal-Glc) (from McBiTec, Strasbourg, France), 6-sialyl-lactose (6'-SL) and 3-sialyl-lactose (3'-SL) (from Carbosynth, Berkshire, U.K.) were used as external standards. For characterization of the sample pretreatment the recovery of the individual oligosaccharides in the samples was determined (bovine milk: 79.9 +/- 5.7 % for 3'-SL, 85.5 +/- 0.5 % for 6'-SL and 96.2 +/- 2.9 % for GalNAc-Gal-Glc; caprine SM-UF permeate: 58.3 +/- 4.0 % for 3'-SL and 66.5 ± 2.5 % for 6'-SL; caprine NF retentates: 89.2 +/- 5.5 % for 3'-SL and 91.6 +/- 2.6 % for 6'-SL).

#### *III.3.2.2 Quantification of other components*

Dry mass was measured by the VDLUFA method C 35.5 (VDLUFA 1995c). Total nitrogen content in the samples were measured by the Kjeldahl method (VDLUFA VI C 30.2) (VDLUFA 1995b). The ash content was measured according to the method VDLUFA VI C 10.2 (VDLUFA 1995a). Determination of the salts Na, K, Mg, Ca and P were performed with slight modifications according to the method VDLUFA III 10.8.1.2 and 10.8.2 (VDLUFA 1976a, 1976b). The citric acid content was measured with a test kit (r-biopharm AG, Darmstadt, Germany).

## III.4 Results and Discussion

### *III.4.1 Enrichment of MOS from bovine milk*

For the further enrichment of MOS and a better permeation of milk salts and residual sugars from bovine milk permeate, an additional NF using a MOS concentrate produced on industrial scale as a basic raw material was performed at pH 5 and pH 7. The production of the MOS retentate on industrial scale was described in our previous study (Altmann et al. 2015) and briefly in materials and methods **(Fig. 3.1a)**. For the second NF on pilot plant scale the spiral wound module DL with a NMWCO of 150-300 Da was applied **(Table 3.1)**. The MOS content was quantified by determination of the main components of the MOS fraction (6'-SL, 3'-SL, GalNAc-Gal-Glc) in bovine milk. The contents of the MOS and of the other solids in the samples determined as average of two different NFs are listed in **Table 3.2 a)** and **b)**. The composition of the initial samples (pre-manufactured bovine NF retentate DOW) at pH 5 and 7 were comparable. No significant differences in the compositions of the initial samples (pre-manufactured bovine NF retentate DOW) and the pH adjusted initial NF retentates at pH 5 and 7 were determined. The concentrates exhibited a dry mass content of 27.3 g/L to 27.9 g/L, which is composed of 3.4 % to 3.7 % MOS, of 34.4 % to 37.0 % mono- and disaccharides, of 14.4 % to 15.1 % citric acid, of 1.0 % to 1.3 % total nitrogen and of 21.23 % to 25.6 % ash. The composition of the initial samples is comparable before and after pH adjustment.

Depending on the envisage targets, a high recovery of MOS and/or a high purity of the retentate can be reached. Application of a further NF process at different pH environments, let to reduced concentrations of mono- and disaccharides as well as of citric acid and ash contents. Because of the concentration factor of 4 during NF, the highest possible MOS content, which could be attained, was the 5-fold of the concentration in the initial sample. In the bovine NF retentate after concentration at pH 5 the dry mass content of 33.1 g/L was slightly higher than in the initial sample. When considering the concentration of the sample during this NF step, it becomes evident that a high amount of solids was separated. The MOS content of 6.9 % in dry mass (2281.8 mg/mL) was doubled compared to the initial sample. The concentration of mono- and disaccharides (17.7 % in dry mass) was lower than in the initial sample, indicating a permeation of the residual sugars from the NF retentate.

**Table 3.2 a)** Concentration of MOS (3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose), mono- and disaccharides (glucose, galactose, lactose), dry mass, citric acid, total nitrogen and ash in bovine NF retentates DOW, NF retentates DL after concentration and NF retentates DL after diafiltration at pH 5 and pH 7 (average of two trials, n=2). The recovery of the standards was: bovine milk: 79.9 +/- 5.7 % for 3'-SL, 85.5 +/- 0.5 % for 6'-SL and 96.2 +/- 2.9 % for GalNAc-Gal-Glc.

| **Sample** | **Bovine NF retentate DOW initial sample** | | | | | | **Bovine NF retentate DL after concentration** | | | | | | **Bovine NF retentate DL after diafiltration** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH 5 | | | pH 7 | | | pH 5 | | | pH 7 | | | pH 5 | | | pH 7 | | |
| **6'-SL [mg/L]** | 110.7 | +/- | 38.6 | 122.8 | +/- | 29.6 | 269.2 | +/- | 91.2 | 221.9 | +/- | 46.1 | 352.9 | +/- | 102.9 | 311.7 | +/- | 78.9 |
| **3'-SL[mg/L]** | 591.0 | +/- | 185.8 | 662.2 | +/- | 134.2 | 1436.8 | +/- | 451.4 | 1446.6 | +/- | 390.9 | 1930.4 | +/- | 544.1 | 1857.0 | +/- | 552.3 |
| **GalNAc-Gal-Glc [mg/L]** | 228.3 | +/- | 23.7 | 255.5 | +/- | 14.2 | 575.7 | +/- | 59.9 | 572.3 | +/- | 46.1 | 730.4 | +/- | 20.6 | 639.9 | +/- | 30.2 |
| **∑ MOS [mg/L]** | 930.0 | | | 1040.5 | | | 2281.8 | | | 2240.8 | | | 3013.7 | | | 2808.5 | | |
| **Lactose [g/L]** | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 | 0.0 | +/- | 0.0 | 0.1 | +/- | 0.0 |
| **Glucose [g/L]** | 4.5 | +/- | 0.5 | 5.3 | +/- | 0.2 | 2.6 | +/- | 1.8 | 4.7 | +/- | 0.3 | 0.1 | +/- | 0.1 | 0.2 | +/- | 0.0 |
| **Galactose [g/L]** | 4.9 | +/- | 0.2 | 5.0 | +/- | 0.1 | 3.1 | +/- | 1.4 | 4.6 | +/- | 0.2 | 0.1 | +/- | 0.0 | 0.2 | +/- | 0.0 |
| **Dry mass [g/L]** | 27.3 | +/- | 0.5 | 27.9 | +/- | 0.6 | 33.1 | +/- | 9.6 | 42.7 | +/- | 0.6 | 21.3 | +/- | 0.5 | 33.7 | +/- | 0.7 |
| **Citric acid [g/L]** | 4.1 | +/- | 1.6 | 4.0 | +/- | 0.3 | 6.1 | +/- | 2.5 | 8.4 | +/- | 0.8 | 2.8 | +/- | 0.2 | 9.9 | +/- | 0.4 |
| **Total nitrogen [g/L]** | 0.3 | +/- | 0.0 | 0.3 | +/- | 0.0 | 0.6 | +/- | 0.0 | 3.6 | +/- | 0.3 | 0.5 | +/- | 0.0 | 0.5 | +/- | 0.0 |
| **Ash [g/L]** | 7.0 | +/- | 0.3 | 5.9 | +/- | 0.0 | 7.4 | +/- | 2.5 | 11.5 | +/- | 0.5 | 4.7 | +/- | 0.3 | 10.6 | +/- | 0.3 |

Even though the citric acid content (18.3 % in dry mass) was slightly higher than at the beginning, about 70 % were separated during the concentration process. In the bovine NF retentate after concentration at pH 7, the dry mass content of 42.7 g/L was higher than in the equivalent sample at pH 5. The MOS content of 5.3 % in dry mass was higher than in the initial sample but about 23 % lower than in the NF retentate after concentration at pH 5. With 19.7 % in dry mass in the NF retentate at pH 7, the amount of citric acid was at the same level than in the corresponding sample at pH 5. The concentration of mono- and disaccharides in NF retentate after concentration at pH 7 was at the same level as at pH 5.

**Table 3.2 b)** Composition of mineral compounds (sodium, potassium, magnesium, calcium, phosphor) in bovine NF retentates DOW and bovine NF retentates DL after diafiltration at pH 5 and pH 7 (average of two trials, n=2).

| **Sample** | **Bovine NF retentate DOW initial sample** | | | | | | **Bovine NF retentate DL after diafiltration** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH 5 | | | pH 7 | | | pH 5 | | | pH 7 | | |
| **Sodium [g/L]** | 0.3 | +/- | 0.0 | 0.4 | +/- | 0.0 | 0.1 | +/- | 0.1 | 0.6 | +/- | 0.0 |
| **Potassium [g/L]** | 1.4 | +/- | 0.0 | 1.4 | +/- | 0.0 | 0.2 | +/- | 0.1 | 2.2 | +/- | 0.0 |
| **Magnesium [g/L]** | 0.3 | +/- | 0.0 | 0.2 | +/- | 0.0 | 0.4 | +/- | 0.1 | 0.6 | +/- | 0.0 |
| **Calcium [g/L]** | 1.2 | +/- | 0.8 | 1.4 | +/- | 0.0 | 0.6 | +/- | 0.4 | 1.5 | +/- | 0.5 |
| **Phosphor [g/L]** | 0.5 | +/- | 0.0 | 0.5 | +/- | 0.0 | 0.6 | +/- | 0.2 | 0.7 | +/- | 0.2 |

The diafiltration was performed with 20 diavolumes. In bovine NF retentate after diafiltration at pH 5 the dry mass was 21.3 g/L and lower than in the initial sample before NF, indicating a high permeation of solids during this wash step. With 3013.7 mg/L, the MOS content contributed 14.1 % to the dry mass and was about 4.1-fold higher than in the initial sample. This is due to a combination of high permeation of solids and the concentration of MOS during NF. The content of mono- and disaccharides was 1.2 % in dry mass, indicating a 99 % permeation of the residual sugars in comparison to the initial sample. The content of citric acid was 13.3 % in dry mass and lower than in the initial sample at pH 5. With 21.8 % in dry mass the concentration of ash was slightly lower than in the initial sample. However, considering the 5-fold concentration, it becomes clear that the majority of ash was separated in the retentate at pH 5. In bovine NF retentate after diafiltration at pH 7 the dry mass was 33.7 g/L. Even though the dry mass decreased during diafiltration indicating a permeation of solids, the content is markedly higher than in the equivalent sample at pH 5. Because of the

lower concentration of MOS (2808.5 g/L) and the higher content of solids, the MOS fraction in the NF retentate at pH 7 was with 8.3 % in dry mass about 40 % lower than in the retentate at pH 5. Reasons for the higher dry mass in the retentate after diafiltration at pH 7 are the distinctly higher contents of citric acid (29.3 % in dry mass) and ash (31.4 % in dry mass).

The results show a higher enrichment of MOS and a better permeation of other solid compounds after NF and diafiltration at pH 5 in comparison to the same process at pH 7. While the permeation of mono- and disaccharides was the same at acidic and neutral pH, the separation of salts was markedly higher in the NF retentate after diafiltration at pH 5. In the SM-UF permeate the fat fraction, the caseins and whey proteins were separated by centrifugation followed by UF. Therefore, the salt content is assumed to be comparable to the concentrations in milk serum. The dissolved salts are presented as ions or molecules (ion pairs e.g. cations with anions) in the milk serum and the SM-UF permeate. At the original pH 6.7 in milk, many salts are only partly ionized (Lucey and Horne 2009; Walstra et al. 1999). While the monovalent cations $Na^+$ and $K^+$ are completely dissociated in the milk serum, the divalent ions $Mg^{2+}$ (64 % $MgCit^-$, 26 % $Mg^{2+}$, 10 % $MgPO_4^-$), $Ca^{2+}$ (55 % Ca-$citrate^-$, 10 % $CaHPO_4$, 35 % $Ca^{2+}$) as well as the counterions citrate (85 % Mg- or Ca-citrate, 14 % $citrate^{3-}$) and phosphate (51 % $H_2PO_4^-$, 39 % $HPO_4^{2-}$, 10 % $CaPO_4^-$ and $MgPO_4^-$) are presented partly ionized at the original pH of milk. Especially the molecules Ca-citrate- and Ca-phosphate are presented at appreciable amounts. In acidic milieu the dissociation of salts is higher (Töpel 2004; Fox and McSweeney 2009), resulting in lower molecular masses. After NF at pH 5 a 79 % higher permeation of citric acid and a 34 % higher permeation of calcium was measured in comparison to the process at pH 7. Moreover, at acidic pH the permeation of sodium was 70 % higher and the permeation of potassium was 88 % higher than at neutral milieu. The higher dissociation of the salts at the acidic pH, especially of citrate and calcium possibly improved the permeation during NF due to its lower molecular masses. According to information of the membrane manufacturer, the pores of the membrane are slightly bigger at acidic milieu than in neutral environment (Alfa Laval Corporate AB). This might be another reason for the better separation of salts at the acidic pH. Rice et al. (2006) evaluated the fouling behavior of a thin film composite flat-sheet membrane (TFC-SR3), which is similar to the thin film composite membrane (SR 50) applied in this study, depending on the pH of the bovine SM-UF permeate (feed solution). During acidic NF at pH 5 the flux decrease was about 20 % lower than at pH 8, indicating lower fouling of the membrane. This was due to the complete

dissociation of calcium at pH 5 (Rice et al. 2006) In our work, the permeate flux during the NF processes was 31.6 L/m$^2$h at pH 5 and slightly higher than at pH 7 with 27.8 L/m$^2$h **(Table 3.1)**. Beside the better separation of the salts at acidic pH environment, the performance of the NF at pH 5 might be beneficial to decrease or even prevent the fouling of the membranes. When the membrane separation process should be performed over a long period of time a low fouling is particularly important.

**Fig. 3.2** indicates the retention of MOS (6'-SL, 3'-SL, GalNAc-Gal-Glc) in NF retentates after concentration and diafiltration at pH 5 and pH 7 using the spiral wound membrane module DL (white, grey and black bars, left ordinate). Furthermore the MOS content in relation to the total sugar content (MOS, lactose, glucose, galactose) is shown for these NF retentates (striped bars, right ordinate). The content of MOS in relation to the total sugar content in the initial sample before NF with the DL (bovine NF retentate DOW) at pH 5 was 8.9 % and at the same level as at pH 7 with 9.2 %. After NF with the DL, the recovery of the three MOS in the NF retentate after concentration at pH 5 and pH 7 was between 47.7 % and 66.0 %. Due to a low dilution of the samples with demineralized water in the amount of the residual volume of the filtration device, the recovery of the MOS within these samples is slightly higher than shown in the figure. The MOS content in relation to the total sugar content in the NF retentate after concentration at pH 5 was with 30.5 % more than 3-fold higher than in the initial sample. This is due to the permeation of mono- and disaccharides and the concentration of MOS during the concentration step. In the NF retentate after concentration at pH 7 a lower MOS content in relation to total sugar content was measured (19.2 %) due to a lower enrichment of MOS. Nevertheless, at pH 7 a good permeation of the mono- and disaccharides was achieved indicating a 2-fold higher MOS content in relation to total sugar content than in the initial sample. The retention of the MOS in the NF retentate after diafiltration at pH 5 was with 79.8 % very high. During diafiltration almost the all mono- and disaccharides permeated resulting in a very high MOS content in relation to the total sugar content of 92.1 % in the NF retentate at pH 5. The diafiltration at pH 7 led to comparable results. The retention of MOS was between 68.9 % and 75.6 % and the MOS content in relation to total sugar content was 87.3 % in the NF retentate after diafiltration at pH 7.

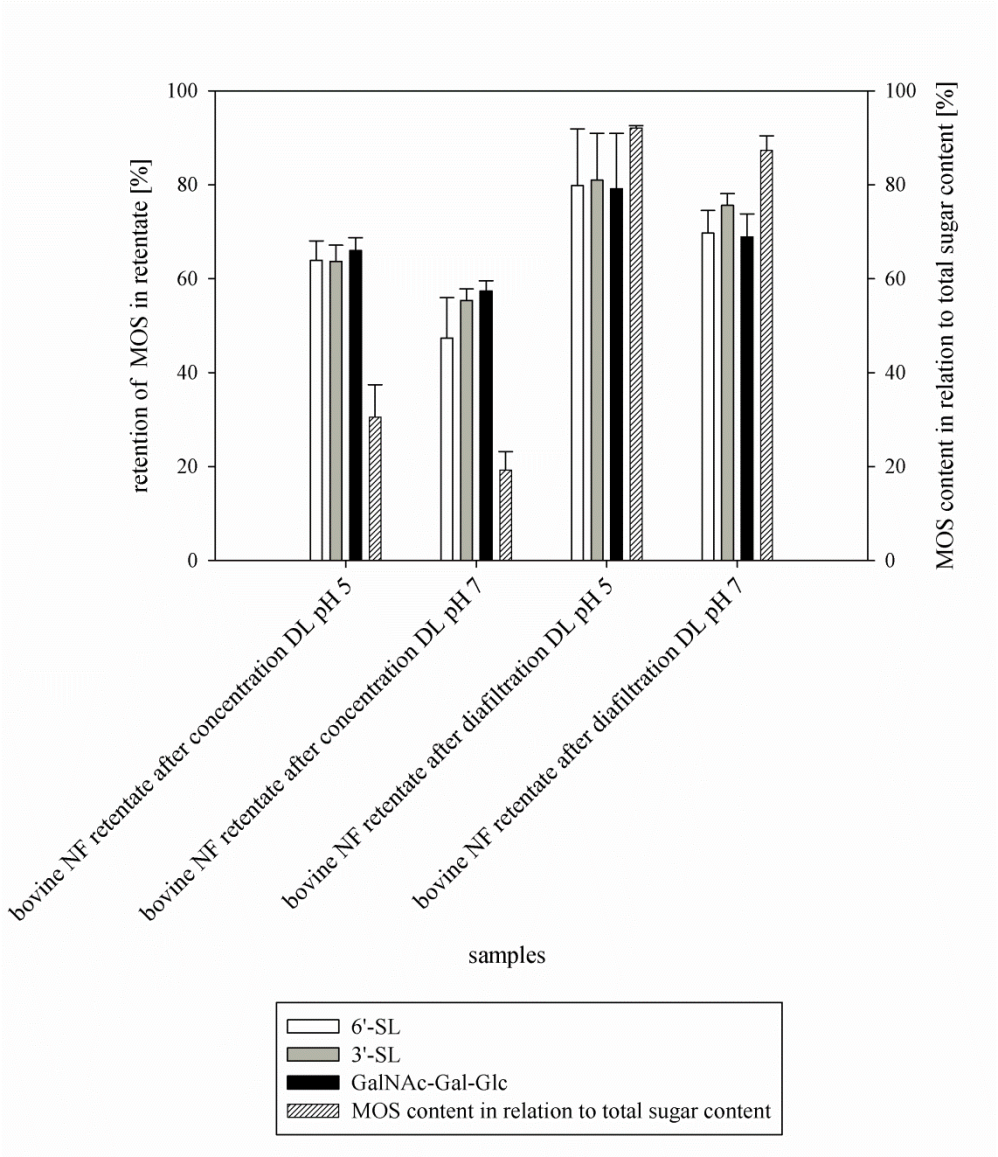



**Fig. 3.2** Retention of MOS by NF of bovine milk at pH 5 and pH 7 [%] (white, grey, and black bars; left ordinate) and MOS content in relation to the total sugar content [%] (striped bars, right ordinate), (average of two trials, n=2, 3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose). MOS content in relation to total sugar content in the initial sample was 8.9 +/- 1.9 % at pH 5 and 9.2 +/- 1.6 % at pH 7.

It can be summarized that the MOS content in relation to total sugar content in the NF retentate after diafiltration at pH 5 and pH 7 was about 9-fold higher than in the corresponding initial samples. During the concentration step the MOS were enriched and

mono- and disaccharides passed the NF membrane. The subsequent diafiltration with high amounts of water resulted in a nearly complete separation of mono- and disaccharides. Therefore, a multiple diafiltration is a key element for the permeation of residual sugars and salts. Because the degree of permeation of mono- and disaccharides was similar after concentration and diafiltration processes at pH 5 and 7, it can be concluded that the pH environment has nearly no influence on the permeation of the residual sugars. The acidic and the neutral MOS were retained at the same level indicating that the pH did not influence the separation behavior of the single MOS, too. However, the pH environment during NF has a high impact on the separation of salts from the MOS. Up to now, only a few studies dealt with the enrichment of MOS by NF of bovine milk. Because of the low concentrations of MOS in bovine milk Sarney et al. (2000) reported about a very small recovery of these oligosaccharides (Sarney et al. 2000). Therefore, a high concentration factor during the NF and the application of high amounts of a readily available source (e.g. a by-product of dairy industry) play a pivotal role for the efficient and economic enrichment of MOS from bovine milk. Mehra et al. (2014) used the available by-product mother liquor (the solution remaining after lactose crystallization and separation of a whey UF permeate) for the enrichment of MOS from bovine milk by NF on industrial scale (Raj Mehra et al. 2014). As described in the introduction, the MOS retentate, produced by this research group, revealed comparable concentrations of MOS, residual sugars and salts as it were achieved in our previous work (Altmann et al. 2015). By using a concentrate as in our study as source for a further NF, a higher enrichment of MOS, a nearly complete separation of mono- and disaccharides and a sufficient permeation of salts was realized.

### *III.4.2 Enrichment of MOS from caprine milk*

The aim of the further studies was, to transfer the process for the enrichment of MOS by NF to other milk sources, e.g. caprine milk. The composition of MOS in goat milk is closer to the profile in human milk, containing more complex oligosaccharides. Oliveria et al. (2015) described a 5- to 9-fold higher concentration of MOS in caprine milk in comparison to bovine milk (D. L. Oliveira et al. 2015). The application of the membrane separation process for the enrichment of MOS in caprine milk was performed on pilot plant scale using the membrane modules DL and SR 50 **(Table 3.1)**. The amounts of the main solids in the samples, determined as average of two different NF trials, are listed in **Table 3.3 a)** and **3.3 b)**.

**Table 3.3 a)** Concentration of MOS (3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose), mono- and disaccharides (glucose, galactose, lactose), dry mass, citric acid, total nitrogen and ash in caprine lactose hydrolyzed SM-UF-permeate and caprine retentates produced by application of the membrane modules SR 50 and DL (average of two trials, n=2), (n.d.= below limit of determination). The NFs were performed at the original pH of the original sample at pH 6.5. The recovery of the standards was: caprine SM-UF permeate: 58.3 +/- 4.0 % for 3'-SL and 66.5 ± 2.5 % for 6'-SL; caprine NF retentates: 89.2 +/- 5.5 % for 3'-SL and 91.6 +/- 2.6 % for 6'-SL.

| **Sample** | **Caprine lactose hydrolyzed SM-UF-permeate** | **Caprine NF retentate SR 50** | **Caprine NF retentate DL** |
|---|---|---|---|
| **6'-SL [mg/L]** | 6.4 +/- 1.0 | 5.3 +/- 1.0 | 6.4 +/- 1.4 |
| **3'-SL[mg/L]** | 12.4 +/- 1.5 | 19.3 +/- 3.3 | 24.0 +/- 2.7 |
| **∑ MOS [mg/L]** | 18.8 | 24.7 | 30.4 |
| **Lactose [g/L]** | n.d. | n.d. | n.d. |
| **Glucose [g/L]** | 18.8 +/- 0.3 | 1.1 +/- 0.1 | 1.0 +/- 0.0 |
| **Galactose [g/L]** | 16.5 +/- 0.3 | 1.0 +/- 0.1 | 0.9 +/- 0.0 |
| **Dry mass [g/L]** | 50.0 +/- 1.3 | 7.1 +/- 1.0 | 6.9 +/- 0.3 |
| **Citric acid [g/L]** | 1.7 +/- 1.0 | 2.3 +/- 0.5 | 2.4 +/- 1.0 |
| **Total nitrogen [g/L]** | 0.5 +/- 0.0 | 0.2 +/- 0.0 | +/- |
| **Ash [g/L]** | 5.5 +/- 0.1 | 1.6 +/- 0.0 | 1.7 +/- 0.1 |

The acidic MOS 3'-SL and 6'-SL present a lower proportion of whole oligosaccharide content in caprine milk than in bovine milk. However, only a few MOS are available as standards required for quantification by HPAEC (Barile et al. 2009), and due to a better comparability between the experiments using milk of different origin, the MOS in caprine milk were also quantified by the standards 3'-SL and 6'-SL. The neutral oligosaccharide GalNAc-Gal-Glc was not detected in caprine milk.

The lactose hydrolyzed skimmed and ultrafiltered milk permeate (SM-UF permeate) exhibited a dry mass content of 50.0 g/L, which is composed of 0.04 % MOS, 70.6 % monosaccharides (glucose and galactose), 3.3 % citric acid and 11.2 % ash. The NF cycles were performed at pH 6.5. The highest possible MOS content, which could be achieved after NF, was the double of the concentration in the initial sample due to the concentration factor of 2. The degree of enrichment of the oligosaccharides and the permeation of the monosaccharides and the salts during NF by using the two membranes were comparable. The caprine NF retentates generated by application of the membranes SR 50 and DL exhibited a dry mass of 6.9 g/L to 7.1 g/L indicating a permeation of solids of 85 to 90 % after concentration and diafiltration.

The MOS contents in the concentrates generated with the membranes SR 50 (24.7 mg/L) and DL (30.4 mg/L) were slightly higher than in the SM-UF permeate. In comparison to the initial sample, the concentrations of monosaccharides and of ash in the NF retentates were markedly lower. During the NF cycles with the SR 50 and DL, only 30 % of the citric acid permeated. The MOS concentration in dry mass was 0.4 % in both NF retentates and 9-fold higher than in the initial sample. Moreover, the dry mass was composed of 28.1 % to 29.8 % monosaccharides, of 31.9 % to 35.1 % citrate and of 24.0 % to 22.4 % ash. The results showed an enrichment of MOS and a permeation of the solids especially of the monosaccharides during the NF process. No significant differences were determined between the both membrane modules SR 50 and DL.

**Table 3.3 b)** Composition of mineral compounds (sodium, potassium, magnesium, calcium, phosphor) in caprine lactose hydrolyzed SM-UF-permeate and caprine retentates produced by application of the membrane modules SR 50 and DL (average of two trials, n=2 (average of two trials, n=2).

| Sample | Caprine lactose hydrolyzed SM-UF-permeate | | | Caprine NF retentate SR 50 | | | Caprine NF retentate DL | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sodium [g/L]** | 0.4 | +/- | 0.0 | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 |
| **Potassium [g/L]** | 1.8 | +/- | 0.1 | 0.4 | +/- | 0.0 | 0.4 | +/- | 0.0 |
| **Magnesium [g/L]** | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 | 0.1 | +/- | 0.0 |
| **Calcium [g/L]** | 0.1 | +/- | 0.0 | 0.4 | +/- | 0.0 | 0.2 | +/- | 0.0 |
| **Phosphor [g/L]** | 0.4 | +/- | 0.1 | 0.3 | +/- | 0.0 | 0.3 | +/- | 0.0 |

**Fig. 3.3** reveals the retention of MOS in NF retentate produced by using the membrane modules SR 50 and DL (white, grey, and black bars; left ordinate). In addition, the MOS content in relation to the quantified sugar compounds (residual sugar: lactose, glucose, glactose; quantified MOS: 6'-SL, 3'-SL, GalNAc-Gal-Glc) is shown (striped bars, right ordinate). The MOS content in relation to the total sugar content in the lactose hydrolyzed SM-UF permeate was 0.1 %. The retention of MOS in the caprine NF retentates generated by application of the two membranes was between 34.9 % and 37.0 % for 6'-SL and 66.1 % to 69.7 % for 3'-SL. The MOS content in relation to total sugar content in the concentrates was between 1.2 % and 1.6 % and 23-fold to 31-fold higher than in the initial sample. This is due to good separation of the oligosaccharides from the residual sugars.

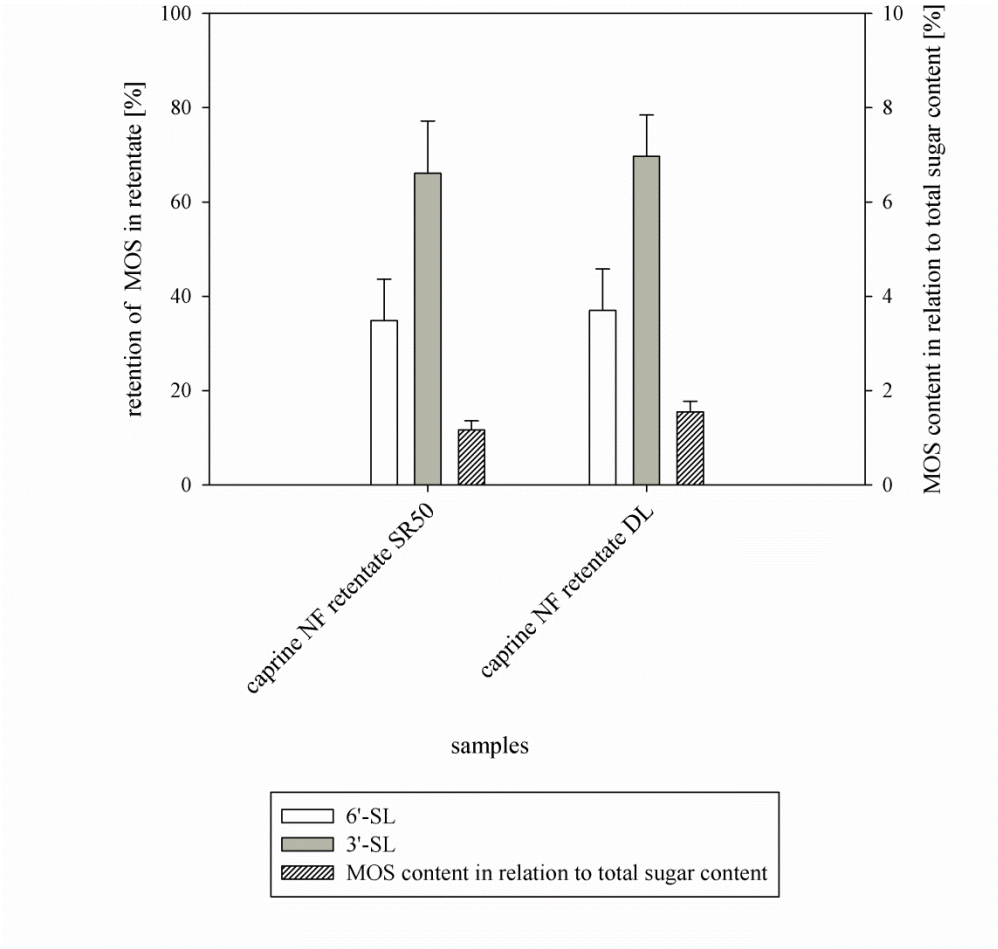



**Fig. 3.3** Retention of MOS by NF of caprine milk [%] (white, grey, and black bars; left ordinate) produced by application of the membrane modules SR 50 and DL and MOS content in relation to the total sugar content [%] (striped bars, right ordinate), (average of two trials, n=2, 3'-SL = 3-sialyl-lactose, 6'-SL = 6-sialyl-lactose, GalNAc-Gal-Glc = N-acetyl-galactosaminyl-lactose). MOS content in relation to total sugar content in the initial sample was 0.05 +/- 0.0 %.

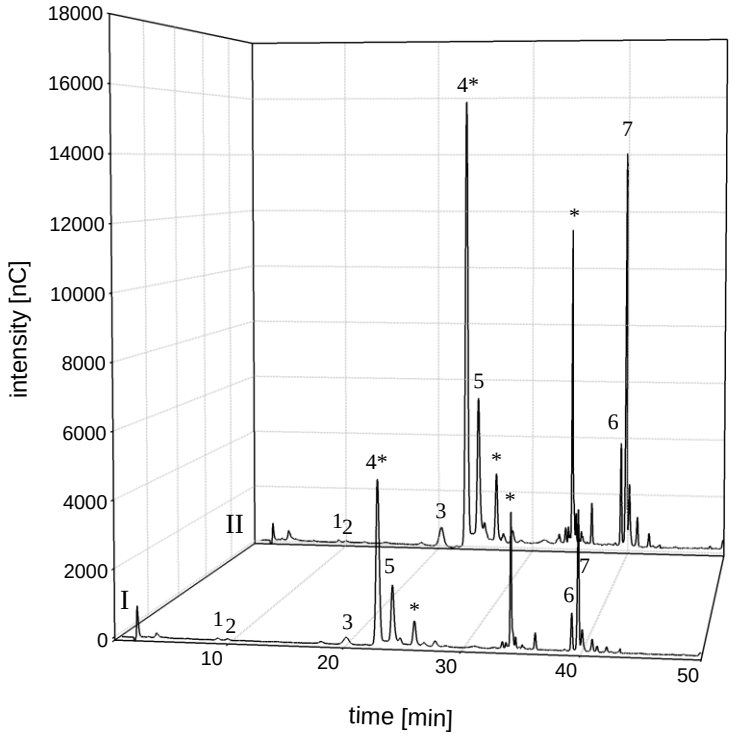
It can be concluded that the membranes SR 50 and DL are suitable for the enrichment of MOS and the separation of the monosaccharides using caprine milk as base product. Even though, the concentrations of oligosaccharides in the NF retentates are low, a markedly increase of the MOS content in relation to total sugar content was achieved. To increase the MOS concentration in the retentate, NF with a higher concentration factor should be conducted. Moreover, the performance of the NF at acidic pH for a better separation of the

salts is recommendable, when a higher purity of the MOS concentrate wants to be achieved. A few more studies were conducted for the enrichment of MOS from caprine milk. Martinez-Ferez et al. (2006) performed a NF of caprine SM-UF permeate with 3 diavolumes for the enrichment of MOS (A. Martinez-Ferez et al. 2006). They achieved a NF retentate with a retention of total oligosaccharides of about 80 %. The MOS content in relation to the residual lactose content in the concentrate was 7.6 % in the caprine NF retentate and higher as achieved in the present study. Other studies reported about a retention of MOS at the same level (Sarney et al. 2000; D. Oliveira et al. 2014). Due to the different analytical methods of MOS quantification, it is hardly possible to compare the results. So far, quantification of total oligosaccharide content was performed by determining the difference of total sugar content to lactose content. In the present study the MOS were quantified by HPAEC using the commercially available standards 3'-SL and 6'-SL. It can be assumed that the total MOS content in the NF retentates is higher due to more different complex oligosaccharides presented in caprine milk. Moreover, the sugars 3'-SL and 6'-SL count with a molecular mass of 655 Da to the smallest oligosaccharides distributed in caprine milk assuming that the retention of the larger MOS is at least as high as of the small MOS. However, for the first time single MOS were quantified in retentates of NF for the enrichment of oligosaccharides from caprine milk.

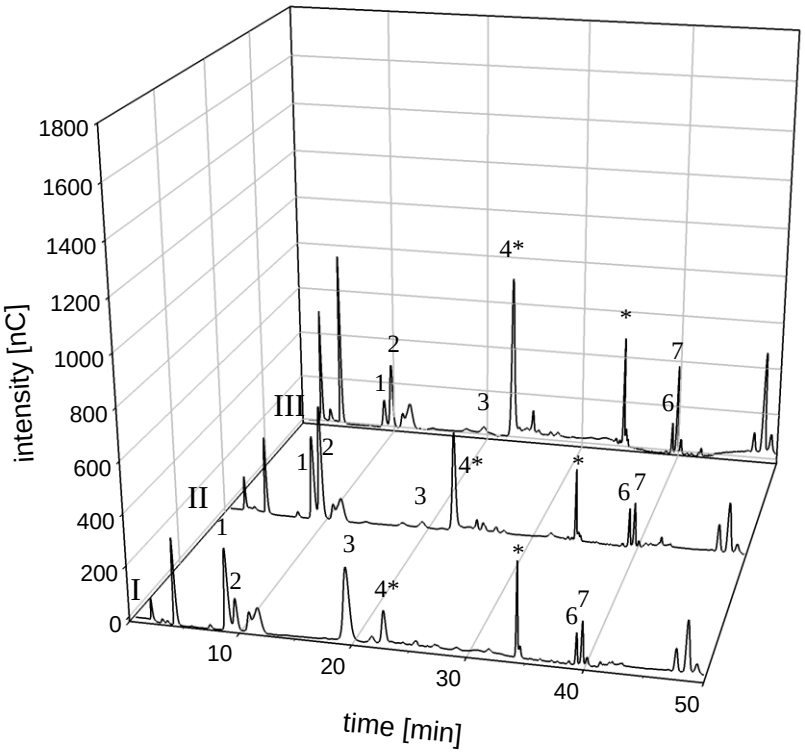
### *III.4.3 Composition of sugars in MOS concentrates*

**Fig. 3.4** indicates the HPAEC chromatograms of the initial samples and the concentrates produced by NF of bovine and caprine milk for the enrichment of MOS. Due to the separation of interfering high amounts of mono- and disaccharides by sample pretreatment with graphitized carbon solid phase extraction, only low residual amounts of galactose (peak 1), glucose (peak 2) and lactose (peak 3) were detected in the bovine and caprine samples. In **Fig. 3. 4 a)** the chromatograms of the initial sample (bovine NF retentate produced by application of the membrane DOW on industrial scale) (I) and of the bovine NF retentate after diafiltration (II) at pH 5 using the membrane module DL are shown.

**Fig. 3.4** HPAEC chromatograms of the initial samples and the retentates of the NFs for the enrichment of MOS from bovine and caprine milk. For separation of mono- and disaccharides samples were purified by graphitized carbon solid-phase extraction. (1= galactose, 2= glucose, 3= lactose, 4= 6'-glalctosyl-lactose, 5= GalNAc-Gal-Glc, 6= 6'-SL, 7= 3'-SL, * = GOS).




a) Chromatograms of the initial sample (bovine NF retentate DOW) (I) and the bovine NF retentate (DL) after diafiltration at pH 5 (II).




b) Chromatograms of the caprine SM-UF permeate before lactose hydrolysis (I), the caprine lactose hydrolyzed SM-UF permeate (II) and the caprine NF retentate generated by the membrane DL.

In the initial sample at pH 5 (I) ,the most abundant MOS GalNAc(α1-3)-Gal(β1-4)-Glc (peak 5, retention time 24.2 min), 6'-SL (peak 6, retention time 39.3 min) and 3'-SL (peak 7, retention time 39.9 min) were detected. Moreover, some galactooligosaccharides (GOS) were detected in this sample indicated as asterisks **(Fig. 3.1 a)**. As described in our previous study these high amounts of GOS (peak 4* = 6'-galaytosyllactose, retention time 22.9 min; peak *, retention time 26.1 min) were synthesized during the lactose hydrolysis with a β-galactosidase from *Kluyveromyces lactis*, due to its transgalactosylation activity (Altmann et al. 2015). The main reaction product of the enzymatic synthesis of GOS during lactose hydrolysis with the applied β-galactosidase (Frenzel et al. 2015) 6'-galactosyllactose could be identified in the NF-retentates (peak 4* in **Fig. 3.4 a**). One GOS (peak *, retention time 34.2 min) was detected in the initial sample before lactose hydrolysis. This galactotriose could be related to

Gal(α1-3)-Gal(β1-4)-Glc or Gal(β1-3)-Gal(β1-4)-Glc (Altmann et al. 2015). In the NF retentate after diafiltration with the membrane DL at pH 5 a markedly increase of the peak areas of the MOS (peak 5-7) and GOS (peak 4* and peaks with asterisks) were detected in comparison to the initial sample without changes in the composition.

**Fig. 3.4 b)** reveals the HPAEC chromatograms of caprine SM-UF permeate before lactose hydrolysis (I), the caprine lactose hydrolyzed SM-UF permeate (II) and the NF retentate produced by using the membrane DL (III). In the SM-UF permeate before lactose hydrolysis the acidic MOS 6'-SL (peak 6, retention time 39.4 min) and 3'-SL (peak 7, retention time 39.9 min) were identified. The neutral MOS GalNAc-Gal-Glc, which was quantified in bovine milk, was not present in caprine milk. Moreover, a galactotriose with the retention time of 6'-galactosyllactose (peak 4*, retention time 22.8 min) and a hexosetrisaccharide (peak *, retention time 34.3 min) were detected in significant amounts in the unhydrolyzed SM-UF permeate. The caprine SM-UF permeate before lactose hydrolysis (I) and after lactose hydrolysis (II) exhibited comparable peak areas of the MOS (peak 6, 7). It can be concluded that the process of enzymatic lactose hydrolysis did not degrade the MOS. Also Sarney at al.(2000) reported that a β-galactosidase from *Aspergillus oryzae* is suitable for lactose hydrolysis without changing the MOS profile in human milk (Sarney et al. 2000). However, a significant increase of the peak area of 6'-galactosyllactose in the caprine SM-UF permeate after lactose hydrolysis indicated a further synthesis of this GOS by the β-galactosidase of *Kluyveromyces lactis*. 6'-galactosyllactose was described to be the main GOS produced by the transgalactosidase

activity of this enzyme (Frenzel et al. 2015). In the caprine retentate produced by NF with the membrane DL (III) an increase of the peak areas of the MOS (peak 6, 7) and GOS (peak 4 and peaks with asterisk) in comparison to the initial samples was detected.

A few studies were performed for the microbial or enzymatic production of MOS presented in human milk. Some of these MOS such as 3'-sialyllactose, 6'-sialyllactose, 2'-fucosayllactose, 3'-fucosyllactose or lacto-N-biose are commercially available (Han et al. 2012). Due to the high complexity of MOS in human milk a lot of research has to be done to create a MOS profile resembling to that of human milk by biotechnological synthesis and for reasonable costs. The synthesized MOS has to meet all the requirements of novel food (D. L. Oliveira et al. 2015).

By the enrichment of MOS during NF of milk from domestic animals, various different complex oligosaccharides could be concentrated. As described before, bovine milk contains mainly the acidic MOS 3'-SL and 6'-SL and only traces of fucosylated MOS. The MOS profile of caprine milk is closer to that of human milk than bovine or ovine milk (A Martinez-Ferez et al. 2006; D. L. Oliveira et al. 2015). With this study, a feasible process for the enrichment of MOS by NF was developed, which could be implemented on industrial scale. The combined addition of MOS concentrates from bovine and caprine milk as functional ingredient is an opportunity for the development of bioactive food products containing different complex oligosaccharides, which are also present in human milk.

## III.5 Conclusion

With this work the influence of the pH value on the degree of enrichment of MOS from bovine milk by NF was studied for the first time. The highest MOS retention and milk salt permeation were achieved with NF at pH 5. The MOS content in dry mass (14.1 %) after diafiltration with the DL membrane was about 5-fold higher than in the initial NF retentate produced with the DOW membrane (3 % in dry mass), which was used as a raw material for this study, and about 140-fold higher than in the bovine lactose hydrolyzed SM-UF permeate (0.1 % in dry mass) described in our previous work. Although the NF at pH 7 resulted in a lower MOS content in dry mass (8.3 %), a markedly enrichment of MOS in the retentate was also achieved. The high MOS content in relation to total sugar content of 87 % to 92 % in the final concentrate produced at pH 5 and pH 7 indicated a nearly complete permeation of mono- and disaccharides, which was about 9-fold higher than in the first NF retentate and about 900-fold higher than in the bovine lactose hydrolyzed SM-UF permeate described in our prior study. Considering the results achieved by the present investigations, an optimized process for production of a MOS concentrate with high purity from bovine milk was developed. Moreover, first studies for the transferability of the optimized process to milk of other breeds were performed. NF for the enrichment of MOS in caprine milk permeate were conducted. In the caprine NF retentates, the MOS concentration in dry mass was 9-fold higher than in the lactose hydrolyzed caprine SM-UF permeate and the MOS content in relation to the total sugar content was 23-fold to 31-fold higher than in the initial sample. Even though a sufficient enrichment of MOS and a separation of the other solids were achieved, a further optimization of the procedure by varying milieu and process conditions should be performed in future.

The enrichment of MOS from whey permeate as by-product of cheese processing could increase the economic value of cheese whey as most of the oligosaccharides and lactose are transferred into the whey. Even though the concentrations of MOS in bovine milk are very low, an enrichment from whey permeate is highly promising due to the high amount of whey available in the dairy industry. Because caprine milk contains an oligosaccharide profile very similar to human milk and due to its higher concentration of these beneficial sugars, caprine whey represents also a valuable source for the enrichment of MOS. The developed two-stage NF process with lactose hydrolyzed SM-UF permeate at acidic conditions could be

implemented as continuous process on industrial scale applying to bovine or caprine cheese whey permeate for the enrichment of MOS. It was shown that the enzymatic hydrolysis of lactose in the initial sample supported a better separation of MOS from the residual sugars. Moreover, the β-galactosidase from *Kluyveromyces lactis* used, synthesized bioactive GOS by transgalactosylation, which were also concentrated during NF. Based on this procedure, the production of a MOS concentrate as a functional ingredient for the food industry should be feasible. Due to the different compositions of the MOS fractions in milk of domestic animals, the combined addition of MOS concentrates attained from bovine and caprine milk to food such as a beverage or infant formula might be beneficial to reproduce the oligosaccharide profile of human milk.

**Acknowledgement**

This research project is funded by the Federal Ministry for Education and Research (BMBF) as part of the competence network "FoCus- Food Chain Plus". NFs on industrial scale were performed in cooperation with the Theo Müller Group, Sachsenmilch, Leppersdorf, Germany.

## III.6 References

Albrecht, S., Lane, A. L., Mariño, K., Al Busadah, K. A., Carrington, S. D., & Hickey, R. M., et al. (2014). A comparative study of free oligosaccharides in the milk of domestic animals. *British Journal of Nutrition, 111*(7), 1313-1328

Altmann, K., Wutkowski, A., Kämpfer, S., Klempt, M., Lorenzen, P., & Clawin-Rädecker, I. (2015). Comparison of the efficiency of different NF membranes for the enrichment of milk oligosaccharides from bovine milk. *European Food Research and Technology, 241* (6), 803-815

Barile, D., Tao, N., Lebrilla, C. B., Coisson, J.-D., Arlorio, M., & German, J. B. (2009). Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *International Dairy Journal, 19*(9), 524-530

Bode, L. (2009). Human milk oligosaccharides: prebiotics and beyond. *Nutrition Reviews, 67*(2), 183-191

Bode, L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology, 22*(9), 1147-1162

Boehm, G., & Stahl, B. (2007). Oligosaccharides from milk. *Journal of Nutrition, 137*(3), 847-849

Bruggink, C., Maurer, R., Herrmann, H., Cavalli, S., & Hoefler, F. (2005). Analysis of carbohydrates by anion exchange chromatography and mass spectrometry. *Journal of Chromatography A, 1085*(1), 104-109

Fox, P. F., & McSweeney, P. (2009). *Advanced dairy chemistry. volume 3: lactose, water, salts and minor constituents* (3. ed.). Ireland: Springer-Verlag.

Frenzel, M., Zerge, K., Clawin-Rädecker, I., & Lorenzen, P. Chr. (2015). Comparison of the galacto-oligosaccharide forming activity of different β-galactosidases. *LWT - Food Science and Technology, 60*(2), 1068-1071

Gopal, P. K., & Gill, H. S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. *British Journal of Nutrition, 84*(supplement 1), 69-74

Han, N. S., Kim, T.-J., Park, Y.-C., Kim, J., & Seo, J.-H. (2012). Biotechnological production of human milk oligosaccharides. *Biotechnology Advances, 30*(6), 1268-1278

Kiskini, A., & Difilippo, E. (2013). Oligosaccharides in goat milk: structure, health effects and isolation. *Cellular & Molecular Biology, 59*(1), 25-30

Kunz, C., & Rudloff, S. (2006). Health promoting aspects of milk oligosaccharides. *International Dairy Journal, 16*(11), 1341-1346

Lucey, J. A., & Horne, D. S. (2009). Milk Salts: Technological Significance. In P. McSweeney, & P. F. Fox, *Advanced Dairy Chemistry Volume 3 Lactose, Water and minor constituents* (351-389). New York: Springer.

Martinez-Ferez, A., Guadix, A., & Guadix, E. M. (2006). Recovery of caprine milk oligosaccharides with ceramic membranes. *Journal of Membrane Science, 276*(1-2), 23-30

Martinez-Ferez, A., Guadix, A., Zapata-Montoya, J. E., & Guadix, E. M. (2008). Influence of transmembrane pressure on the separation of caprine milk oligosaccharides from protein by cross-flow ultrafiltration. *International Journal of Dairy Technology, 61*(4), 333-339

Martinez-Ferez, A., Rudloff, S., Guadix, A., Henkel, C. A., Pohlentz, G., Boza, J. J., et al. (2006). Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal, 16*(2), 173-181

Mehra, R., Barile, D., Marotta, M., Lebrilla, C. B., Chu, C., & German, J. B. (2014). Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. *PLoS One, 9*(5)

Mehra, R., & Kelly, P. (2006). Milk oligosaccharides: Structural and technological aspects. *International Dairy Journal, 16*(11), 1334-1340

Oliveira, D., Wilbey, R. A., Grandison, A., & Roseiro, L. (2014). Natural caprine whey oligosaccharides separated by membrane technology and profile evaluation by capillary electrophoresis. *Food and Bioprocess Technology, 7*(3), 915-920

Oliveira, D. L., Wilbey, R. A., Grandison, A. S., Duarte, L. C., & Roseiro, L. B. (2012). Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. *International Dairy Journal, 24*(2), 102-106

Oliveira, D. L., Wilbey, R. A., Grandison, A. S., & Roseiro, L. B. (2015). Milk oligosaccharides: A review. *International Journal of Dairy Technology, 68*(3), 305-321

Rice, G., Kentish, S., O'Connor, A., Stevens, G., Lawrence, N., & Barber, A. (2006). Fouling behaviour during the nanofiltration of dairy ultrafiltration permeate. *Desalination, 199*(1–3), 239-241

Sarney, D. B., Hale, C., Frankel, G., & Vulfson, E. N. (2000). A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. *Biotechnology and Bioengineering, 69*(4), 461-467

ten Bruggencate, S. J. M., Bovee-Oudenhoven, I. M. J., Feitsma, A. L., van Hoffen, E., & Schoterman, M. H. C. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutrition Reviews, 72*(6), 377-389

Thum, C., Cookson, A., McNabb, W. C., Roy, N. C., & Otter, D. (2015). Composition and enrichment of caprine milk oligosaccharides from New Zealand Saanen goat cheese whey. *Journal of Food Composition and Analysis, 42*, 30-37

Töpel, A. (2004). *Chemie und Physik der Milch: Naturstoff-Rohstoff-Lebensmittel*. Hamburg: Behr's Verlag.

Urashima, T., Saito, T., Nakamura, T., & Messer, M. (2001). Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal, 18*(5), 357-371

VDLUFA (1976a). 10.8.1 Nassaufschluss unter Druck. In C. Naumann, & R. Basser, *Die chemische Untersuchung von Futtermitteln*. Darmstadt, Germany: VDLUFA.

VDLUFA (1976b). 10.8.2 Bestimmung von ausgewählten Elementen in pflanzlichem Material und Futtermitteln mit ICP-OES. In C. Naumann, & R. Basser, *Chemische Untersuchung von Futtermitteln*. Darmstadt, Germany: VDLUFA.

VDLUFA (1995a). C 10.2 Bestimmung der Gesamtasche. In O. Siegel, *Chemische, physikalische und mikrobiologische Untersuchungsverfahren für Milch, Milchprodukte und Molkereihilfsstoffe*. Darmsatdt: VDLUFA, Germany.

VDLUFA (1995b). C 30.2 Bestimmung des Gesamt-Stickstoffgehaltes von Milch und Milchprodukten nach Kjeldahl. In O. Siegel, *Chemie, pysikalische und mikrobiologische Untersuchungsverfahren für Milch, Milchprodukte und Molkereihilfsstoffe*. Darmstadt: VDLUFA, Germany.

VDLUFA (1995c). C 35.5 Bestimmung des Tockenmassegehaltes von flüssigen Milchprodukten. In O. Siegel, *Chemische, physikalische und mikrobiologische*

*UNtersuchungsverfahren für Milch, Milchprodukte und Molkereihilfsstoffe*. Darmstadt: VDLUFA, Germany.

Walstra, P., Geurts, T. J., Noomen, A., Jellema, A., & Boekel, M. A. J. S. v. (1999). *Dairy Technology- Princoples of Milk Properties and Processes*. Basel: Marcel, Dekker, Inc.

Zerge, K. (2014). *Charakterisierung und Gewinnung von Oligosacchariden als potenielle funktionelle Lebensmittelinhaltsstoffe*. Doctoral Thesis, Technical University Dresden, Germany

# Chapter IV

## Generation and identification of anti-inflammatory peptides from bovine β-casein using enzyme preparations from cod and hog

Karina Altmann*, Adam Wutkowski, Martin Klempt, Ingrid Clawin-Rädecker, Hans Meisel, Peter Chr. Lorenzen

*Accepted in Journal of the Science of Food and Agriculture*

*February 2016*

Max Rubner-Institut Kiel, Department of Safety and Quality of Milk and Fish Products, Hermann-Weigmann-Straße 1, 24103 Kiel, Germany

*corresponding author

## IV. 1 Abstract

**BACKGROUND:** Inflammation is a contributing factor for the development of various chronic diseases. However, there is only little information about anti-inflammatory peptides from food products. The aim of the present study was the generation and identification of potentially anti-inflammatory peptides from bovine β-casein with enzyme preparations from cod and hog. Furthermore, the potential of Cod Trypsin, derived from fishery by-products, to produce this bioactive peptides for replacement of non-food grade TPCK treated porcine Trypsin enzyme preparation was evaluated.

**RESULTS:** Potentially anti-inflammatory peptides were obtained by hydrolysis of β-casein with the tryptic enzyme preparations cod trypsin, porcine trypsin (tosyl phenylalanyl chloromethyl ketone (TPCK)-treated) and a porcine trypsin and chymotrypsin preparation (PTN 6.0 S). Proteolysates generated with enzyme preparations containing mainly chymotryptic activity (Cryotin, Cryotin F) did not exhibit any effect.

**CONCLUSION:** The more chymotryptic enzyme activity is present, the lower is the potentially anti-inflammatory activity of the hydrolysates in HEK$^{nf\kappa b\text{-}RE}$ cells. Comparable peptides were produced by application of porcine Trypsin (TPCK) and Cod Trypsin. Therefore, the enzyme preparation Cod Trypsin can replace the non-food grade porcine enzyme preparation Trypsin (TPCK) for the generation of potentially anti-inflammatory peptides from β-casein.

**Keywords**

Enzymatic hydrolysis, β-casein hydrolysates, tryptic/chymotryptic enzymes, anti-inflammatory peptides, identification of peptides

## IV.2 Introduction

Milk proteins, and especially β-casein, are precursors of many biologically active peptides [1, 2]. Milk-derived bioactive peptides are inactive within the sequence of the parent protein and can be released by enzymatic hydrolysis during food processing or gastrointestinal digestion [1]. These peptides may demonstrate various biological activities, such as antioxidative, ACE inhibitory, antimicrobial, antihypertensive, and opioid activities [3]. The field of bioactive peptides has been extensively reported [4], but studies dealing with anti-inflammatory food-derived peptides are much less prevalent [5-8].

Inflammation is a response to infection, irritation, and injury. It is involved in the development of various chronic diseases, including diabetes, cancer, asthma, and obesity [9]. NFκB is a homo- and heterodimeric transcription factor that plays a key role in inflammatory process, and is responsible for expression and regulation of genes involved in inflammation, immunity, and apoptosis [10]. Stimulation with the pro-inflammatory cytokine TNF-α triggers most often the canonical NF-κ-B signaling pathway [11] by activation of IκB-kinase complex (IKK complex). IKK complex phosphorylates N-terminal serine residues on IκB, which gets degraded by proteasomes and releases NFκB. The liberated transcription factor translocates into the nucleus to induce expression of inflammatory genes [10-13].

Processing of fish and fishery products requires the removal of skin, head, bones and viscera, which are termed by-products. While most of this organic material is still wasted, there is increased awareness of utilizing marine by-products as sources of industrial enzymes. The pyloric caeca, which is part of the viscera, is rich in digestive enzymes, such as the serine proteases trypsin and chymotrypsin [14]. Proteolytic fish enzymes have already been applied in food processing, for example, as a substitute for rennet in cheese manufacturing, for the removal of oxidized flavor from milk, and for accelerating cheese ripening [14-17]. Nevertheless, the application of fish enzymes in food processing is so far still limited and their use as a food-processing tool has to be further studied.

Therefore, the aim of the present work was to generate and identify anti-inflammatory peptides from bovine β-casein with enzyme preparations from cod fish, and for comparison from hog, containing different activities of trypsin and chymotrypsin. Recently, a group of large, hydrophobic peptides generated by hydrolysis of β-casein with porcine trypsin (TPCK)

showed anti-inflammatory effects by TNF-α mediated activation of NFκB in HEK$^{nfκb\text{-}RE}$ cells [18]. This enzyme preparation is not suitable for human consumption and therefore, an alternative source is needed. cod trypsin derived from fishery by-products may replace porcine trypsin (TPCK).

## IV.3 Materials and methods

### *IV.3.1 Isolation of β-casein from rennet casein*

Isolation of β-casein from rennet casein was performed according to Le Magnen and Maugas (1995) with minor modifications [19]. Briefly, a 5 % rennet casein solution (w/w) (Fonterra, Auckland, New Zeeland) in demineralized water (pH 9) was chilled to 0.2- 0.4°C. For precipitation of $\alpha_S$- and para-κ-casein, the pH of the solution was adjusted to 4.6 by dropwise addition of 0.1 mmol/L HCl. Precipitated $\alpha_S$- and para-κ-caseins were separated from solution by filtration (curd draining bag, pore size 0.3 mm). β-casein was precipitated by slowly warming the solution to 40°C. The precipitate was separated, dissolved in demineralized water, solubilized by adjusting the pH to 7.0 (25°C), and lyophilized. The freeze-dried β-casein isolate consisted of 83- 85 % β-casein (determined by polyacrylamide gel electrophoresis densitometry) and exhibited a protein content of 87- 89 % (Kjeldahl method, N= 6.38).

### *IV.3.2 Enzymes and hydrolysates*

For hydrolysis of β-casein, the enzyme activities of the enzyme preparations applied **(Table 4.1)** were determined with the azocasein assay, according to Reichad et al. 1990 [20] with minor modifications. In brief, azocasein (Sigma Chemical Co., St. Louis, USA) was dissolved at a final substrate concentration of 0.5 % (w/v) in 0.1 M phosphate buffer (pH 7.0). Substrate (400 µL) was mixed with 100 µL enzyme preparation of different dilutions. After incubation at 37°C for 1 h, the reaction was stopped by addition of 150 µL of 20 % trichloroacetic acid. The tubes were allowed to stand in an ice-bath for 30 min, and were then centrifuged at 13,000 g for 2 min. Supernatant (500 µL) was removed from each tube and mixed with 500 µL of 1 M NaOH. The absorbance was determined at 435 nm. Control samples were treated in the same manner, except that, instead of enzyme preparation, 100 µL phosphate of buffer was added to the substrate. The color- concentration relationship of azocasein and its digestion products obeys Beer's law, and for the calculation of enzyme activity, an extinction coefficient of 34 L $mol^{-1}$ $cm^{-1}$ was used. One Azo unit is defined as the amount of the enzyme

that catalyzes the conversion of 1 micro mole of substrate per minute at 37°C. Enzyme activities for the enzyme preparations applied are listed in **Table 4.1**.

**Table 4.1** Sources of enzyme preparations and enzyme activities applied for hydrolysis of a 5 % β-casein solution at pH 7.8 and 37 °C for 3- 4 h. Tryptic/ chymotryptic ratio was calculated from the activities of enzyme preparations according to the manufacturers. For the β-casein hydrolyses, nearly same enzyme activity in Azo units/g β-casein was chosen. [a]U= unit, [b]BAEE = Nα-Benzoyl-L-arginine ethylster, [c]USP = United States Pharmacopeia unit, [d] GPR = N-Succinyl-Gly-Pro-Arg-p-nitroanilide, [e] AAPF = N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, [f] Trypsin EC = 3.4.21.4, [g] Chymotrypsin EC = 3.4.21.1

| Enzyme preparation Trade name | Origin | Activity (according to the manufacturer) [U [a]/g enzyme peparation] | | Tryptic [f]/ chymotryptic [g] ratio | Azo U/ g or ml enzyme preparation |
|---|---|---|---|---|---|
| | | trypsin | chymotrypsin | | |
| Trypsin (TPCK) | Hog | $40 \cdot 10^3$ BAEE [b] U/g | - | 1 / 0 | $190 \cdot 10^3$ Azo U/ g |
| PTN 6.0 S | Hog | $1450 \cdot 10^3$ USP [c] U/g | $78 \cdot 10^3$ USP U | 1 / 0.05 | $62.54 \cdot 10^3$ Azo U/g |
| Cod trypsin | Cod fish | 165 GPR [d] U/g | - | 1 / 0 | 159.74 Azo U/ml |
| Cryotin | Cod fish | 137.4 GPR U/g | 27.4 AAPF [e] U/g | 1 / 0.2 | 344.12 Azo U/ml |
| Cryotin F | Cod fish | 160 GPR U/g | 331 AAPF U/g | 1 / 2.06 | 2082.76 Azo U/ml |

For preparation of the hydrolysates, proteolysis of a 5 % β-casein solutions (w/w) with different tryptic (EC 3.4.21.4) and chymotryptic (EC 3.4.21.1) enzyme preparations from hog (trypsin TPCK-treated, Merck KGaA, Darmstadt, Germany, enzyme substrate-ratio of 40 BAEE U/g β-casein for trypsin activity; PTN 6.0 S ®, Novozymes A/S, Bagsvaerd, Denmark, enzyme-substrate-ratio of 1450 USP U/g β-casein for trypsin activity and 78 USP U/g β-casein for chymotrypsin activity) and Cod fish *(Gadus morhua)* (cod trypsin ®, enzyme substrate-ratio of 165 GPR U/g β-casein for trypsin activity; Cryotin ®, enzyme substrate-ratio of 6,87 GPR U/g β-casein for trypsin activity and 1,37 AAPF U/g β-casein for chymotrypsin activity; Cryotin F ®, enzyme substrate-ratio of 16 GPR U/g β-casein for trypsin activity and 33 AAPF U/g β-casein for chymotrypsin activity; all enzymes from North Ltd., Reykjavík, Iceland) were performed. For further information about enzyme activities from the manufacturer see **Table 4.1**. Incubation was carried out at 37°C and pH-stat conditions of 7.8 (0.1 M NaOH) for 4 hours with porcine enzymes PTN 6.0S and porcine trypsin (TPCK), and for 3 hours with fish enzymes cod trypsin, Cryotin, Cryotin F, respectively [21, 22]. To stop the enzymatic reaction, the hydrolysates were heated batchwise for

10 min at 90°C. The whole hydrolysates were fractionated by ultrafiltration applying a membrane with a Nominal Molecular Weight Cut-Off (NMWCO) of 5 kDa (Koch Membrane Systems, Wilmington, MA, USA). The peptide fractions (fraction I = whole proteolysate, fraction II = peptide fraction > 5 kDa, fraction III = peptide fraction < 5 kDa) were lyophilized and stored at 20°C until further analysis.

### *IV.3.3 Separation and identification of peptides by HPLC-ESI-MS$^n$ (LC-MS)*

Identification and separation of the β-casein-derived peptides was performed using online high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS$^n$) using an ion-trap mass spectrometer LTQ XL (Thermo Scientific Inc., San Jose, CA). Lyophilized peptide fractions were dissolved in 20 % (v/v) acetonitrile (ACN) and 0.1 % (v/v) formic acid (FA). The solution was filtered by applying centrifugal devices (Pall Nanosep 10 kDa Omega) for 10 min at 13000 rpm in a centrifuge (Biofuge Fresco; Heraeus Instruments), and analyzed on a polymeric reversed phase column (PLRP-S-300Å, 5 µm, 150 x 2.1 mm, Agilent Technologies, Waldbronn, Germany). For chromatographic separation of peptides, 0.1 % (v/v) FA in ultrapure water (solvent A) and 0.1 % (v/v) FA in ACN (solvent B) were used. A linear gradient from 3 % to 60 % solvent B over 40 min at a flow rate of 0.2 mL/min was applied followed by a column wash step with 90 % solvent B for 5 min and re-equilibration at the initial conditions for at least 10 min.

Mass spectra were generated in the positive ionization mode in the full scan range (150 up to 2000 m/z) and a data-dependent scan with fragmentation of the five most intense ions (activation type = CID, normalized collision energy = 35.0 eV, isolation width of 2 and an activation Q of 0.250). ESI spray voltage was set to 3.5 kV; capillary temperature was 275 °C. Data acquisition and processing was performed with Xcalibur version 2.0.7 SP1 (Thermo Scientific Inc., San Jose, CA). Molecular masses were determined from the measured m/z ions by ProMass$^{TM}$Deconvolution 2.5 (Thermo Scientific Inc., San Jose, CA). The processed files were subsequently searched against an in-house *Bos taurus* database (originally downloaded from: http://uniprot.org release October 2012) extended to include all natural variants of ß-casein, using the search algorithm SEQUEST and MASCOT on Proteome Discoverer 1.4 (Thermo Scientific Inc., San Jose, CA). The search was performed choosing

Trypsin (semi) as the enzyme with two missed cleavages allowed. As variable modifications, oxidation (M) and phosphorylation (S, T, Y) were applied. Peptide precursor mass tolerance was set at 0.5 Da. The false discovery rate was set at 0.05. The evaluation of the identified peptides was performed manually. Briefly, all peptides with at least two precursor masses in a fully MS, must have at least five (for small peptides up to 6 aminoacids three) isotopically resolved y-,b-, or a-ions match to theoretical peptide fragments to be considered positive. Furthermore, the major peaks of these peptides with intensities of higher than 10- 20 % of the maximum intensity in the MS/MS must match theoretical peptide fragments. All peptides modified by phosphorylation were considered positive if the modification had been previously described in the literature and no peptide with the same precursor mass has been found by Proteome Discoverer. The peptides identified in this study are listed in **Table 4.2**.

### *IV.3.4 Evaluation of anti-inflammatory effect via NFκB activation in HEK$^{nfκb\text{-}RE}$ cells*

HEK$^{nfκb\text{-}RE}$ cells [18] were cultivated at 5 % $CO_2$ in DMEM medium (PAA, Cölbe, Germany) supplemented with 10 % fetal calf serum (FCS) (PAA, Cölbe, Germany). Lyophilised peptide fractions I- III (10 mg/mL) were dissolved in culture medium and sterile filtered. HEK$^{nfκb\text{-}RE}$ cells were seeded in white (clear bottom) 96-well plates at a density of 10,000 cells/ well. After overnight incubation, cells were infected with 0.2 ng/mL TNF-α and incubated with peptide fractions (final concentration 5 mg/mL). For control cells were treated with 0.2 ng/mL TNF-α without peptide sample, with peptide sample without TNF-α, and only with DMEM medium without peptides and TNF-α. After 24 hours, medium was removed, cells were washed twice with PBS and lysed by freezing and defrosting twice. Luciferase assay was started by incubation of cells with 45 µL of Beetle Lysis-Juice (PJK, Kleinblittersdorf, Germany) for 10 min. The light product developed by luciferin degradation was measured over a period of 1 second inae Chameleon plate reader (Hidex, Turku, Finland). Quadruplicate incubations and measurements were considered to be one experiment. The data are expressed as arbitrary units of luciferase activity of HEK$^{nfκb\text{-}RE}$ cells compared with the control (value of cells with peptide sample without TNF-α stated as 1).

### *IV.3.5 Statistical analysis*

Statistical significance values of relative luciferase activity between different proteolysates and TNF-α stimulation alone were determined using Kruskal- Wallis one-way analysis followed by the Dunns multiple comparison test with the software SigmaPlot 11.0 (SigmaPlot Software, Systat Software Inc., USA). The data are expressed as the arithmetic means ± standard deviation (SD) of at least three independent experiments. Values are reported in relation to the luciferase activity of untreated cells.

## IV.4 Results and discussion

### *IV.4.1 Identification of peptides in tryptic/chymotryptic hydrolysates*

For enzymatic hydrolysis, five enzyme preparations from hog and cod containing different tryptic and chymotryptic activities were applied **(Table 4.1)**. The enzyme preparations porcine trypsin (TPCK) and cod trypsin exhibit only tryptic activity, while PTN 6.0 S (tryptic/chymotryptic ratio 1/0.05), Cryotin (tryptic/chymotryptic ratio 1/0.2) and Cryotin F (tryptic/chymotryptic ratio 1/2.06) exhibit increasing chymotryptic activity. β-Casein hydrolyses were performed by applying comparable enzyme activities, as determined by the azocasein assay.

Total hydrolysate (I), peptides > 5 kDa (II), and peptides < 5 kDa (III) were characterized by LC-MS **(Fig. 4.1 a-c)**. **Fig. 4.1 a)** shows LC-MS chromatograms of the total hydrolysates (fraction I). The peptide profiles of the total hydrolysates generated with cod trypsin and porcine trypsin (TPCK) are very similar because both enzyme preparations exhibit only tryptic activity. The total hydrolysate produced with PTN 6.0 S exhibits a slightly different peptide spectrum with additional small peaks due to a low chymotryptic activity in this enzyme preparation. Proteolysates (fraction I) generated with Cryotin and Cryotin F exhibit completely different peptide profiles because of high chymotryptic activity. In contrast, to the results described above, the proteolysates generated with Cryotin and Cryotin F showed only a few peptides with high retention times (>25 min).

Ultrafiltration with a 5 kDa membrane resulted in nearly the same peptide patterns and retention times in the retentate and permeate, but the patterns exhibited quite different peak areas. Peptide profiles of the 5 kDa retentates (fraction II) **(Fig. 4.1 b)** showed an enrichment of some fragments. Peptides with molecular masses of 4823.8 Da to 7402.2 Da (peptides 17-20) in the 5 kDa retentates produced by application of cod trypsin, porcine trypsin, and PTN 6.0 S displayed higher peak areas in comparison with their corresponding total hydrolysates. The peak areas of small peptides in the retentates of cod trypsin, porcine trypsin (TPCK), and PTN 6.0 S, numbered with 1 to 16, are reduced compared to their total hydrolysates.

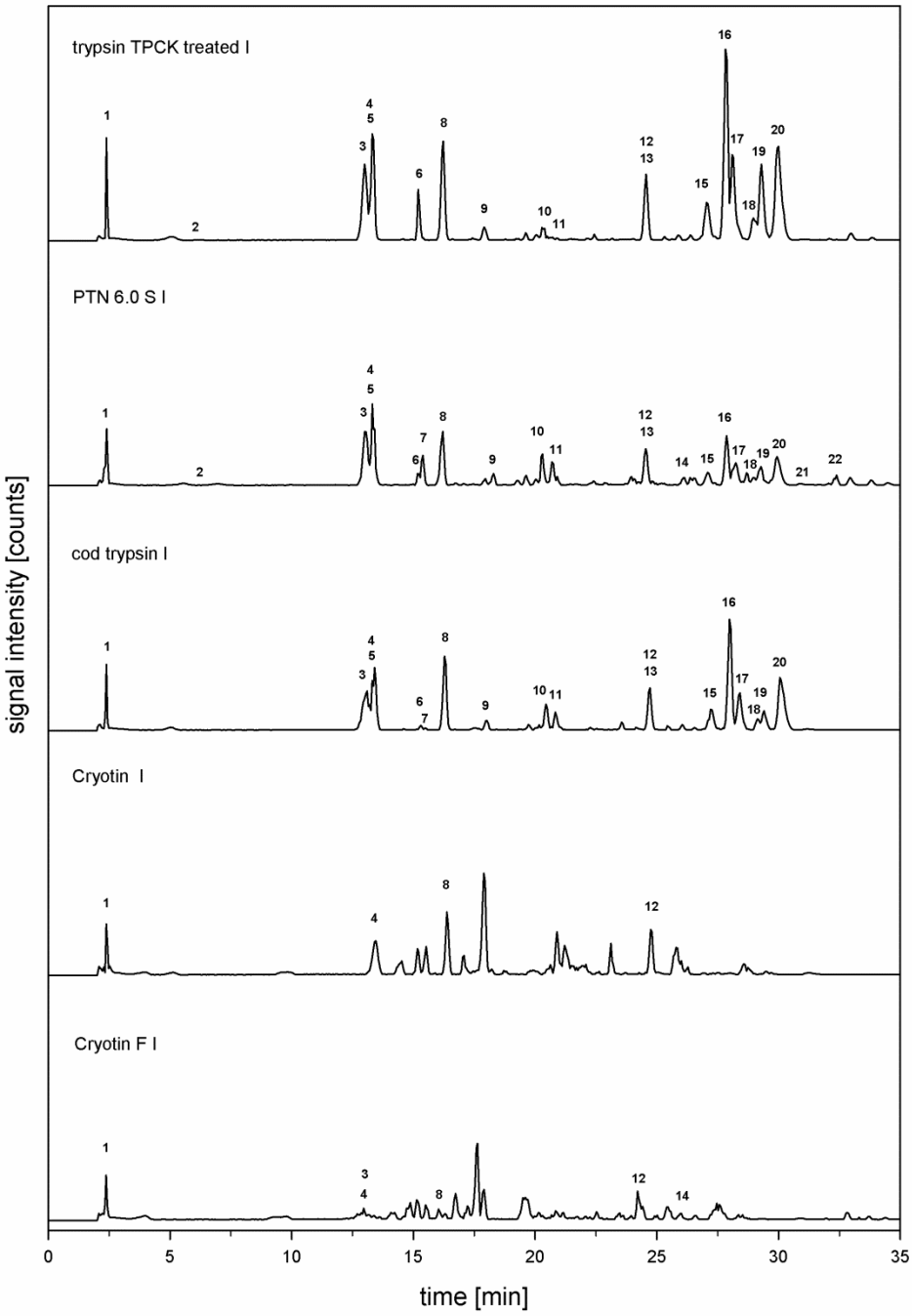



**Fig. 4.1 a).** LC-MS profiles of total β-casein hydrolysates (fraction I) generated with the enzyme preparations cod trypsin (CT), trypsin TPCK-treated (T), PTN 6.0S (P), and Cryotin F (CF). Visible peaks of the main 26 β-casein fragments focused **(Table 4.2)** are numbered with 1- 22 in the chromatogram.

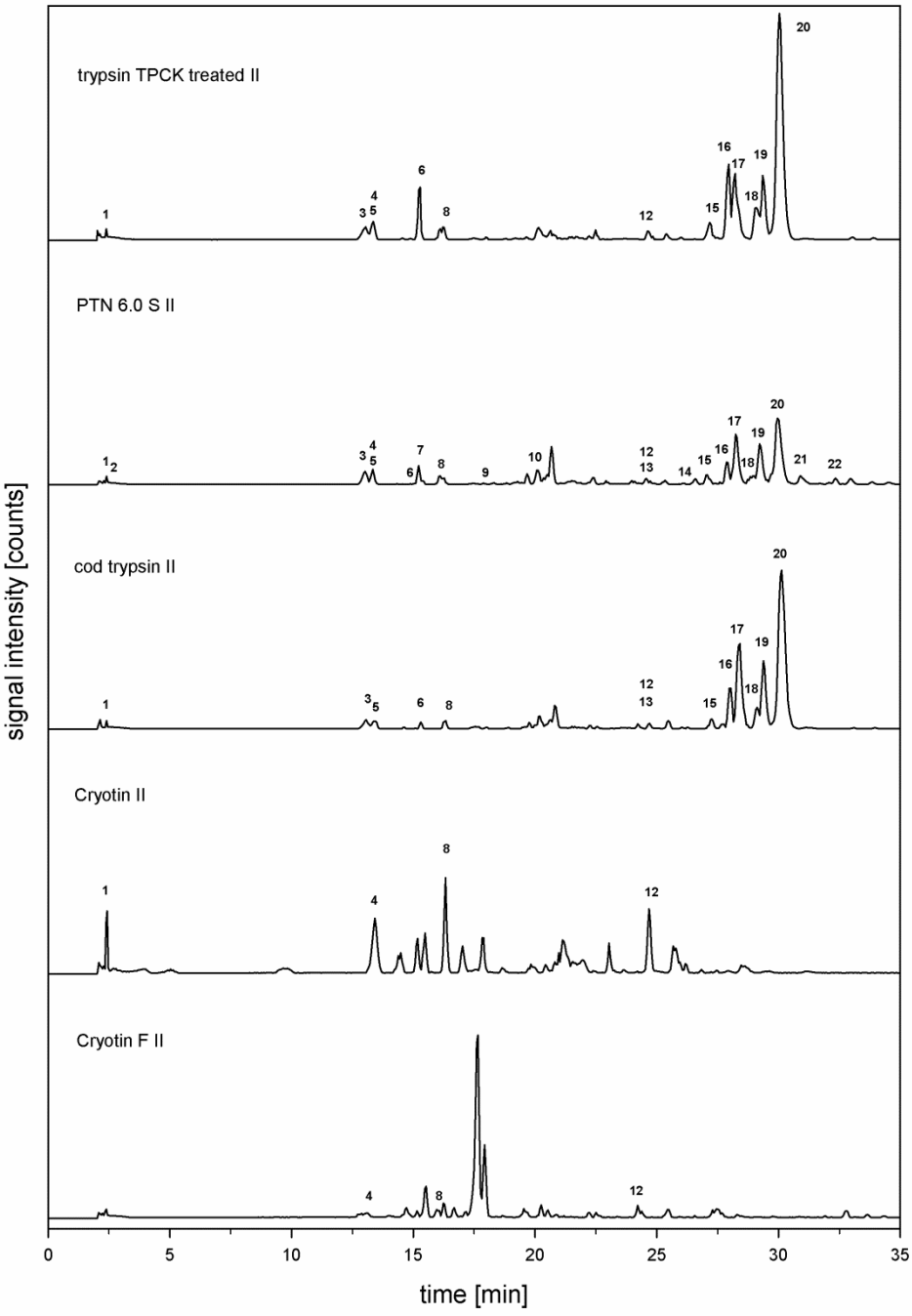



**Fig. 4.1 b)** LC-MS profiles of retentates 5 kDa (fraction II) generated with the enzyme preparations cod trypsin (CT), porcine trypsin (TPCK) (T), PTN 6.0S (P), and Cryotin F (CF). Visible peaks of the main 26 β-casein fragments focused **(Table 4.2)** are numbered with 1- 22 in the chromatogram.

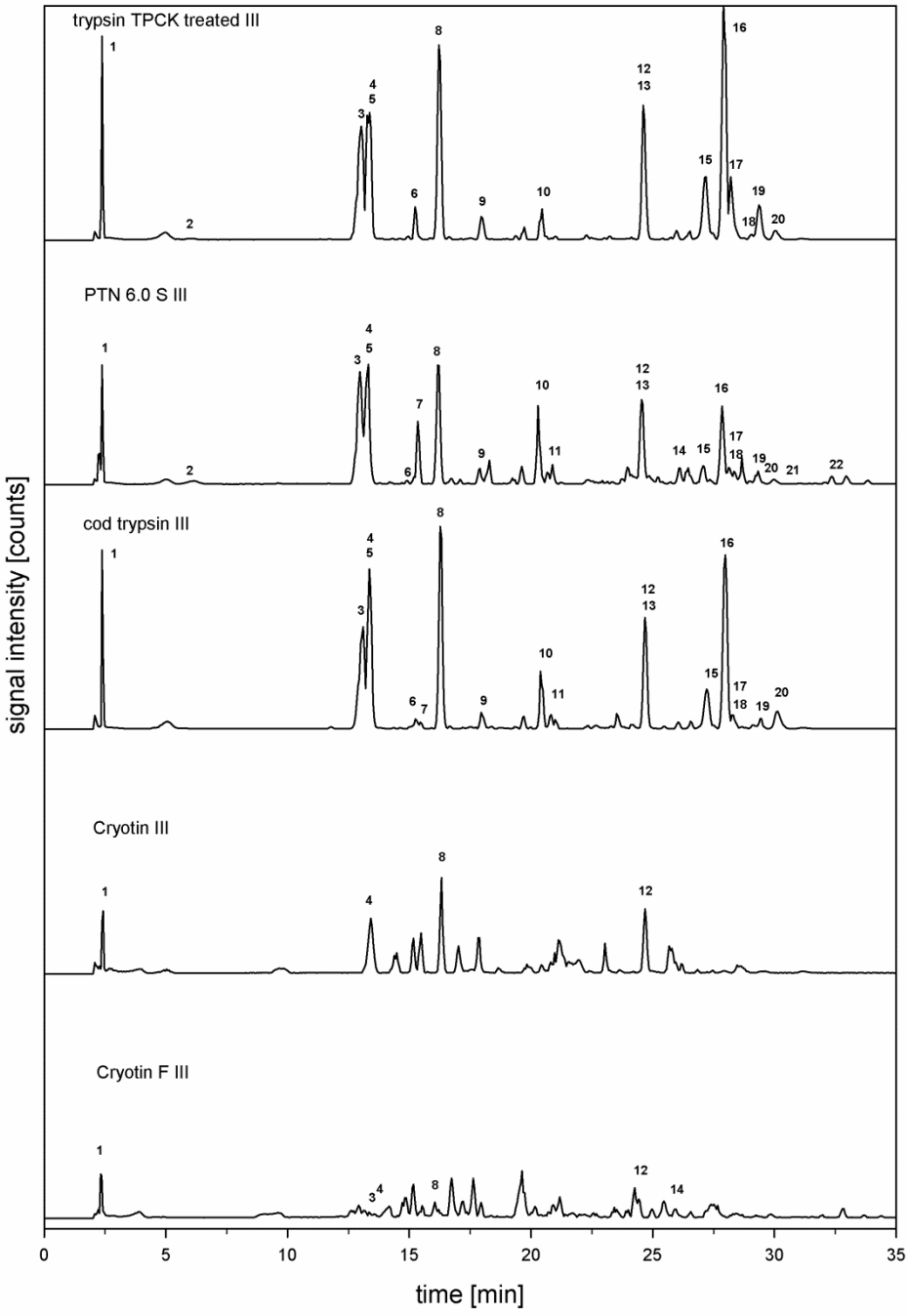



**Fig. 4.1 c)** LC-MS profiles of permeates 5 kDa (fraction III) generated with the enzyme preparations cod trypsin (CT), porcine trypsin (TPCK) (T), PTN 6.0S (P), and Cryotin F (CF). Visible peaks of the main 26 β-casein fragments focused **(Table 4.2)** are numbered with 1- 22 in the chromatogram.

The chromatograms **(Fig. 4.1 c)** of the 5 kDa permeates (fraction III) produced by application of the enzymes cod trypsin, porcine trypsin (TPCK), and PTN 6.0 S are far comparable. Small peptides, numbered with 1 to 16, are concentrated and peak areas of fragments with high molecular mass, numbered with 17 to 22, are reduced in comparison to their total hydrolysates.

The peptides in hydrolysates were identified by applying Proteome Discoverer 1.4 and the database Uniprot. **Table 4.2** displays the peptides characterized in fractions I- III of hydrolysates incubated with cod trypsin (CT), porcine trypsin (TPCK) (T), PTN 6.0S (P), Cryotin (C), and Cryotin F (CF). We concentrated on 26 main peptides presented in hydrolysates generated with cod trypsin, porcine trypsin, and PTN 6.0 S. The main 26 peptides are numbered in **Table 4.2** with 1- 22. In peak 17 and 19, three different peptides were co-eluting with same retention time in each case, and were therefore marked with only one number. **Figure 4.1A- C** show LC-MS chromatograms of the β-casein hydrolysates, in which peaks are numbered with the corresponding peptides from **Table 4.2**. The β-casein fragments exhibit masses ranging from about 640 Da to 7500 Da (MH+) and retention times from 2.5 min to 33 min. Because commercially available rennet casein was used for isolation of β-casein, peptide sequences of the genetic variants of β-casein $A^1$ (peptides 17(1- 3)), $A^2$ (peptides 13, 19(1- 3)), and B (peptide 9) [23, 24] were found. The identified peptides of hydrolysates generated with the enzyme preparations cod trypsin and porcine trypsin (TPCK), which contain only tryptic activity, are very similar. Therefore, it can be assumed that trypsin from cod and from hog have comparable substrate specificities. Furthermore, Bunkenborg et al. 2013 described that trypsin derived from cod and hog hydrolyze peptide bonds at the carboxylic end of the amino acid residue arginine (R) and lysine (K) [25]. Peptides 14, 21, and 22 occurred only in fractions generated with PTN 6.0 S because of the chymotryptic side activity in this enzyme preparation. Although all β-casein fragments shown in **Table 4.2** (with the exception of peptide 3) exert more cleavage sites for chymotrypsin, hydrolysis with PTN 6.0 S resulted mainly in tryptic β-casein fragments. Even though, PTN 6.0 S exhibits low chymotryptic activity **(Table 4.1)**, this enzyme preparation reveals mainly tryptic activity. Hydrolysates from chymotryptic proteolysis with Cryotin and Cryotin F displayed completely different peptide profiles containing more small peptides. The corresponding proteolysates contained only peptides 1, 4, 8, and 12, which were also presented in hydrolysates generated with cod trypsin, porcine trypsin (TPCK), and PTN 6.0S. In addition, with Cryotin F, only

peptides 3 and 14 were produced. Because of the high chymotryptic activity of the preparations Cryotin and Cryotin F, β-casein is degraded into other smaller peptides.

**Table 4.2** Identification of the main peptides of total β-casein hydrolysate (I), peptides > 5 kDa (II) and peptides < 5 kDa (III) generated with different proteolytic enzymes by HPLC-ESI-MS$^n$. The focus is on main tryptic β-casein fragments generated by cod trypsin, porcine trypsin, and PTN 6.0 S. [a] cod trypsin, [b] porcine trypsin (TPCK), [c] PTN 6.0 S, [d] Cryotin, [e] Cryotin F, ∑ = phosphoserine

| Peak | β-CN fragment | Primary structure | *(m/z)* $(M+H^+)$ | CT [a] I | CT II | CT III | T [b] I | T II | T III | P [c] I | P II | P III | C [d] I | C II | C III | CF I | CF II | CF III |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | β-CN A (f 100-105) | EAMAPK | 646.3 | + | + | + | + | | + | + | + | + | + | + | + | + | | + |
| **2** | β-CN A (f 98-105) | VKEAMAPK | 873.6 | | | | + | | + | + | + | + | | | | | | |
| **3** | β-CN A (f 177-183) | AVPYPQR | 830.4 | + | + | + | + | + | + | + | + | + | | | | + | | + |
| **4** | β-CN A (f 106-113) | HKEMPFPK | 1013.6 | + | | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **5** | β-CN A (f 170-176) | VLPVPQK | 780.5 | + | + | + | + | + | + | + | + | + | | | | | | |
| **6** | β-CN A (f 33-48) | FQ∑EEQQQTEDELQDK | 2062.0 | + | + | + | + | + | + | + | + | + | | | | | | |
| **7** | β-CN A (f 194-202) | QEPVLGPVR | 994.6 | + | | + | | | | + | + | + | | | | | | |
| **8** | β-CN A (f 108-113) | EMPFPK | 748.3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **9** | β-CN B (f 114-122) | YPVEPFTER | 1137.2 | + | | + | + | | + | + | + | + | | | | | | |
| **10** | β-CN A (f 191-202) | LLYQEPVLGPVR | 1383.9 | + | | + | + | | + | + | + | + | | | | | | |
| **11** | β-CN A (f 184-190) | DMPIQAF | 820.9 | + | | + | + | | | + | | + | | | | | | |
| **12** | β-CN A (f 203-209) | GPFPIIV | 742.5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **13** | β-CN A$^2$ (f 49-68) | IHPFAQTQSLVYPFPGPIPN | 2223.3 | + | + | + | + | | + | + | + | + | | | | | | |
| **14** | β-CN A (f 194-209) | QEPVLGPVRGPFPIIV | 1717.5 | | | | | | | + | + | + | | | | + | | + |
| **15** | β-CN A (f 69-97) | SLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 3114.3 | + | + | + | + | + | + | + | + | + | | | | | | |
| **16** | β-CN A (f 184-202) | DMPIQAFLLYQEPVLGPVR | 2186.4 | + | + | + | + | + | + | + | + | + | | | | | | |
| **17(1)** | β-CN A$^1$ (f 33-97) | FQ∑EEQQQTEDELQDKIHPFAQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 4863.8 | + | | + | | | | + | + | + | | | | | | |
| **17(2)** | β-CN A$^1$ (f 53-97) | AQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 5359.8 | | | + | + | + | + | + | + | + | | | | | | |
| **17(3)** | β-CN A$^1$ (f 49-97 ) | IHPFAQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 7402.2 | + | + | + | + | + | + | + | + | | | | | | | |
| **18** | β-CN A (f 123-169) | QSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK | 5310.4 | + | + | + | + | + | + | + | + | + | | | | | | |
| **19(1)** | β-CN A$^2$ (f 33-97) | FQ∑EEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 7362.5 | + | + | + | + | + | + | + | + | | | | | | | |
| **19(2)** | β-CN A$^2$ (f 53-97) | AQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 4823.8 | + | | + | | | | + | + | + | | | | | | |
| **19(3)** | β-CN A$^2$ (f 49-97) | IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK | 5319.5 | | | + | + | + | + | + | + | + | | | | | | |
| **20** | β-CN A (f 114-169) | YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK | 6361.7 | + | + | + | + | + | + | + | + | + | | | | | | |
| **21** | β-CN A (f 114-165) | YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVL | 5729.1 | | | | | | | + | + | + | | | | | | |
| **22** | β-CN A (f 184-209) | DMPIQAFLLYQEPVLGPVRGPFPIIV | 2910.1 | | | | | | | + | + | + | | | | | | |

### *IV.4.2 Potentially anti-inflammatory effect of peptide fractions in HEK$^{nfκb\text{-}RE}$ cells*

The evaluation of the potentially anti-inflammatory effect of β-casein hydrolysates generated with the enzyme preparations cod trypsin, porcine trypsin, PTN 6.0 S, Cryotin, and Cryotin F was performed with TNF-α stimulated HEK$^{nfκb\text{-}RE}$ cells **(Fig. 4.2A- C)**. These cells exhibited a TNF-α concentration-dependent expression of luciferase, which was linear between 0.2 and 12.8 ng/mL TNF-α. Treatment of cells with 0.4 ng/mL TNF-α resulted in increased luciferase activity by a factor of 9.0 ± 2.3 (mean ± SD) compared with unstimulated controls without TNF-α addition.

**Fig. 4.2 (a)**

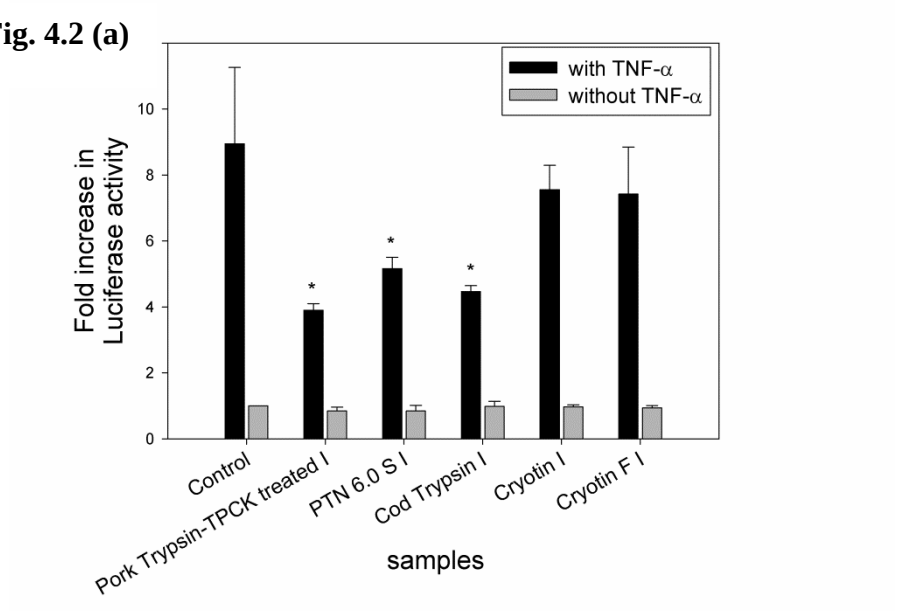


**Fig. 4.2 (b)**

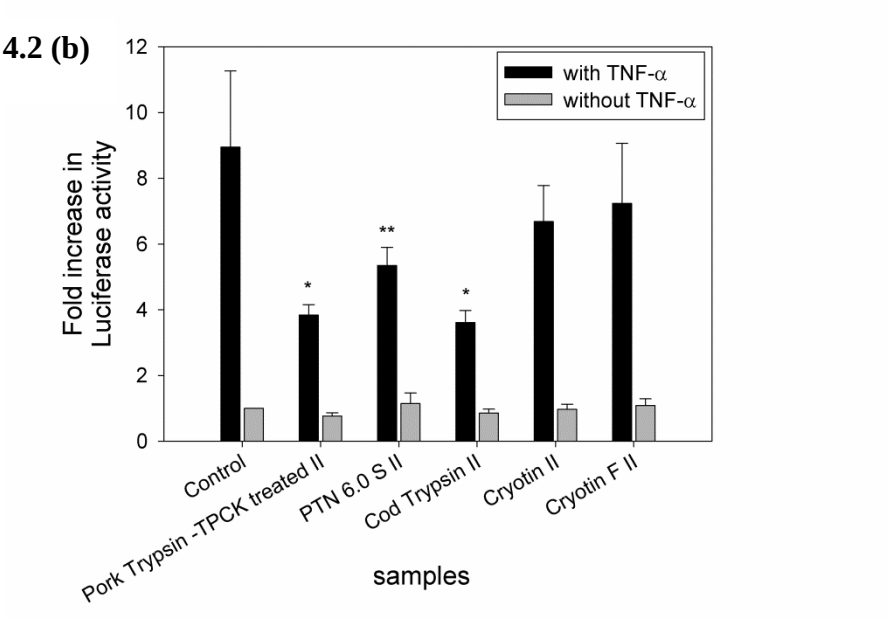



**Fig. 4.2 (c)**

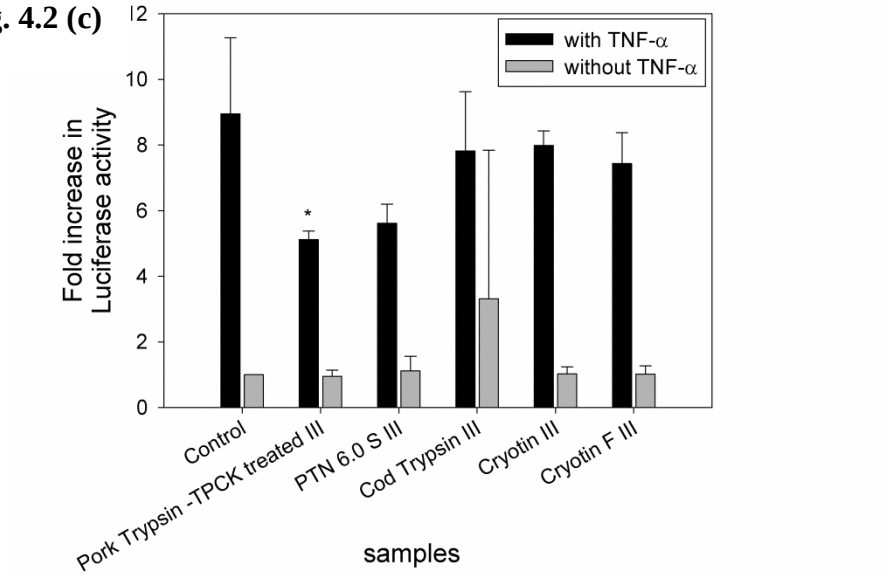



**Fig. 4.2.** Potentially anti-inflammatory activity of (a) total β-casein hydrolysate (fraction I), (b) 5 kDa retentates (fraction II), and (c) 5 kDa permeates (fraction III), generated with porcine trypsin, cod trypsin, PTN 6.0 S, Cryotin, and Cryotin F in TNF-α-induced HEK$^{nfκB\text{-}RE}$ cells measured as luciferase activity. Luminescence of treated cells is shown as fold increase compared with the untreated control. Values are means ± standard deviations (SD) of two hydrolysate productions with at least three independent replicates of cell incubation and luciferase activity determination. Significance values were calculated using Kruskal-Wallis one-way analysis followed by the Dunn's multiple comparison test. Significance values were determined in comparison with TNF-α treatment: *$P < 0.05$, **$P < 0.01$.

The total hydrolysates produced by application of the enzyme preparations cod trypsin, porcine trypsin, and PTN 6.0 S revealed reduced luciferase activity, and therefore, decreased NFκB activation in stimulated HEK$^{nfκb\text{-}RE}$ cells **(Fig. 4.2A)**. Addition of the total hydrolysate generated with cod trypsin to stimulated cells resulted in a reduction of the luciferase activity of about 50 % from 9.0 ± 2.3 to 4.5 ± 0.2. Applying the total hydrolysate generated with porcine trypsin (TPCK) led to a comparable reduction of the luciferase activity of about 50 % (3.9 ± 0.2). Treatment of cells with the total proteolysate prepared by PTN 6.0 S showed a reduction of the luciferase activity of about 40 % (5.2 ± 0.3). Total hydrolysates generated with the enzyme preparations Cryotin and Cryotin F did not reveal any effect on luciferase activity.

Incubation of cells with the 5 kDa retentates of proteolysates generated by cod trypsin and porcine trypsin (TPCK) **(Fig. 4.2B)** exhibited a decrease in luciferase activity within the same range compared to the total hydrolysates, resulting in a reduction of TNF-α stimulation over 50 % from 9.0 ± 2.3 (control) to 3.6 ± 0.4 and 3.8 ± 0.3, respectively. As with the whole hydrolysate, a reduction of the luciferase activity of about 40 % was determined by treatment of cells with the 5 kDa retentate of the hydrolysates prepared by PTN 6.0 S (5.4 ± 0.6). As shown in **Fig. 4.2 A**, the 5 kDa retentates produced with Cryotin and Cryotin F also did not reveal any anti-inflammatory activity in cells.

The luciferase activity was also decreased by about 40 % after addition of the 5 kDa permeates of the hydrolysate generated by porcine trypsin (TPCK) (5.1 ± 2.3) and PTN 6.0S (5.6 ± 0.6) **(Fig. 4.2C)**. However, there was no significant decrease in the luciferase activity, when the permeate of the proteolysate generated by cod trypsin was applied. The reason for the high luciferase activity in HEK$^{nfκb\text{-}RE}$ cells after treatment with the 5 kDa permeate produced by cod trypsin, with or without addition of TNF-α, is not yet known. Applying the 5 kDa permeates prepared by Cryotin and Cryotin F did not exhibit any effect on luciferase activity **(Fig. 4.2C)**. This may be due to the fact that most peptides from hydrolysates liberated by the enzyme preparations cod trypsin, porcine trypsin-TPCK treated and PTN 6.0 S are not present in proteolysates generated by the preparations Cryotin and Cryotin F. The most tryptic peptides were degraded when β-casein was hydrolyzed with the enzyme preparations Cryotin and Cryotin F. Recently, a study evaluated the potentially anti-inflammatory activity of peptides smaller than 1 kDa (permeate 1 kDa) from a β-casein

hydrolysate generated with porcine trypsin (TPCK) [18]. In this study, the proteolysate was ultrafiltrated with a 5 kDa membrane and the 5 kDa permeate was ultrafiltrated with a 1 kDa membrane. The tryptic β-casein fraction, which contained peptides smaller than 1 kDa, did not show any anti-inflammatory effect in TNF-α stimulated HEK$^{nfκb\text{-}RE}$ cells. Therefore, in the present study, a further fractionation of the 5 kDa permeates using a 1 kDa membrane filtration was not performed.

The egg-derived peptide IRW showed anti-inflammatory activity in TNF-α stimulated endothelial cells by reducing the production of the inflammatory marker MCP-1. The extent of TNF-α stimulation was reduced by about 40 % when cells were treated with 50 µmol/L of IRW [26]. Moreover, the peptides IRW and IQW derived from ovotransferrin showed a reduction in oxidative stress by a decrease of superoxide production in TNF-α stimulated human umbilical vein endothelial cells [27]. The reduction of luciferase activity by a decrease of NFκB activity in HEK$^{nfκb\text{-}RE}$ cells, found in the present study with tryptic casein hydrolysate, was in the same order of magnitude as data reported in the literature. It can be concluded that the β-casein hydrolysates generated with the enzyme preparations cod trypsin, porcine trypsin (TPCK), and PTN 6.0S exhibit the same potential of anti-inflammatory activity.

## IV.5 Conclusion

Potentially anti-inflammatory peptides were produced from β-casein by hydrolysis with the tryptic enzyme preparations cod trypsin, porcine trypsin (TPCK), and PTN 6.0 S. The whole hydrolysates (fraction I) and the UF-retentates > 5 kDa (fraction II) from the preparations cod trypsin and porcine trypsin (TPCK) exhibited a 50 % reduction of luciferase activity, which was the highest potentially anti-inflammatory effect in TNF-α induced HEK$^{nfkb\text{-}RE}$ cells. In conclusion, the generation of anti-inflammatory peptides by hydrolysis of β-casein with cod trypsin, as a by-product of the fishery industry, could be a new scope of application in food processing. Cod trypsin can replace the non-food grade porcine enzyme preparation trypsin (TPCK) in this process as anti-inflammatory activity was highest in hydrolysates generated with enzyme preparations cod trypsin and trypsin (TPCK) containing tryptic but no chymotryptic activity. By application of PTN 6.0 S with a tryptic/chymotryptic ratio of 1/0.05, the anti-inflammatory activity was slightly lower. Proteolysates prepared by incubation with the preparations Cryotin (tryptic/chymotryptic ratio 1/0.2) and Cryotin F (tryptic/chymotryptic ratio 1/2.06) did not show any reduction in luciferase activity. The more chymotryptic enzyme activity is present, the lower the potentially anti-inflammatory activity of the hydrolysates in TNF-α induced HEK$^{nfkb\text{-}RE}$ cells.

Biologically active short peptides, which are not degraded during gastrointestinal digestion, can be absorbed intact and elicit their biological activity at target areas. It is assumed that bioactive peptides may be absorbed mainly via paracellular diffusion or via carrier mediated transport across the cell monolayer [1]. As shown in a previous study, peptides < 1 kDa generated with porcine trypsin (TPCK) did not exhibit potentially anti-inflammatory activity in HEK$^{nfkb\text{-}RE}$ cells [18]. Therefore, it can be assumed that potentially anti-inflammatory activity is based upon longer peptides > 1 kDa. It is not to be expected that long oligopeptides would show potentially anti-inflammatory activity, because of degradation during gastrointestinal digestion. However, the oligopeptides identified in tryptic β-casein hydrolysate **(Table 4.2)** may be precursor for the liberation of smaller bioactive peptides, which are released by enzymatic hydrolysis in the body. The bioactivity of long-chain peptides as precursors has been described before [1]. It is possible that oligopeptides containing a bioactive sequence display their biological effect by passing through the cell monolayer and reaching potential sites of action in the body, where the protected active sequence is released from the precursor

molecule. For example, a precursor of casomorphin-7 has been found in the plasma of newborn calves after their first milk intake. This pre-casomorphins could reach opioid receptors located in the nervous, endocrine, and immune systems of the organism [1].

## ABBREVIATIONS USED

TPCK, tosyl phenylalanyl chloromethyl ketone; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; TNF-α, tumor necrosis factor alpha; $HEK^{nfkb\text{-}RE}$ cells, human embryotic kidney cells; IKK complex, IκB-kinase complex; NMWCO, Nominal Molecular Weight Cut-Off

## ACKNOWLEDGMENT

This research project is funded by the Federal Ministry for Education and Research (BMBF) as part of the competence network “FoCus- Food Chain Plus”.

## IV. 6 References


1. Meisel H, Biochemical properties of peptides encrypted in bovine milk proteins. *Curr. Med Chem* **12:** 1905-1919 (2005).
2. Boutrou R, Gaudichon C, Dupont D, Jardin J, Airinei G, Marsset-Baglieri A, Benamouzig R, Tome D, Leonil J, Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *Am J Clin Nutr* **97:** 1314-1323 (2013).
3. Meisel H, Biochemical properties of regulatory peptides derived from milk proteins. *Pept Sci* **43:** 119-128 (1997).
4. Dziuba B and Dziuba M, Milk proteins-derived bioactive peptides in dairy products: molecular, biological and methodological aspects. *Acta Sci Pol Technol Aliment* **13:** 5-25 (2014).
5. Espeche Turbay MB, de Moreno de LeBlanc A, Perdigón G, Savoy de Giori G, Hebert EM, β-Casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. *J. Dairy Sci* **95:** 1108-1118 (2012).
6. Nielsen DS, Theil PK, Larsen LB, Purup S, Effect of milk hydrolysates on inflammation markers and drug-induced transcriptional alterations in cell-based models. *J Anim Sci* **90** (Suppl 4): 403-5 (2012).
7. Piccolomini A F, Iskandar MM, Lands L C, Kubow S, High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food Nutr Res* **56:** 1-10 (2012).
8. Tavares TG, Spindola H, Longato G, Pintado ME, Carvalho JE, Malcata FX, Antinociceptive and anti-inflammatory effects of novel dietary protein hydrolysate produced from whey by proteases of *Cynara cardunculus*. *Int Dairy J* **32:** 156-162 (2013).
9. Millán-Linares Model C, Yust Mdel M.; Alcaide-Hidalgo JM, Millán F, Pedroche J, Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway. *Food Chem* **151:** 141-147 (2014).

10. Xu M, Chen ZJ, Ubiquitin mediated regulation of protein kinases in NFkB signaling. In *Regulation of organelle and cell compartment signaling,* ed. by Bradshaw RA, Dennis EA. Academic Press, London, pp. 71-82 (2011)

11. Zelová H, Hošek J, TNF-α signalling and inflammation: interactions between old acquaintances. *Inflammation Res.* **62:** 641-651 (2013).

12. Li H, *The role of RIP ubiquitination in the TNF-α signaling pathway*. Doctoral thesis, University at Buffalo, Proquest, Ann Arbor MI (2007).

13. Rahman MM, McFadden G, Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog.* **2:** 66-77 (2006).

14. Gudmundsdottir A, Palsdottir HM, Atlantic cod trypsins: From basic research to practical applications. *Mar Biotechnol* **7:** 77-88 (2005).

15. Rossano R, Larocca M, Riccio P, Digestive enzymes of the crustaceans *Munida* and their application in cheese manufacturing: a review. *Mar Drugs* **9:** 1220-1231 (2011).

16. Stefansson B, Helgadóttir L, Olafsdottir S, Gudmundsdottir Á, Bjarnason JB, Characterization of cold-adapted Atlantic cod *(Gadus morhua)* trypsin I — Kinetic parameters, autolysis and thermal stability. *Comp Biochem and Physiol, Part B.* **155:** 186-194 (2010).

17. Shahidi F, Janak Kamil YVA, Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Sci Technol* **12:** 435-464 (2001).

18. Malinowski J, Klempt M, Clawin-Rädecker I, Lorenzen PC, Meisel H, Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. *Food Chem* **165:** 129-133 (2014).

19. Le Magnen C, Maugas J-J, Method for obtaining beta casein. United States Patent 5397577 (1995).

20. Reichard U, Buttner S, Eiffert H, Staib F, Ruchel R, Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *J Med Microbiol* **33:** 243-51 (1990).

21. Lorenzen PChr, Kiesner C, Goepfert A, Schlimme E, Properties of strong-heated foods: in vitro proteolysability of sterilised milk. *Milchwiss-Milk Sci Int* **54:** 442-446 (1999).

22. Lorenzen PChr, Schlimme E, The plastein reaction: properties in comparison with simple proteolysis. *Milchwiss-Milk Sci Int* **47:** 499-504 (1992).

23. Gallinat JL, Qanbari S. Drögemüller C, Pimentel ECG, Thaller G, Tetens J, DNA-based identification of novel bovine casein gene variants. *J Dairy Sci* **96:** 699-709 (2013).

24. Farrell HM Jr, Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK, Hicks CL, Hollar CM, Ng-Kwai-Hang, K. F.; Swaisgood, H. E., Nomenclature of the proteins of cows' milk—sixth revision. *J Dairy Sci* **87:** 1641-1674 (2004).

25. Bunkenborg J, Espadas G, Molina H, Cutting edge proteomics: benchmarking of six commercial trypsins. *J Proteome Res* **12:** 3631-3641 (2013).

26. Huang W, Chakrabarti S, Majumder K, Jiang Y, Davidge ST, Wu J, Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *J Agric Food Chem* **58:** 10840-10846 (2010).

27. Majumder K, Chakrabarti S, Davidge ST, Wu J, Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *J Agric Food Chem* **61:** 2120-2129 (2013).

# Chapter V Conclusions

This work is focused on the production of functional food ingredients of the carbohydrate and protein fractions of milk. With the present investigations an enrichment of bioactive oligosaccharides and a generation of bioactive peptides from milk were realized. A characterization of the beneficial health effects of the oligosaccharides and peptides was performed by evaluation of the NFκB activity in $HEK^{nfkb\text{-}RE}$ cells *in vitro*.

## V.1 Development of procedures for the enrichment of MOS by NF

For the enrichment of MOS by membrane filtration, an initial screening and a comparison of the efficiency of different NF membranes were performed on laboratory scale *(Manuscript 1, chapter II)*. The best results were achieved by application of NF membranes with a NMWCO of 150-400 Da. A retention of MOS (6'-SL, 3'-SL, GalNAc-Gal-Glc) of 49 % to 84 % was accomplished. In consideration of the results from laboratory scale NF, the process was transferred to pilot plant and industrial scales. The same level of retention was achieved by application of the spiral wound modules SR 50 and DL on pilot plant scale and by the membrane DOW on industrial scale NF. A 100-fold increase in the MOS content in relation to the total sugar content (10.6 %) compared to the initial sample (0.1 %) was achieved after NF on industrial scale by application of the membrane DOW. The NF retentate produced on industrial scale was composed of 2.6 % MOS, 28.3 % mono- and disaccharides (glucose, galactose, lactose), 23.5 % citric acid and 24.7 % ash. A screening of the efficiency of different NF membranes for the enrichment of MOS on different scales of production was performed for the first time.

Furthermore, the NF process for the enrichment of MOS was optimized, achieving a better permeation of milk salts and residual sugars during NF at pH 5 and pH 7 by using the previously concentrated NF retentate from the preceding experiment as a raw material *(Manuscript II, chapter III)*. After NF and diafiltration with the membrane module DL on pilot plant scale, a high retention of MOS of 68 % to 79 % was achieved at acidic and neutral milieu. The high MOS content in relation to total sugar content of 87.3 % to 92.7 % in the final NF retentate at pH 5 and 7 (9-fold higher than in the first NF retentate, 900-fold higher than in the initial sample) indicated a nearly complete permeation of mono- and disaccharides.

Generally, a higher enrichment of MOS in dry mass (5-fold higher than in the first NF retentate, 140-fold higher than in the initial sample) and a higher permeation of salts were achieved by NF at pH 5. The dry mass of the NF retentate at pH 5 was composed of 14.1 % MOS, 1.2 % mono- and disaccharides, 13.3 % citric acid and 21.8 % ash. Moreover, the first study on the transferability of the optimized process to caprine milk resulted in a markedly increase in the MOS content in relation to total sugar content in retentate (23- to 31-fold higher than in the initial sample) of NF at neutral milieu. The influence of the pH on the degree of enrichment of MOS by NF was evaluated for the first time.

The results obtained demonstrate that a two-stage NF process is highly suitable for the enrichment of MOS. Even though the concentration of oligosaccharides in milk of domestic animals is very low *(chapter I.1.2.1)* (Martinez-Ferez et al. 2006; Albrecht et al. 2014), high degrees of MOS enrichment were achieved by the developed NF process. The membrane filtration technology is widely applied in the dairy industry for the fractionation of milk components, e.g. caseinmicelles, WPC, WPI or lactose *(chapter I.1.2.5)* (Mehra et al. 2014) as well as for concentration and desalination of products e.g. by NF (Carić et al. 2009). Moreover, membrane filtration and evaporation techniques can be combined whereby the first concentration and desalination is often done by membrane filtration followed by evaporation (Carić et al. 2009). Because the technological devices are available, the implementation of the NF procedure for the enrichment of MOS is feasible without high acquisition costs and effort. Furthermore, the NF process can be performed continuously for 24 h a day using a readily available dairy stream. Whey-UF permeate or SM-UF permeate are produced in high amounts and are valuable sources for the enrichment of MOS *(chapter I.1.2.5)* (Barile et al. 2009). NF at acidic milieu resulted in a higher permeation of milk salts, especially of calcium, sodium and potassium, but also of citric acid *(Manuscript II, chapter III*). While at pH 7 the divalent salts are only partly ionized, the dissociation of the minerals is higher at acidic milieu, resulting in lower molecular masses (Lucey and Horne 2009; Walstra et al. 1999; Fox and McSweeney 2009). Therefore, the NF at pH 5 might promote a higher permeation of milk salts. Moreover, Rice et al. (2006) detected a markedly lower decrease in permeate flux during NF of bovine SM-UF permeate at pH 5 compared to the process at pH 8, indicating a lower fouling of the membrane (Rice et al. 2006). In our investigations, the permeate flux was also slightly higher during NF at pH 5 compared to pH 7. The high permeate flux of 31.6 $L/m^2h$ at pH 5 resulted in very short process time of the NF. Therefore, the permeate flux and

the membrane fouling behavior cannot be evaluated. The NF at acidic milieu promotes a higher permeation of the milk salts and might be beneficial to lower the fouling of the membrane. Especially when NF is performed continuously over a long period of time, a decreased fouling of the membrane is mandatory for a successful and effective performance.

Our investigations on laboratory scale showed a better separation of MOS from the residual sugars (lactose, glucose, galactose) when lactose was hydrolyzed in the SM-UF permeate before NF *(Manuscript 1,chapter II)*. Because the MOS fraction in milk of domestic animals is mainly composed of trisaccharides (e.g. 3'-SL, 6'-SL = 655 Da, GalNAc-Gal-Glc = 546 Da), the selective permeation of the residual sugars was higher when lactose was hydrolyzed. Moreover, during the lactose hydrolysis with a β-galactosidase from *Kluyveromyces lactis*, GOS were synthesized. During NF, the valuable GOS were enriched in appreciable amounts *(chapter II, III)*, representing a second bioactive sugar component in the retentate. Therefore, lactose hydrolysis of SM-UF permeate is a beneficial step to gain a NF retentate of high purity containing MOS and synthesized GOS. The NF permeate as a by-product of the MOS enrichment contained high amounts of glucose and galactose. These sugars may be used as fermentation products, e.g. for ethanol production. A concentration of the NF permeate e.g. by reverse osmosis (RO) before fermentation might be necessary, due to high dilution during diafiltration (DF). In a recent study, NF of mother liquor without lactose hydrolysis was performed for the concentration of MOS (Mehra et al. 2014). By the application of mother liquor as a raw material, crystalline lactose was obtained as a valuable dairy product before MOS enrichment. However, without lactose hydrolysis no GOS were synthesized.

In consideration of the achieved results for the enrichment of MOS from bovine lactose hydrolyzed SM-UF permeate at acidic milieu and the first investigations for the transferability of this NF process to lactose hydrolyzed caprine SM-UF permeate at neutral milieu, the enrichment of MOS from caprine milk should be performed at acidic milieu to increase the milk salt permeation. Furthermore, NF of caprine milk permeate should be performed with a higher concentration factor (CF) and diafiltration (DF) might be conducted with a higher amount of diavolumes (DV) to achieve a higher degree of MOS enrichment and a higher purity of the concentrate.

Whey permeates or SM-UF permeates derived from bovine and caprine milk present highly valuable sources for the enrichment of MOS by NF. Even though the concentrations of MOS

in bovine milk are very low, a concentration of MOS is highly promising due to the high amounts of whey available in the dairy industry. Caprine milk contains a MOS profile closer to that of human milk than bovine milk. Therefore, the combined addition of MOS concentrates produced from bovine and caprine milk as a functional ingredient to food might be beneficial to reproduce the oligosaccharide profile of milk.

In conclusion, an efficient and optimized procedure for the enrichment of MOS by NF on industrial scale was developed resulting in a high retention of MOS and a high purity of the NF retentate due to high permeation of residual sugars and milk salts. Implementation of this developed process in food industry could enable the production of NF retentates with high MOS concentrations for the application as functional food ingredients.

### V.2 Development of procedures for the generation of anti-inflammatory peptides

By hydrolysis of β-casein with the enzyme preparations cod trypsin, porcine trypsin (TPCK) and PTN 6.0 S, potentially anti-inflammatory peptides were generated and identified. The highest potentially anti-inflammatory effect was detected in total β-casein hydrolysates (fraction I) and in the UF retentates > 5 kDa (fraction II) produced by the enzyme preparations cod trypsin and porcine trypsin (TPCK), exhibiting a reduction in luciferase activity of 50 % in TNF-α induced $HEK^{nfκb\text{-}RE}$ cells. Due to the comparable extent of reduction of luciferase activity and the almost identical peptide profile, cod trypsin, as a by-product of the fish manufacturing industry, can replace the non-food grade porcine enzyme preparation trypsin (TPCK). A new field of application for cod trypsin may be the generation of anti-inflammatory peptides from bovine β-casein. Hydrolysis of β-casein with the porcine enzyme preparation PTN 6.0 S (tryptic/chymotryptic ratio 1/0.05) resulted in a slightly lower anti-inflammatory activity, whereas the application of the fish enzyme preparations Cryotin (tryptic/chymotryptic ratio 1/0.2) and Cryotin F (tryptic/chymotryptic ratio 2.06) did not show any reduction of luciferase activity. It can be concluded that the higher the chymotryptic activity the lower the potentially anti-inflammatory activity of the hydrolysates in TNF-α induced $HEK^{nfκb\text{-}RE}$ cells.

The identified peptides in the potentially anti-inflammatory hydrolysates exhibited masses from 640 to 7500 Da **(Table 4.2)**. Recently, a study showed that a peptide fraction < 1 kDa hydrolyzed by porcine trypsin (TPCK) did not exhibit potentially anti-inflammatory effect in

HEK$^{nfκb\text{-}RE}$ cells (Malinowski et al. 2014). It can be supposed that the anti-inflammatory activity of the tryptic β-casein hydrolysates is based on peptides > 1 kDa. Because of degradation during gastrointestinal digestion, peptides with high molecular mass are not to be expected to exhibit potentially anti-inflammatory activity.

The identification of peptides derived from dietary proteins in humans *in vivo* is very difficult. Boutrou et al. (2013) identified peptides in the jejunum of humans who ingested caseins and whey proteins within a period of 0 h to 6 h after intake. Most casein-derived peptides in the jejunal effluents had a molecular size of 450-1800 Da. Fragments with a molecular size of 750-1050 Da were predominant during 6 h after ingestion, whereas the number of peptides exhibiting a mass of 1050-1800 Da started to decrease after 3 h of digestion. A few peptides ranging in size between 1800-3000 Da were present up to 2.5 h after consumption, indicating a degradation by longer digestion times. Moreover, the absorption of β-casein-derived fragments up to a size of 4400 Da has been shown by identification of the peptide β-CN (f 170-209) in the jejunal effluents (Boutrou et al. 2013).

In our investigation *(Manuscript 3, chapter IV)*, various potentially anti-inflammatory peptides derived from β-casein exhibited molecular masses in the range of 640-3200 Da (peptides 1-16, 22) **(Table IV.2)**. The fragment β-CN (f 184-202) with the primary structure DMPIQAFLLYQEPVLGPVR is described to exhibit potentially anti-inflammatory activity by the reduction of NFκB activity measured by the luciferase activity in HEK$^{nfκb\text{-}RE}$ cells *in vitro* (Malinowski et al. 2014). This anti-inflammatory peptide was also generated in our study by tryptic hydrolysis with the enzyme preparations cod trypsin, porcine trypsin (TPCK) and the porcine PTN 6.0 S *(Manuscript 3, chapter IV)*. Considering the results of Boutrou et al. (2013), it is possible that these peptides of the potentially anti-inflammatory β-casein hydrolysate are absorbed completely. Moreover, β-casein fragments were liberated which reveal other bioactivities. During the hydrolysis of β-casein with the tryptic enzyme preparation cod trypsin, porcine trypsin (TPCK) and the porcine tryptic and chymotryptic PTN 6.0 S, two anti-oxidative peptides (β-CN (f 98-105) peptide 2, β-CN (f 170-176) peptide 5), one ACE inhibitory peptide (β-CN (f 108-113) peptide 8) and one peptide exhibiting anti-oxidative and opioid activity (β-CN (f 177-183) peptide 3) were released **(Table IV.2, Fig. 1.8)**. Furthermore, in the potentially anti-inflammatory β-casein hydrolysates, fragments with a molecular size of 4800-7500 Da were identified (peptides 17-21) **(Table IV.2)**. A

degradation of these peptides during the gastrointestinal digestion is expected. However, all β-casein-derived tryptic peptides and especially high molecular weight fragments might function as a precursor of smaller bioactive peptides during the gastrointestinal digestion. Indeed, various ACE inhibitory, opioid and anti-oxidative peptides are encrypted in the primary structure of the tryptic peptides generated in our investigation **(Fig. 1.8, Table IV.2)**. Also Meisel et al. (2005) described that bioactivity of long-chain peptides may rely on smaller fragments liberated by enzymatic fragmentation. It is probable that inactive peptides containing the latent bioactive sequence enter the blood stream and elicit their effects after enzymatic release of the protected active sequence. For instance, a precursor of β-casomorphin-7 (β-CN (f 60-66)) has been found in the plasma of calves after their first milk intake. This pre-casomorphin could reach target areas where the active sequence gets liberated (Meisel 2005). The identified peptides in the potentially anti-inflammatory β-casein hydrolysates generated by tryptic proteolysis by applying cod trypsin, porcine trypsin (TPCK) and PTN 6.0 S might also serve as a precursor for the liberation of smaller anti-inflammatory β-casein fragments.

As a result, the development of a procedure for the generation of potentially anti-inflammatory peptides from β-casein by application of tryptic enzyme preparations from cod and hog was successful. An establishment of this process on high scale production should be feasible enabling the provision of a functional ingredient for food industry.

## V.3 Characterization of the biological activity of enriched MOS concentrates and generated β-casein hydrolysates

The evaluation of the bioactivity of the MOS concentrates produced by NF and the tryptic and chymotryptic β-casein hydrolysates *(Manuscript 3, chapter IV)* was performed in TNF-α stimulated $HEK^{nfkb\text{-}RE}$ cells *in vitro (chapter I.1.4).*

SM-UF permeate and the MOS enriched retentates generated by NF on pilot plant and industrial scales exhibited immunostimulatory activity by the increase in luciferase activity in comparison to the control *(Manuscript 1, chapter II)*. Moreover treatment of cells with the MOS standards 3'-SL and 6'-SL resulted in a significant up-regulation of luciferase activity.

Incubation of the cells with lactose showed no significant effect on luciferase activity in comparison to the control. It has to be considered that the NF retentates may contain further substances such as peptides, milk salts and GOS, which may influence the NFκB activity in the cells.

The evaluation of the anti-inflammatory effect of β-casein hydrolysates generated with the enzyme preparations cod trypsin, porcine trypsin and PTN 6.0 S exhibited a decrease in luciferase activity of about 50 % in comparison to the control. The tryptic and chymotryptic enzyme preparations Cryotin and Cryotin F showed no significant reduction in luciferase activity.

The cell assay for the determination of the activity of the immune regulating transcription factor NFκB in HEK$^{nfkb\text{-}RE}$ cells may provide a first indication for the immunostimulatory activity of MOS and the anti-inflammatory activity of tryptic β-casein hydrolysates. It has to be considered that *in vitro* cell assays do not ensure that the same effect is revealed in the human body *in vivo*. However, for a first evaluation of the biological activity, the determination of the NFκB activity in HEK$^{nfkb\text{-}RE}$ cells is highly suitable.

## V.4 Outlook

In the present work, procedures were developed for the enrichment of MOS and the generation of anti-inflammatory β-casein hydrolysates. The characterization of the biological activity of the products was performed by the evaluation of the NFκB activity in HEK$^{nfkb\text{-}RE}$ cells *in vitro*. An important next step should be the determination of the biological effect of these products in human clinical trials *in vivo*. Recently, an intervention study for the evaluation of the anti-inflammatory effect of the total β-casein hydrolysate generated with the porcine enzyme preparation PTN 6.0 S *in vivo* has been performed in cooperation with Universitätsklinikum Schleswig-Holstein (UKSH) in Kiel. The outcome of the trial is not known yet, as the evaluation of the results is still being performed. Moreover, an intervention study for the determination of the immunostimulatory activity of the MOS concentrates should be performed.

To provide sufficient amounts of the bioactive products for clinical trials and to realize the application of these products as functional ingredients in food, the developed procedures should be suitable for the transfer into industrial scale production. The enrichment of MOS by NF was established on industrial scale. However, the innovative procedures developed for desalting have to be transferred to industrial scale. Also the generation of anti-inflammatory peptides from β-casein should be transferred to large scale production.

## V.5 References

Albrecht S, Lane AL, Mariño K, Al Busadah KA, Carrington SD, Hickey RM & Rudd PM (2014) A comparative study of free oligosaccharides in the milk of domestic animals. *British Journal of Nutrition* 111(7), 1313-1328.

Barile D, Tao N, Lebrilla CB, Coisson J-D, Arlorio M & German JB (2009) Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *International Dairy Journal* 19(9), 524-530.

Boutrou R, Gaudichon C, Dupont D, Jardin J, Airinei G, Marsset-Baglieri A, Benamouzig R, Tome D & Leonil J (2013) Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition* 97(6), 1314-1323.

Carić M, Akkerman JC, Milanović S, Kentish SE & Tamime AY (2009) Technology of Evaporators, Membrane Processing and Dryers. In: *Dairy Powders and Concentrated Products*. 99-148. Wiley-Blackwell.

Fox PF & McSweeney P (2009) *Advanced Dairy Chemistry- Volume 3: Lactose, water, salts and minor constituents*. 3. edn. Springer, Ireland.

Lucey JA & Horne DS (2009) Milk Salts: Technological Significance. In: McSweeney P & Fox PF (eds) *Advanced Dairy Chemistry Volume 3 Lactose, Water and minor constituents*. 351-389. Springer, New York.

Malinowski J, Klempt M, Clawin-Rädecker I, Lorenzen PC & Meisel H (2014) Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. *Food Chemistry* 165(0), 129-133.

Martinez-Ferez A, Rudloff S, Guadix A, Henkel CA, Pohlentz G, Boza JJ, Guadix EM & Kunz C (2006) Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal* 16(2), 173-181.

Mehra R, Barile D, Marotta M, Lebrilla CB, Chu C & German JB (2014) Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. *PLoS One* 9(5).

Meisel H (2005) Biochemical properties of peptides encrypted in bovine milk proteins. *Current Medicinal Chemistry* 12(16), 1905-1919.

Rice G, Kentish S, O'Connor A, Stevens G, Lawrence N & Barber A (2006) Fouling behaviour during the nanofiltration of dairy ultrafiltration permeate. *Desalination* 199(1–3), 239-241.

Walstra P, Geurts TJ, Noomen A, Jellema A & Boekel MAJSv (1999) *Dairy Technology-Principles of Milk Properties and Processes*. Marcel, Dekker, Inc., Basel.