Research Article

Journal of PeptideScience

Received: 15 April 2009 Revised: 3 September 2009 Accepted: 7 September 2009 Published online in Wiley Interscience: 10 November 2009

(www.interscience.com) DOI 10.1002/psc.1190

Colostrum and bioactive, colostral peptides differentially modulate the innate immune response of intestinal epithelial cells

Ann Louise Worsøe Jørgensen,[a] Helle Risdahl Juul-Madsen[b] and Jan Stagsted[a]*

Characterization and identification of peptides with bioactivity from food have received considerable interest recently since such bioactive components must be adequately documented if they are part of functional food claims. We have characterized peptides from colostrum or those generated by a simulated gastrointestinal digest (GI) and tested them for bioactivity using murine intestinal (mIC_{c12}) cells and compared with bioactivity of intact colostrum. The peptides were recovered in the permeate after dialysis. The presence of peptides in the permeate was confirmed by C_{18} RP-HPLC, determination of free amino termini and MALDI MS. The bioactivity of the intact colostrum and colostral peptides in the permeate was tested using mIC_{c12} cells stimulated in the absence or presence of different bacterial ligands that mediate cellular activation through stimulation of Toll-like receptors (TLR). Whereas intact colostrum generally reduced TLR-mediated signaling, the isolated peptides seemed to either stimulate or reduce the immune response depending on the bacterial ligand used for stimulation. Interestingly, the most potent bioactive peptides originated from nondigested colostrum, which had only been subject to endogenous protease activity. Identified peptides in the nondigested colostrum originated exclusively from the casein fraction of colostrum as shown by MALDI MS/MS identification. Thus, multiple components with different bioactivities towards the innate immune response appear in bovine colostrum. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: colostrum; bioactive peptides; NF-κB stimulation; gastrointestinal digest; innate immunity; TLR; LPS; PGN; PAM_3CSK_4

Introduction

Colostrum is the first food for newborn mammals, and it is assumed to have a major impact on the future health of the individual [1,2]. Colostrum protects the mucosal surface against bacterial challenge during bacterial colonization of the neonatal intestine. Several proteins in colostrum are present in elevated concentration compared to milk [3] and many of these proteins modulate the immune response and/or have antimicrobial activities, e.g. lactoferrin [4,5], immunoglobulins [6], toll-like receptors (TLRs) [7–9] and CD14 [6,10–12].

The neonate has an immature adaptive immune system, and it therefore relies on an effective innate response. Central to this response are the TLR receptors present on the cell surface or inside the cells depending on cell type [13,14]. TLR receptors are members of the pattern recognition receptor (PRR) family, which recognize bacteria-derived components containing unique pathogen-associated molecular patterns (PAMPS). The extracellular domains of TLR facilitate recognition of PAMPS, whereas the intracellular domains mediate signaling leading to production of proinflammatory cytokines. The homology of TLRs across species is high; however, some differences in the signaling cascade are observed depending on the cell type [15,16].

Earlier studies analyzing the immune regulating effects of milk and colostrum have typically measured secretion of cyto- and chemokines by immune competent cells following stimulation with bacterial ligands. These studies have shown that milk-derived CD14 mediates the secretion of IL-8, tumor necrosis factor (TNF-α) and neutrophil activator-78 when exposed to lipopolysaccharide (LPS) [11]. LeBouder and coworkers have shown that immune signaling by intestinal cells in the presence of milk can be either negatively regulated when mediated by TLR2 and TLR3, or positively regulated when signaling is facilitated by TLR4 and TLR5 as determined by measuring IL-8, TNF-α and IL-6 expression [9]. They identified a proteinaceous component $>$80 kDa to be responsible for the effect on TLR4.

Colostrum and milk-derived proteins are precursors for many potentially biologically active peptides, which are inactive when they are embedded as part of the intact protein [17,18]. These peptides are released by digestion with proteases and are presumed to become active during passage through the gastrointestinal (GI) tract. Some proteins present in colostrum pass intact through

* *Correspondence to: Jan Stagsted, Department of Food Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark. E-mail: jan.stagsted@agrsci.dk*

a *Department of Food Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark*

b *Department of Animal Health, Welfare and Nutrition, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark*

Abbreviations used: *CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; PGN, peptidoglycan; LPS, lipopolysaccharide; ReLPS, rough lipopolysaccharide; Pam_3CSK_4, [(S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH]; PBS, phosphate buffered saline; RLU, relative luciferase unit; GI, gastro-intestinal; TLR, toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; LMW, low molecular weight; HMW, high molecular weight.*

the neonatal intestine, e.g. secretory immunoglobulin A [10], lactoferrin and α-antitrypsin [19], whereas others are digested by proteases released in the GI tract, thereby making these peptides accessible and reactive within the intestinal environment. A wide range of bioactive, milk-derived peptides have been described, from antithrombotic, opioid-like, antihypertensive, nutritional and immunomodulatory to antimicrobial [3,19–22]. Most bioactive peptides are derived from caseins whereas only a few derived from whey proteins have been described. Hitherto, studies of immunomodulatory effects of milk-derived bioactive peptides on intestinal cells are limited.

Two main strategies have been used to identify and characterize biologically active peptides: (i) isolation from *in vivo* GI digests of precursor proteins, and (ii) isolation from *in vitro* enzymatic digests of precursor proteins. Studies of peptides from *in vivo* digestion require collection of intestinal content. This approach has been used to compare the gastric protease activity of caseins from ingested colostrum of mouse origin and milk of human origin [23,24].

In vitro digestion has been used extensively for studying formation of potentially bioactive peptides from complex food matrices [24–28]. Following digestion, the peptides are purified and tested for bioactivity. Digestion of proteins has either been carried out by fermentation with microorganisms or by addition of proteases, e.g. pepsin combined with Corolase PP, a mixture of pancreatic enzymes, or pancreatic fluids [25,26,28]. Fermentation with microorganisms during production of yogurt or cheese [26,27,29] results in peptides originating from both bacteria and the food matrix, making interpretation more complex, particularly in immunomodulatory assays due to the presence of bacterial endotoxins. Pancreatic enzymes generate a variety of peptides that become potentially bioavailable during subsequent passage through the small intestine, but the resulting peptides may be hard to purify and identify due to the wide range of sizes as well as the many different protease cleavage sites.

Using *in vitro* generated peptides in bioassays without further purification may be a challenge because of the remaining enzymatic activity of the added proteases, although these may be eliminated by e.g. boiling [26,30], ultrafiltration [28], RP-HPLC [27,31], or acidification or alkalinization of the digest [31]. Inactivation of the proteases by boiling is very efficient, but heating may induce unwanted side reactions of the peptides [32], which could reduce bioactivity. Ultrafiltration is a good way to isolate peptides, but it is tedious, and normally requires a subsequent concentration step. Acidification and alkalinization of the digest are also efficient, but readjusting pH during measurement of bioactivity may allow some enzymes to refold and become active again.

The aim of this study was to isolate peptides from colostrum before or after digestion with pancreatic proteases *in vitro*. A simple procedure using a dialysis bag with water immersed in colostrum with or without pepsin, followed by addition of pancreatic proteases was used to recover peptides for characterization by RP-HPLC and MALDI ToF/ToF MS, and for testing of bioactivity. The bioactivity was evaluated by incubation of colostrum or peptides from colostrum with murine intestinal epithelial cells (mIC_{c12}) transfected with the luciferase gene under control of the NF-κB promoter together with bacterial PAMPS (LPS, peptidoglycan (PGN) and [(*S*)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-(*S*)-Lys_4-OH • 3HCl] (Pam_3CSK_4), which are recognized by distinct TLRs.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium, HAM F-12 nutrient mixture, glutamine, fetal bovine serum and *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid were bought from GIBCO (Invitrogen, Paisley, United Kingdom). Dialysis tubing (Spectra/Por Dialysis Membrane, 3.5 Da cut-off) was purchased from Spectrum Laboratories, Inc., CA. Pepsin from porcine stomach mucosa (453 units/mg solid), *a*-cyano-4-hydroxycinnamic acid (CHCA; MALDI MS grade), formic acid (98%) and ATP; (approx. 99%) were acquired from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Insulin, dexamethasone, selenium, transferrin, triiodothyronine, epidermal growth factor, D-glucose, hygromycin B and penicillin/streptomycin stock were of culture grade from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Corolase PP was from AB Enzymes (Darmstadt, Germany). Fluorescamine was purchased from Acros Organics (Geel, Belgium). The mIC_{c12} cells were a kind gift from Dr M W Hornef. Borate, disodiumhydrogenphosphate dodecahydrate and potassium chloride ($\geq$99.5%) were bought from Merck (Darmstadt, Germany) and sodium chloride was from J. T. Baker (Deventer, Netherlands). Leucine ($\geq$99%), 2,5-dihydroxybenzoic acid (DHB; $\geq$99%), PGN from *Staphylococcus aureus*, and LPS ($\leq$3% protein) were purchased from Fluka Chemika (Buchs, Schweizerland). Acetonitrile was of HPLC grade from Rathburn Chemicals Ltd (Walkerburn, Scotland). GELoader T.I.P.S (20 µl) was bought from Eppendorf AG (Hamburg, Germany) and C_{18} column material (Poros 50 R2) was from PerSeptive Biosystems (MA, USA). Synthetic Pam_3CSK_4 and rough lipopolysaccharide (ReLPS) were bought from Apotech Corporation via Axxora (Lausen, Switzerland). The time stable ATP determination kit was purchased from Biaffin GmbH & Co KG (Kassel, Germany). Trichloroacetic acid (>99%) and sodium azide were purchased from AppliChem (Darmstadt, Germany). Acrodisc PF Syringe Filters (0.8/0.2 µm Super membrane, nonpyrogenic) were from Pall Newquay, United Kingdom.

Conditions for mIC_{c12} Cells

mIC_{c12} cells stably expressing the luciferase reporter gene under control of the NF-κB promoter were routinely cultivated as described [33–35]. The mIC_{c12} cells were grown in presence of 50 µg/ml hygromycin B as well as pencillin/streptomycin. Approximately 10^3 cells were seeded in each well of a 96 well plate (optical bottom plate, polymer base white with lid, cell culture sterile PS, Nunc, NY, USA) and grown for 2 days in medium without hygromycin B but with pencillin/streptomycin. Ligands were resuspended as follows: 3 µg/ml PGN in phosphate buffered saline (PBS), 1 mg/ml Pam_3CSK_4 1 mg/ml ReLPS in PBS and 3 mg/ml LPS in cell medium unless stated otherwise. Ligands, colostrum and content of dialysis bags were filtered through 0.2 µm sterile filters, diluted and applied to the cells in growth medium without hygromycin B. Following stimulation for 4 h, the cells were washed with ice-cold PBS followed by addition of 20 µl of water to each well and freezing over night at −20 °C to lyse the cells.

Determination of Luciferase Activity

The luciferase assay was prepared as described by the manufacturer and all reagents were allowed to equilibrate to room temperature before use. The luciferin containing substrate (30 µl) was applied to each well followed by 50 µl 2 mM ATP in PBS.

Journal of PeptideScience

Bubbles were removed by centrifugation at 781 × *g* for 3 min. After incubation for 15 min at room temperature luciferase activity was measured using a liquid scintillation counter (1450 Microbeta, Wallac Trilux, Wallay Oy, Turku, Finland). The luciferase activity of stimulated samples was normalized to that of untreated cells and expressed as relative luciferase units (RLU).

Statistical Analysis

Data of the relative luciferace units were subjected to statistical analysis by the Generalized Linear Models (GLM) procedure of the SAS software, ver. 9.2 (SAS Institute Inc., Cary, NC, USA). The data were tested for variance homogeneity by the Bartlett test, and no possible outlier values could be identified. The normality of the data distribution was tested and resulted in transformation of the data by the log(10) function. A two-way model of analysis was used including the main effects of sample, defined by type and concentration, and day as class variables together with interaction between these. Where no interactive effect was found, the model was reduced correspondingly by exclusion of the interaction from the model. The LS-means were calculated and differences regarded as significant at minimum 95% level ($P \leq 0.05$). Differences were classified by the Ryan-Einot-Gabriel-Welsch multiple range test.

Colostrum and Proteolytic Digestion

Colostrum from first milking was obtained from the herd of Holstein-Friesian dairy cows at Research Center Foulum, Aarhus University, Denmark, and immediately frozen. The colostrum was then thawed just before use, diluted 1 : 1 with water and defatted by centrifugation for 30 min at 1500 × *g*, which also removed debris and cells.

Pepsin (0.3 g) and sodium azide (0.02% for prevention of bacterial contamination during the incubation) were added to 1 l of diluted colostrum and pH adjusted to 1.2 with 6N HCl followed by incubation at 37 °C for 1 h. pH was then adjusted to 7.4 with 6 N NaOH followed by addition of 0.7 g Corolase PP. Dialysis bags (3.5 kDa cut-off) containing 20 ml deionized water for sampling of low molecular weight (LMW) components were added to the digests, and incubation continued for up to 27 h at 37 °C. The number of dialysis bags corresponded to maximally 10% dilution of the LMW components of the colostrum. The nondigested colostrum samples were treated similarly, but without addition of proteases.

Determination of Free Amino Groups and Characterization of Peptides by Capillary HPLC

Fractions of the dialysates were precipitated with 12% (v/v) TCA and analyzed for the concentration of *N*-termini as described previously [36,37]. TCA supernatant (1 µl) was injected on a reverse-phased C_{18} column (Agilent Zorbax 300SB, 150 × 0.5 mm, 300 Å, 5 µm) coupled to a capillary HPLC system (Agilent Technologies 2000, Waldbronn, Germany) consisting of a capillary pump with split flow, autosampler, valve, micro-vacuum degasser, thermostated column compartment, variable wavelength detector and the software Agilent Chemstation. Column temperature was 20 °C, and the flow rate was 10 µl/min. Detection was monitored at 210 and 280 nm. Samples were loaded in 5% formic acid and eluted with a linear gradient (45 min) of 80% acetonitrile, 5% formic acid.

Identification of Peptides by MALDI ToF/ToF MS

Handmade C_{18} columns were created in gel-loading pipette tips as described previously [38]. The columns were equilibrated with 20 µl 5% formic acid. A 30 µl sample of the permeate obtained from dialysis of nondigested colostrum was applied to the pipette tip followed by washing with 20 µl 5% formic acid. Bound peptides were then sequentially eluted with 25, 50, 75 and 95% acetonitrile in 5% formic acid directly onto the target (MTP AnchorChip 600/384, Bruker Daltonics, Bremen, Germany) followed by addition of 0.5 µl 20 mg/ml DHB in 50% acetonitrile or 10 mg/ml CHCA in 70% acetonitrile.

The samples were analyzed by a MALDI ToF Tandem Mass Spectrometer Ultraflex ToF-ToF (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser in positive mode. The instrument was operated in reflector mode with an accelerating voltage of 25 kV. Laser pulse energy was adjusted using the calibration standard, and all subsequent acquisitions were performed with those settings. All spectra represent the sum of 5 × 200 shots, which were manually selected on the sample, and were analyzed by the Flex Analysis version 2.4 software (Bruker Daltonics, Bremen, Germany).

Results and Discussion

Stimulation of Cells with Bacterial Endotoxins

Murine mIC$_{c12}$ cells respond to bacterial endotoxins by stimulation of the NF-κB pathway. This cell line is derived from intestinal crypt cells of epithelial origin, which makes them potential targets during intestinal passage of food-derived molecules that may affect receptors involved in the innate immune response.

Dose-response relationships for PGN, Pam_3CSK_4, LPS and ReLPS were determined as relative luciferase activity of the mIC$_{c12}$ cells as a measure for NF-κB activation (Figure 1). Maximal NF-κB activation was obtained with 1 µg/ml PGN or 10 µg/ml Pam_3CSK_4, whereas 10 ng/ml of either rough or smooth LPS was sufficient for sixfold relative NF-κB activation above that obtained in absence of any stimulation. Interestingly, maximal stimulation with Pam_3CSK_4 was almost 12-fold and significantly higher than that obtained for the other ligands.

Other studies have shown that mIC$_{c12}$ cells respond to ligands that bind to and activate TLR [34,35,39–41]. Hornef and coworkers have shown that secretion of the chemokine, MIP-2, by mIC$_{c12}$ cells was highest after stimulation with 1 ng/ml LPS for 3–6 h [34,35]. Besides active TLR4 [34,35], mIC$_{c12}$ cells also express TLR2 and TLR5 as determined by mRNA analysis [41]. The receptor heterodimer TLR1/-2 is necessary for stimulation by Pam_3CSK_4 [42,43] while PGN stimulates both TLR2 and nucleotide oligomerization domain (NOD) receptors [44–46]. Pam_3CSK_4 has been shown to activate NF-κB expression in mIC$_{c12}$ cells at a concentration of 300 ng/ml after 3 h of stimulation, although mRNA expression of the TLR1 receptor has not been directly shown in this cell line [41].

Preliminary experiments indicated that filtration of the PGN suspension through 0.45 µm filters eliminated stimulation (data not shown), indicating that large aggregates of PGN were responsible for the stimulation. However, TLR2-mediated stimulation with large PGN aggregates may be controversial, since recent findings suggest that TLR2 is stimulated only by soluble PGN in monocytes [46], although others find that both soluble and insoluble PGN stimulate via TLR2 [47]. Recently, another publication showed that TLR2 in intestinal cells mediate internalization of PGN, but that

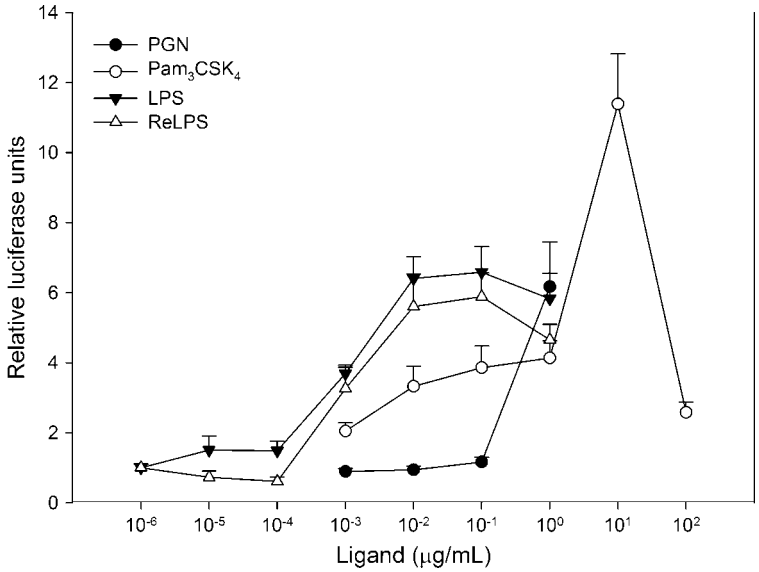

Figure 1. Stimulation of mIC_{c12} cells with PGN, Pam_3CSK_4, LPS and ReLPS. Confluent monolayers of mIC_{c12} cells were incubated with different concentrations of ligands for 4 h, and analyzed for luciferase activity. Data are means ± SEM from at least three different experiments performed with triplicates.

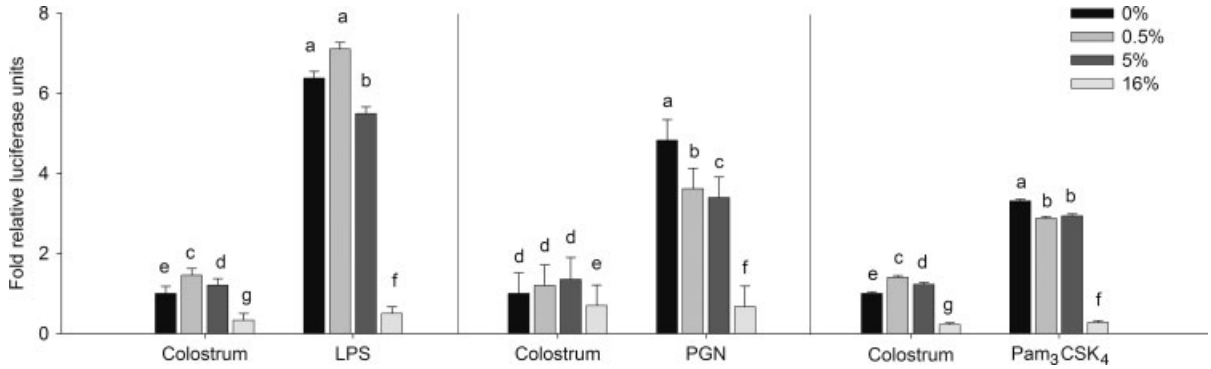

Figure 2. Stimulation of mIC_{c12} cells in absence or presence of LPS, PGN or Pam_3CSK_4 and different concentrations of colostrum (0, 0.5%, 5 or 16%). Data are means ± SEM from at least three different experiments performed with triplicates. Means within each column with different letters differ significantly ($P < 0.001$).

signaling occurs through binding to intracellular NOD2 receptors [48]. TLR2 and NOD have both been shown to mediate signaling by PGN in human cells [44]. In this study, we cannot distinguish whether stimulation with PGN occurred through TLR2 or whether intracellular NOD also contributed to the observed stimulation. The minimal concentration of LPS required to achieve maximal stimulation seems to be similar to that observed previously [34,35], and in our hands both LPS and ReLPS gave similar stimulation, although others have observed a higher response to ReLPS than to LPS in TLR4/MD2 responsive cells [49].

The time to reach maximal stimulation of mIC_{c12} cells with PGN, Pam_3CSK_4 or LPS was determined to be 4–6 h, irrespective of which ligand was used for stimulation (data not shown). We have used 4 h stimulation in the presence of 300 ng/ml LPS, 1 μg/ml PGN, or 10 μg/ml Pam_3CSK_4 for evaluation of bioactivity of colostrum and colostral peptides in the following experiments.

Bioactivity of Colostrum

Only few bioactive components from colostrum have been described, although several proteins involved in the immune function are known to be up regulated in colostrum, e.g. CD14 [11–13], lactoferrin [5] and immunoglobulins [6]. Håversen and coworkers have shown that lactoferrin negatively regulates NF-κB activation and production of cytokines when monocytes are stimulated with LPS [5]. Using intestinal cells, it has been shown that lactoferrin also reduced production of cytokines following stimulation with *Escherichia coli* [4]. An interaction between lactoferrin and LPS has been suggested [50], which also implies that lactoferrin activates monocytes via both TLR4-dependent and -independent signaling pathways [7]. CD14 from human milk has been shown to increase production of various cytokines, e.g. IL-8, TNF–α and epithelial neutrophil activator, in LPS-activated human intestinal cells [11,12]. The acute phase protein, LBP, which is not elevated in colostrum compared to milk, also reduced production of proinflammatory cytokines in the presence of LPS [11]. LeBouder and coworkers found that TLR-mediated cytokine expression was affected by milk after stimulating intestinal cells with bacterial ligands [9].

NF-κB stimulation of mIC_{c12} cells incubated with different concentrations of colostrum in the absence or presence of LPS, PGN or Pam_3CSK_4 is shown in Figure 2. Colostrum alone had

Journal of PeptideScience

significant effects on basal NF-κB activity except for experiments with PGN which in general had high standard errors. Addition of low concentrations (0.5 and 5%) of colostrum modestly increased activity of luciferase above that obtained in absence of colostrum, but a high concentration (16%) of colostrum abrogated the signal completely. In contrast, colostrum decreased stimulation by all three bacterial ligands, although the highest concentration of colostrum again completely abrogated the signal. The inhibitory effect appeared more potent in the presence of PGN and Pam_3CSK_4, since inhibition was also observed even at 0.5%, corresponding to a 200-fold dilution of colostrum.

Our results indicate a suppression of NF-κB activity by addition of colostrum, which is in accordance with experiments with lactoferrin and NF-κB stimulation published previously [4,5]. In monocytic cells, Håversen and coworkers found that lactoferrin down regulated cytokine expression in a dose-dependent manner, and that lactoferrin was internalized. It is not clear whether TLR on the cell surface or inside the cells is responsible for this effect [34,35]. Following a 4-h stimulation of intestinal cells with lactoferrin and *E. coli,* Berlutti and coworkers [4] showed that NF-κB activation decreased from 4.8 to 1, which largely corresponds to the down regulation in presence of LPS observed in Figure 2. Contrary to the suppression of NF-κB activity by colostrum that we have observed, increased levels of IL-6 and TNF-α production by monocytes have been reported in presence of lactoferrin [7]. This effect was independent of LPS but dependent on TLR4. Human milk stimulated IL-8 secretion by human intestinal cells in presence of LPS [9,12]; however, this effect was ascribed to CD14 because an anti-CD14 antibody reduced the response.

Based on the apparent, but complex bioactivity of whole colostrum, we next decided to obtain the LMW fraction and to generate a simulated GI digest of colostrum for testing.

Isolation and Characterization of Peptides from Colostrum

Generation of bioactive peptides from caseins and β-lactoglobulin has previously been studied using fermentated milk hydrolysates, which were ultrafiltrated to remove proteins followed by HPLC and identification by MS [28]. Other researchers have studied the release of albutensins from serum albumin after a simulated GI digest, which indicated that bioactivity of the albutensins was reduced by addition of a mixture of pancreatic enzymes [30].

We isolated LMW components from colostrum by dialysis into water-containing dialysis bags added to the colostrum. This simple setup allows elimination of particulate material, including casein and lipid micelles, as well as high molecular weight (HMW) components present in colostrum that otherwise would interfere with subsequent analyses. It also confined the enzymes that were added to simulate GI digestion, i.e. pepsin and pancreatic proteases, and allowed easy recovery of generated LMW compounds in the permeate.

Formation of peptides by pepsin and pancreatic enzymes was determined by reaction of fluorescamine with free amino groups as shown in Table 1. Initially, the content of peptides in colostrum was low, but addition of proteases increased the amount of peptides. The samples for determination of free amino groups and RP-HPLC were precipitated with TCA prior to analysis. The resulting white protein precipitates disappeared with increasing time of digestion (data not shown). After 1 h digestion by pepsin at pH 1.2, the pH was increased to 7.4, and prolonged digestion with pancreatic proteases for up to 27 h to simulate intestinal passage caused an increase in the amount of peptides in the dialysate, similar to that recovered in the permeate. Samples that were not digested with pepsin prior to addition of pancreatic proteases had lower content (~85%) of peptides than those digested with pepsin.

Table 1. Formation of *n*-termini (mM) in colostrum by enzymatic digestion

Sample	Permeate	Dialysate
− Pepsin 1 h	ND	0.25 ± 0.01
+ Pepsin 1 h	ND	1.06 ± 0.02
− Pepsin + pancreatic enzymes	14.7 ± 0.1	14.9 ± 0.1
+ Pepsin + pancreatic enzymes	17.5 ± 0.1	17.2 ± 0.2

ND, not determined.

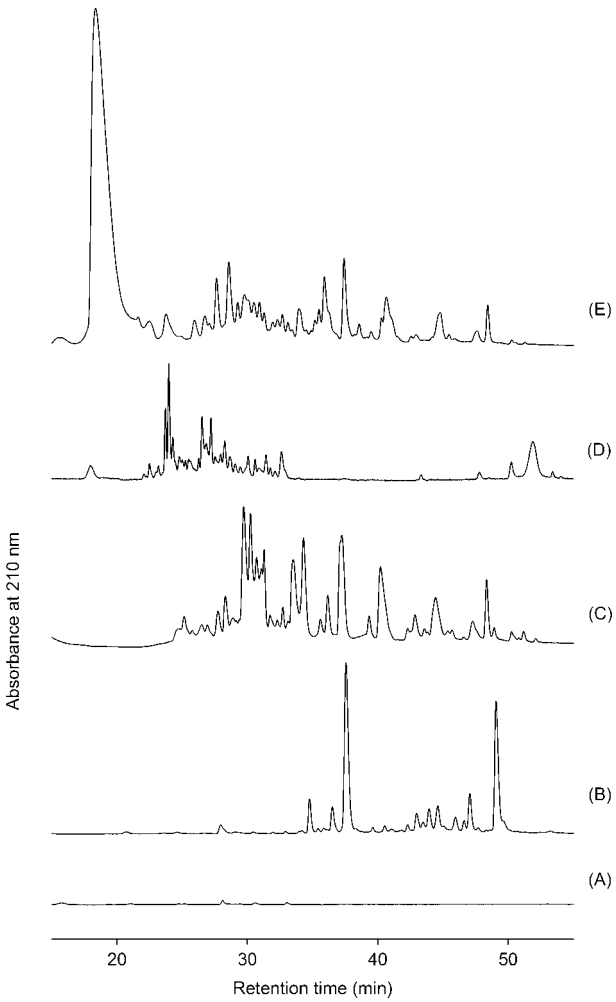

Figure 3. HPLC profiles of TCA supernatants from dialysates and permeates of colostrum. (A) Colostrum dialysate without pepsin 1 h, (B) Colostrum dialysate with pepsin 1 h, (C) Permeate of GI digested colostrum 27 h, (D) Permeate of nondigested colostrum; (E) Dialysate 27 h.

Peptides recovered after TCA precipitation were further characterized by C_{18} chromatography on a capillary HPLC system (Figure 3). The chromatograms of peptides obtained from colostrum after 1 h incubation without (3A) or with pepsin (3B) show that very low amounts of peptides are present in colostrum in accordance with the results of Table 1, but that digestion with pepsin resulted in substantially more signal from several peaks

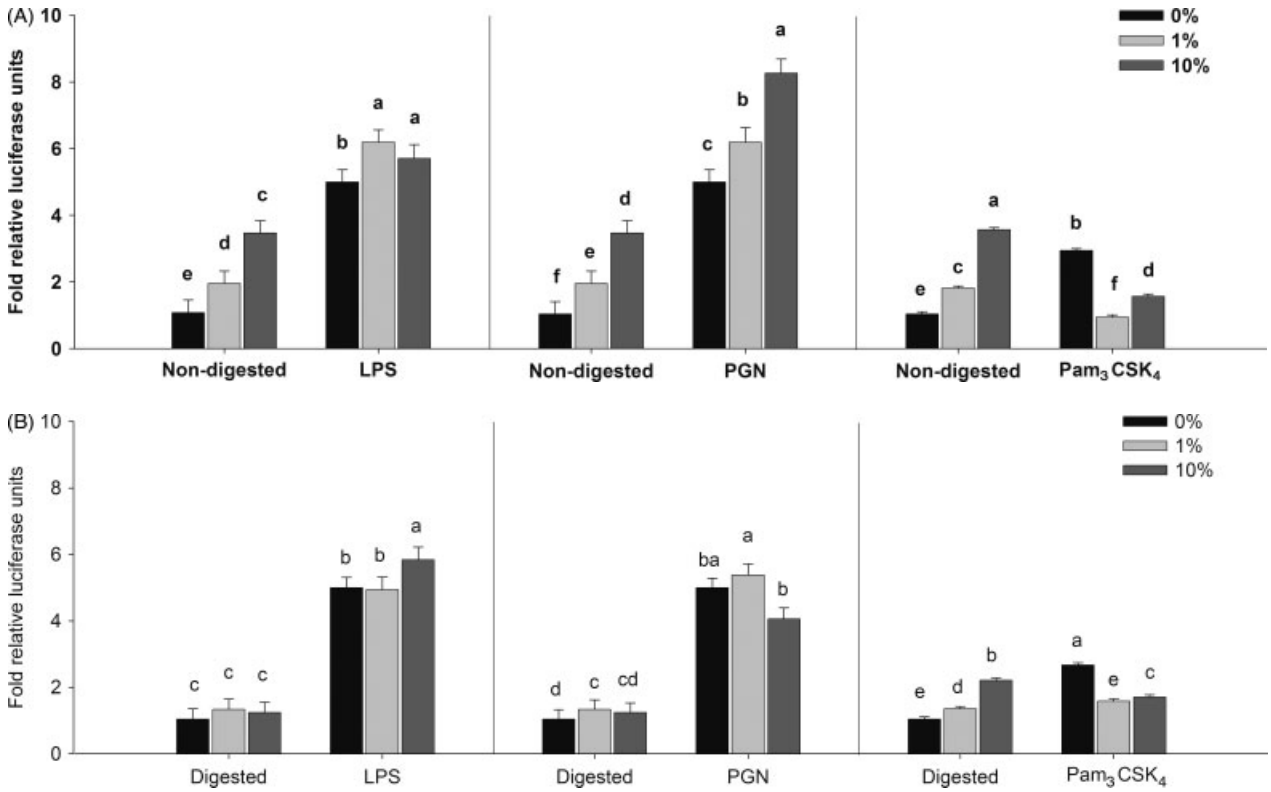

Figure 4. (A) Stimulation of mIC$_{c12}$ cells incubated with peptides from colostrum present in the nondigested permeate (at 0, 1, or 10%) either in absence or presence of LPS, PGN or Pam$_3$CSK$_4$. Data are means ± SEM from at least three different experiments performed with triplicates. Means within each column with different letters differ significantly ($P < 0.001$). (B) Stimulation of mIC$_{c12}$ cells incubated with peptides from colostrum present in the GI digested permeate (at 0, 1 or 10%) either in absence or presence of LPS, PGN or Pam$_3$CSK$_4$. Data are means ± SEM from at least three different experiments performed with triplicates. Means within each column with different letters differ significantly ($P < 0.001$).

in the chromatogram. The peptide profiles of colostrum after prolonged incubation in presence (3C) as well as absence (3D) of pancreatic proteases indicate new formation of peptides, although the profiles are substantially different. The occurrence of multiple peptides in nondigested colostrum (3D) is surprising as it must be due to activity of endogenous proteases. Figure 3(E) shows the absorbance profile of digested colostrum dialysate, where one or more strongly absorbing components eluted at a low concentration of acetonitrile, while these were not present in the permeate.

Bioactivity of Peptides from the Permeate

Several putative bioactive peptides from milk have been characterized and shown to originate from caseins [3,19,20,51], but there are few studies on effect of peptides from colostrum on intestinal health. Studies with milk have been focused on generation of peptides by probiotics, e.g. *Lactobacillus* or *Bifidobacterium* [25–28], and their inhibitory effect on ACE inhibition or antimicrobial activities [26–28].

Bioactivity of LMW components present in nondigested colostrum permeate (Figure 4(A)) or a simulated GI digest of colostrum permeate (Figure 4(B)) was assessed using the mIC$_{c12}$ cells incubated in the absence or presence of LPS, PGN, or Pam$_3$CSK$_4$. The data show that colostrum-derived LMW components stimulated NF-κB activity up to three-fold in a dose-dependent fashion. Addition of PGN together with nondigested colostrum permeate resulted in a dose-dependent increase in stimulation above that obtained with PGN alone, but this effect was much lower in presence of LPS. In contrast, nondigested colostrum permeate resulted in a significant suppression of Pam$_3$CSK$_4$-stimulated NF-κB activity. Whereas the activity of whole colostrum (Figure 2) reduced stimulation by bacterial ligands, the effects of the LMW permeate from nondigested colostrum were obviously more complex.

The data in Figure 4(B) indicate that digested colostrum permeate mediated no or only slight stimulation of NF-κB activity alone, in contrast to the large stimulation observed for nondigested colostrum permeate. In presence of LPS, the nondigested and digested permeates had small but similar effects, whereas the relatively large stimulation by nondigested permeate observed in presence of PGN was absent in the digested permeate. The decreases in NF-κB activity in presence of Pam$_3$CSK$_4$ observed at high concentrations (1 and 10%) of digested permeate were similar to those observed for nondigested permeate.

Apparently, addition of proteases removed the stimulatory components observed in absence of bacterial endotoxins and in presence of PGN from the LMW permeate indicating that the active component(s) were peptide related. Furthermore, comparison of the effects of whole colostrum (Figure 2) with those of the LMW permeate, particularly that obtained from nondigested colostrum (Figure 4(A)), gave unexpected results since whole colostrum caused a concentration dependent suppression but the permeate caused an increased NF-κB activity. Also, the different effects observed in presence of the three bacterial ligands were complex and may in part be due to the receptors that are present inside or on the surface of the mIC$_{c12}$ cells. Both PGN and Pam$_3$CSK$_4$ mediate signaling through TLR2, but PGN may also mediate signaling

Journal of PeptideScience

through NOD [44–46] and Pam_3CSK_4 uses a complex of TLR2 and TLR1 [52,53] for signaling; this may explain the opposite regulation of NF-κB stimulation caused by these ligands and the permeates.

Because addition of proteases removed the stimulatory components observed in the absence of bacterial endotoxins (Figure 4(B)), stimulation of the cells observed in Figure 4(A) could not have been caused by pyrogen-contaminated water or other contaminants, e.g. β-D-glucans derived from the dialysis membranes. Specific effects of the colostral LMV permeate are also indicated by the differential effects on cells dependent on addition of exogenous LPS, PGN or Pam_3CSK_4.

Identification of Peptides Present in Nondigested Colostrum Permeate

In order to identify the possible bioactive peptides in the nondigested colostrum permeate, C_{18} chromatography was carried out. Bound peptides were eluted with two different matrices on a target for MALDI ToF/ToF MS. The resulting mass spectra, when applying the matrix DHB, are shown in Figure 5. Figure 5(A) shows the number of peptides present in the permeate of nondigested colostrum. Elution with four different concentrations of acetonitrile; 25, 50, 75 and 95%, is shown in Figure 5(B)–(E), respectively. No proper ions were present in the mass spectrum shown in Figure 5(A). Applying the sample to C_{18} chromatography followed by elution with four different concentrations of acetonitrile gave many ion signals, peaking in numbers and intensity when eluting with 50% acetonitrile (Figure 5(C)). The amount of ions and the pattern present when applying the CHCA matrix for elution were similar to that obtained for DHB (data not shown). Ions with a signal to noise ratio of at least 20 were fragmented to obtain sequence and identity (Table 2). Peptides with known immunomodulatory or antimicrobial activity are highlighted with brackets/parentheses.

Surprisingly, all detected peptide fragments in the nondigested colostrum permeate originate from caseins indicating that endogenous proteases were present in colostrum. Previous studies of proteases in bovine colostrum have indicated the presence of cathepsin D, cysteine protease, an unidentified protease [54] and plasmin [55], as well as amino- and carboxypeptidases in milk [56,57]. A study with murine colostrum has indicated that colostrum-derived caseins fragmented more readily than milk-derived caseins [23]. Since caseins are the most abundant proteins in both milk and colostrum, peptides from these proteins would also be expected to dominate the MS signal [3] resulting in low or no detection of less abundant peptides, a situation which MS handles relatively poorly.

A previous study [58] has shown that a phosphorylated β-casein fragment 1–28 enhanced proliferation and IL-6 secretion in murine spleen cells through TLR4, but this β-casein peptide was not detected in this study, maybe because of less favorable conditions for detection of phosphopeptides with our setup. Some of the identified peptides in Table 2 have previously been ascribed immunomodulatory and/or antimicrobial activities [59,60]. As described previously, the casein-derived peptides with antimicrobial activities are mainly active towards bacteria like *Klebsiella pneumoniae* [61–63].

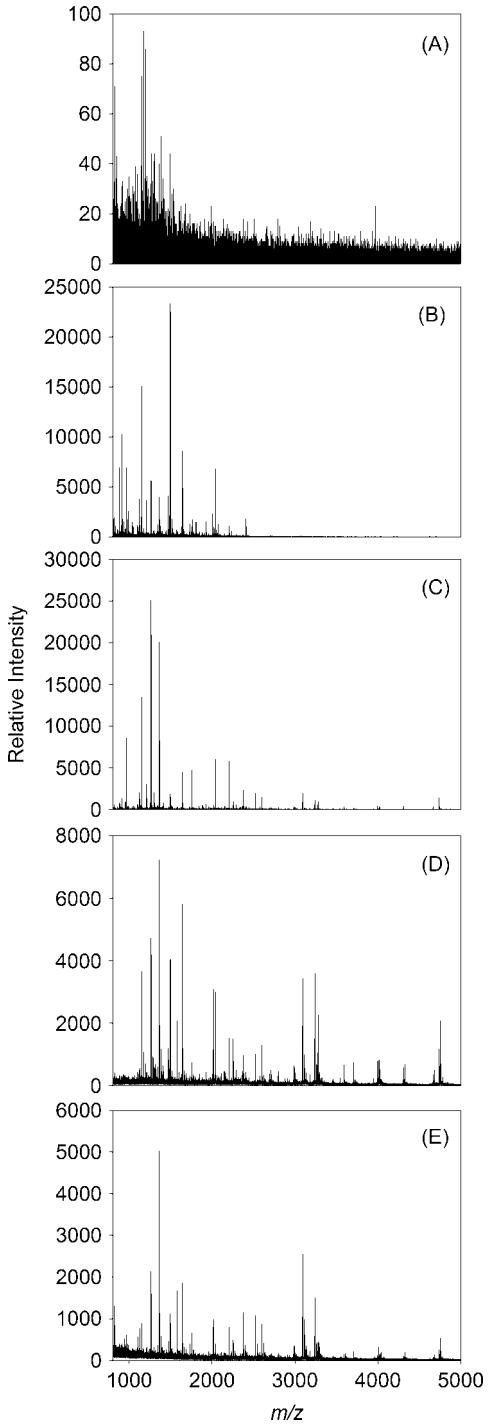

Figure 5. MALDI ToF/ToF MS spectra of nondigested colostrum permeate. (A) Nondigested colostrum permeate applied straight on the target. (B), (C), (D) and (E): Nondigested colostrum permeate eluted from C_{18} column material by 25, 50, 75 and 95% acetonitrile, respectively. DHB was applied to the samples as matrix for MALDI ToF/ToF MS.

Conclusion

Isolation of putative bioactive molecules from colostrum or a simulated GI digest of colostrum using the permeates from dialysis was successful. The concentration of *N*-termini increased with time of digestion and addition of pepsin, and so did complexity of the peaks present in the reverse-phase chromatograms. Whereas whole colostrum generally inhibited signaling by bacterial ligands, the LMW permeate from colostrum was shown to stimulate signaling in absence of ligands and in presence of PGN, but inhibit signaling from Pam_3CSK_4. There was no or little effect of

Table 2. Peptides in nondigested colostrum permeate

$[M + H]^+$	Sequence	Fragment	Matrix
α_{s1}-casein			
1494.799	FVAPFPEVFGKEK	24–36	CHCA
1641.868	FFVAPFPEVFGKEK	23–36	CHCA
2148.085	([IKHQGLPQEVLNENLLRF])	6–23	CHCA
2291.101	FSDIPNPIGSENSGK [TTMPLW]	181–199	DHB
α_{s2}-casein			
1927.065	YQGPIVLNPWDQVKRN	100–115	DHB
2040.103	LYQGPIVLNPWDQVKRN	99–115	CHCA
2203.166	YLYQGPIVLNPWDQVKRN	98–115	CHCA
β-casein			
879.443	MPFPKYP	109–115	CHCA
945.471	AVPYPQRD	177–184	CHCA
1099.680	LVYPFPGPIP	58–67	DHB
1130.693	LHLPLPLLQS	133–142	CHCA
1124.601	VVVPPFLQPE	82–91	DHB
1151.694	([VLGPVRGPFPI])	198–207	CHCA
1173.544	AVPYPQRDMP	177–186	CHCA
1204.607	MPFPKYPVEP	109–118	CHCA
1213.642	LVYPFPGPIPN	58–68	DHB
1264.778	([VLGPVRGPFPII])	197–208	CHCA
1300.688	LVYPFPGPIPNS	58–69	DHB
1363.846	([VLGPVRGPFPIIV])	197–209	CHCA
1411.765	VVVPPFLQPEVMG	82–94	DHB
1469.692	WMHQPHQPLPPT	143–154	CHCA
1485.694	AVPYPQRDMPIQA	177–189	CHCA
1574.879	LTLTDVENLHLPLP	125–149	DHB
1632.758	AVPYPQRDMPIQAF	177–190	CHCA
1752.932	LVYPFPGPIPNSLPQN	58–73	DHB
2016.138	LTLTDVENLHLPLPLLQS	125–152	CHCA
2107.133	[LL(YQEPVLGPVRGPFPIIV])	191–209	CHCA
2375.333	GPIPNSLPQNIPPLTQTPVVVPP	65–86	DHB
2409.024	FQSEEQQQT[a] EDEL	32–45	CHCA
2522.402	GPIPNSLPQNIPPLTQTPVVVPPF	65–87	DHB
2600.412	LVYPFPGPIPNSLPQNIPPLTQTP	58–81	DHB
3091.723	LVYPFPGPIPNSLPQNIPPLTQTPVVVPP	58–86	DHB
3113.485	NIPPLTQTPVVVPPFLQPEVMGVSKVKEA	83–101	CHCA
3238.791	LVYPFPGPIPNSLPQNIPPLTQTPVVVPPF	58–87	DHB
3276.770	GPIPNSLPQNIPPLTQTPVVVPPFLQPEVMG	65–94	DHB
3590.965	GPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK	65–97	DHB
3706.029	LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPE	58–91	DHB
3993.160	LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMG	58–94	DHB
4734.598	LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEA	58–101	CHCA

'[]' and '()' indicate part of sequence previously identified as having immunomodulating or antimicrobial bioactivities, respectively, as reviewed in Refs 3,19.
[a] Phosphorylation.

the LMW permeate on signaling activity by LPS. Peptides in the nondigested colostrum permeate were identified to originate from caseins, and some of these peptides have previously been shown to have immunomodulatory and/or antimicrobial activities.

Acknowledgements

This work was supported by the Danish Strategic Research Council Grant 2101-04-0014. The authors thank Karsten Skjødt, University of Southern Denmark, and Lars Østergaard Pedersen, Lundbeck A/S, for discussions of results, and Marianne Hammershøj, Aarhus University, for assisting with the statistical analysis.

References

1 Plat J, Mensink RP. Food components and immune function. *Curr. Opin. Lipidol.* 2005; **16**: 31–37.
2 Conroy ME, Walker WA. Intestinal immune health. *Nestle. Nutr. Workshop Ser. Pediatr. Program.* 2008; **62**: 111–121.

3 Korhonen H, Pihlanto A. Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Curr. Pharm. Des.* 2007; **13**: 829–843.
4 Berlutti F, Schippa S, Morea C, Sarli S, Perfetto B, Donnarumma G, Valenti P. Lactoferrin downregulates pro-inflammatory cytokines upexpressed in intestinal epithelial cells infected with invasive or noninvasive *Escherichia coli* strains. *Biochem. Cell Biol.* 2006; **84**: 351–357. DOI:10.1139/O06-039.
5 Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. *Cell. Immunol.* 2002; **220**: 83–95. DOI:10.1016/S0008-8749(03)00006-6.
6 Filipp D, Alizadeh-Khiavi K, Richardson C, Palma A, Paredes N, Takeuchi O, Akira S, Julius M. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. *Proc. Natl. Acad. Sci. USA* 2001; **98**: 603–608.
7 Curran CS, Demick KP, Mansfield JM. Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways. *Cell. Immunol.* 2006; **242**: 23–30. DOI:10.1016/j.cellimm.2006.08.006.
8 LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M, Griffin GE, Ferrara P, Schiffrin EJ, Morgan BP, Labeta MO. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *J. Immunol.* 2003; **171**: 6680–6689.
9 LeBouder E, Rey-Nores JE, Raby AC, Affolter M, Vidal K, Thornton CA, Labeta MO. Modulation of neonatal microbial recognition: tlr-mediated innate immune responses are specifically and differentially modulated by human milk. *J. Immunol.* 2006; **176**: 3742–3752.
10 Blais DR, Harrold J, Altosaar I. Killing the messenger in the nick of time: persistence of breast milk sCD14 in the neonatal gastrointestinal tract. *Peditar. Res.* 2006; **59**: 371–376. DOI:10.1203/01.pdr.0000199907.61549.94.
11 Vidal K, Labeta MO, Schiffrin EJ, Donnet-Hughes A. Soluble CD14 in human breast milk and its role in innate immune responses. *Acta Odontol. Scand.* 2001; **59**: 330–334.
12 Labeta MO, Vidal K, Nores JE, Arias M, Vita N, Morgan BP, Guillemot JC, Loyaux D, Ferrara P, Schmid D, Affolter M, Borysiewicz LK, Donnet-Hughes A, Schiffrin EJ. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J. Exp. Med.* 2000; **191**: 1807–1812.
13 Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* 2006; **27**: 352–357. DOI:10.1016/j.it.2006.06.003.
14 Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 2007; **7**: 179–190. DOI:10.1038/nri2038.
15 Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. *J. Immunol.* 2005; **174**: 4453–4460.
16 Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* 2007; **7**: 379–390. DOI:10.1038/nri2075.
17 Clare DA, Swaisgood HE. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 2000; **83**: 1187–1195.
18 Pihlanto A, Korhonen H. Bioactive peptides and proteins. *Adv. Food Nutr. Res.* 2003; **47**: 175–276. DOI:10.1016/S1043-4526(03)47004-6.
19 Silva SV, Malcata FX. Caseins as source of bioactive peptides. *Int. Dairy J.* 2005; **15**: 1–15. DOI:10.1016/j.idairyj.2004.04.009.
20 Meisel H. Biochemical properties of peptides encrypted in bovine milk proteins. *Curr. Med. Chem.* 2005; **12**: 1905–1919.
21 Hong F, Ming L, Yi S, Zhanxia L, Yongquan W, Chi L. The antihypertensive effect of peptides: a novel alternative to drugs? *Peptides* 2008; **29**: 1062–1071. DOI:10.1016/j.peptides.2008.02.005.
22 Otte JM, Kiehne K, Herzig KH. Antimicrobial peptides in innate immunity of the human intestine. *Jpn. J. Gastroenterol.* 2003; **38**: 717–726. DOI:10.1007/s00535-003-1136-5.
23 Yoneda M, Shiraishi J, Kuraishi T, Aoki F, Imakawa K, Sakai S. Gastric proteinase digestion of caseins in newborn pups of the mouse. *J. Dairy Sci.* 2001; **84**: 1851–1855.
24 Ferranti P, Traisci MV, Picariello G, Nasi A, Boschi V, Siervo M, Falconi C, Chianese L, Addeo F. Casein proteolysis in human milk: tracing the pattern of casein breakdown and the formation of potential bioactive peptides. *J. Dairy Res.* 2004; **71**: 74–87. DOI: 10.1017/S0022029903006599.
25 Miquel E, Gomez JA, Alegria A, Barbera R, Farre R, Recio I. Identification of casein phosphopeptides released after simulated digestion of milk-based infant formulas. *J. Agric. Food Chem.* 2005; **53**: 3426–3433. DOI:10.1021/jf0482111.
26 Ohsawa K, Satsu H, Ohki K, Enjoh M, Takano T, Shimizu M. Producibility and digestibility of antihypertensive beta-casein tripeptides, val-pro-pro and ile-pro-pro, in the gastrointestinal tract: analyses using an *in vitro* model of mammalian gastrointestinal digestion. *J. Agric. Food Chem.* 2008; **56**: 854–858. DOI:10.1021/jf072671n.
27 Minervini F, Algaron F, Rizzello CG, Fox PF, Monnet V, Gobbetti M. Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. *Appl. Environ. Microbiol.* 2003; **69**: 5297–5305. DOI: 10.1128/AEM.69.9.5297–5305.2003.
28 Hernandez-Ledesma B, Amigo L, Ramos M, Recio I. Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. *J. Chromatogr. A* 2004; **1049**: 107–114. DOI:10.1016/j.chroma.2004.07.025.
29 Prioult G, Pecquet S, Fliss I. Stimulation of interleukin-10 production by acidic beta-lactoglobulin-derived peptides hydrolyzed with *Lactobacillus paracasei* NCC2461 peptidases. *Clin. Diagn. Lab. Immunol.* 2004; **11**: 266–271. DOI: 10.1128/CDLI.11.2.266–271.2004.
30 De Noni I, Floris R. Specific release of Albutensins from bovine and human serum albumin. *Int. Dairy J.* 2007; **17**: 504–512. DOI:10.1016/j.idairyj.2006.06.022.
31 Schmelzer CE, Schops R, Reynell L, Ulbrich-Hofmann R, Neubert RH, Raith K. Peptic digestion of beta-casein. Time course and fate of possible bioactive peptides. *J. Chromatogr. A* 2007; **1166**: 108–115. DOI:10.1016/j.chroma.2007.08.015.
32 Klostermeyer H, Reimerdes EH. Heat induced crosslinks in milk proteins and consequences for the milk system. *Adv. Exp. Med. Biol.* 1977; **86B**: 263–275.
33 Bens M, Bogdanova A, Cluzeaud F, Miquerol L, Kerneis S, Kraehenbuhl JP, Kahn A, Pringault E, Vandewalle A. Transimmortalized mouse intestinal cells (m-ICc12) that maintain a crypt phenotype. *Am. J. Physiol.* 1996; **270**: C1666–C1674.
34 Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* 2002; **195**: 559–570. DOI: 10.1084/jem.20011788.
35 Hornef MW, Normark BH, Vandewalle A, Normark S. Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. *J. Exp. Med.* 2003; **198**: 1225–1235. DOI: 10.1084/jem.20022194.
36 Larsen LB, Rasmussen MD, Bjerring M, Nielsen JH. Proteases and protein degradation in milk from cows infected with *Streptococcus uberis*. *Int. Dairy J.* 2004; **14**: 899–907. DOI:10.1016/j.idairyj.2004.03.006.
37 Wiking L, Frøst MB, Larsen LB, Nielsen JH. Effects of storage conditions on lipolysis, proteolysis and sensory attributes in high quality raw milk. *Milchwissenschaft* 2002; **57**: 190–193.
38 Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 1999; **34**: 105–116.
39 Lotz M, Konig T, Menard S, Gutle D, Bogdan C, Hornef MW. Cytokine-mediated control of lipopolysaccharide-induced activation of small intestinal epithelial cells. *Immunology* 2007; **122**: 306–315. DOI: 10.1111/j.1365–2567.2007.02639.x.
40 Lotz M, Gutle D, Walther S, Menard S, Bogdan C, Hornef MW. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *J. Exp. Med.* 2006; **203**: 973–984. DOI: 10.1084/jem.20050625.
41 van Aubel RA, Keestra AM, Krooshoop DJ, van Eden W, van Putten JP. Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. *Mol. Immunol.* 2007; **44**: 3702–3714. DOI:10.1016/j.molimm.2007.04.001.
42 Manukyan M, Triantafilou K, Triantafilou M, Mackie A, Nilsen N, Espevik T, Wiesmuller KH, Ulmer AJ, Heine H. Binding of lipopeptide to CD14 induces physical proximity of CD14, TLR2 and TLR1. *Eur. J. Immunol.* 2005; **35**: 911–921. DOI:10.1002/eji.200425336.

43 Nakata T, Yasuda M, Fujita M, Kataoka H, Kiura K, Sano H, Shibata K. CD14 directly binds to triacylated lipopeptides and facilitates recognition of the lipopeptides by the receptor complex of Toll-like receptors 2 and 1 without binding to the complex. *Cell. Microbiol.* 2006; **8**: 1899–1909. DOI:10.1111/j.1462–5822.2006.00756.x.
44 Takada H, Uehara A. Enhancement of TLR-mediated innate immune responses by peptidoglycans through NOD signaling. *Curr. Pharm. Des.* 2006; **12**: 4163–4172.
45 Uehara A, Fujimoto Y, Fukase K, Takada H. Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol. Immunol.* 2007; **44**: 3100–3111. DOI:10.1016/j.molimm.2007.02.007.
46 Natsuka M, Uehara A, Shuhua Y, Echigo S, Takada H. A polymer-type water-soluble peptidoglycan exhibited both Toll-like receptor 2- and NOD2-agonistic activities, resulting in synergistic activation of human monocytic cells. *J. Innate. Immun.* 2008; **14**: 298–308. DOI:10.1177/175342908096518.
47 Dziarski R, Gupta D. *Staphylococcus aureus* peptidoglycan is a toll-like receptor 2 activator: a reevaluation. *Infect. Immun.* 2005; **73**: 5212–5216.DOI: 10.1128/IAI.73.8.5212–5216.2005.
48 Wu L, Feng BS, He SH, Zheng PY, Croitoru K, Yang PC. Bacterial peptidoglycan breaks down intestinal tolerance via mast cell activation: the role of TLR2 and NOD2. *Immunol. Cell Biol.* 2007; **85**: 538–545. DOI:10.1038/sj.icb.7100079.
49 Huber M, Kalis C, Keck S, Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Beutler B, Galanos C, Freudenberg MA. R-form LPS, the master key to the activation ofTLR4/MD-2-positive cells. *Eur. J. Immunol.* 2006; **36**: 701–711.DOI:10.1002/eji.200535593.
50 Elass-Rochard E, Roseanu A, Legrand D, Trif M, Salmon V, Motas C, Montreuil J, Spik G. Lactoferrin-lipopolysaccharide interaction: involvement of the 28–34 loop region of human lactoferrin in the high-affinity binding to Escherichia coli 055B5 lipopolysaccharide. *Biochem. J.* 1995; **312**(Pt 3): 839–845.
51 Hernandez-Ledesma B, Recio I, Amigo L. Beta-lactoglobulin as source of bioactive peptides. *Amino Acids* 2007; **35**: 257–265. DOI:10.1007/s00726-007-0585-1.
52 Shimizu T, Kida Y, Kuwano K. Triacylated lipoproteins derived from Mycoplasma pneumoniae activate nuclear factor-kappaB through toll-like receptors 1 and 2. *Immunology* 2007; **121**: 473–483. DOI: 10.1111/j.1365–2567.2007.02594.x.
53 Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 2007; **130**: 1071–1082. DOI:10.1016/j.cell.2007.09.008.
54 Larsen LB, McSweeney PLH, Hayes MG, Andersen JB, Ingvartsen KL, Kelly AL. Variation in activity and heterogeneity of bovine milk proteases with stage of lactation and somatic cell count. *Int. Dairy J.* 2006; **16**: 1–8. DOI:10.1016/j.idairyj.2005.01.009.
55 Madsen BD, Rasmussen MD, Nielsen MO, Wiking L, Larsen LB. Physical properties of mammary secretions in relation to chemical changes during transition from colostrum to milk. *J. Dairy Res.* 2004; **71**: 263–272. DOI: 10.1017/S0022029904000263.
56 Reimerdes EH, Petersen F, Kielwein G. Milk proteinases. 9. Proteinases from casein micelles, milk serum, cow's blood serum and pseudomonas fluorescens. *Milchwissenschaft* 1979; **34**: 551.
57 De Simone C, Picariello G, Mamone G, Stiuso P, Dicitore A, Vanacore D, Chianese L, Addeo F, Ferranti P. Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *J. Pept. Sci.* 2009; **15**: 251–258.
58 Tobita K, Kawahara T, Otani H. Bovine beta-casein (1–28), a casein phosphopeptide, enhances proliferation and IL-6 expression of mouse CD19+ cells via Toll-like receptor 4. *J. Agric. Food Chem.* 2006; **54**: 8013–8017. DOI:10.1021/jf0610864.
59 Ganjam LS, Thornton WH Jr, Marshall RT, MacDonald RS. Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *J. Dairy Sci.* 1997; **80**: 2325–2329.
60 Matar C, Valdez JC, Medina M, Rachid M, Perdigon G. Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *J. Dairy Res.* 2001; **68**: 601–609. DOI: 10.1017/S0022029901005143.
61 Parker F, Migliore-Samour D, Floc'h F, Zerial A, Werner GH, Jolles J, Casaretto M, Zahn H, Jolles P. Immunostimulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. *Eur. J. Biochem.* 1984; **145**: 677–682. DOI:10.1111/j.1432-1033.1984.tb08609.x.
62 Migliore-Samour D, Floc'h F, Jolles P. Biologically active casein peptides implicated in immunomodulation. *J. Dairy Res.* 1989; **56**: 357–362.
63 Sandre C, Gleizes A, Forestier F, Gorges-Kergot R, Chilmonczyk S, Leonil J, Moreau MC, Labarre C. A peptide derived from bovine beta-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *J. Nutr.* 2001; **131**: 2936–2942.

