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Milk-derived proteins and peptides in clinical trials

Białka i peptydy pochodzące z mleka w badaniach klinicznych

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Summary

Clinical trials are reviewed, involving proteins and peptides derived from milk (predominantly bovine), with the exception of lactoferrin, which will be the subject of another article. The most explored milk fraction is α -lactalbumin (LA), which is often applied with glycomacropeptide (GMP) – a casein degradation product. These milk constituents are used in health-promoting infant and adult formulae as well as in a modified form (HAMLET) to treat cancer. Lactoperoxidase (LCP) is used as an additive to mouth hygiene products and as a salivary substitute. Casein derivatives are applied, in addition, in the dry mouth syndrome. On the other hand, casein hydrolysates, containing active tripeptides, found application in hypertension and in type 2 diabetes. Lysozyme is routinely used for food conservation and in pharmaceutical products. It was successfully used in premature infants with concomitant diseases to improve health parameters. When used as prophylaxis in patients with scheduled surgery, it significantly reduced the incidence of hepatitis resulting from blood transfusion. Lysozyme was also used in infected children as an antimicrobial agent showing synergistic effects in combination with different antibiotics. Proline-rich polypeptide (PRP) was introduced to therapy of Alzheimer's disease patients. The therapeutic value of PRP was proved in several clinical trials and supported by studies on its mechanism of action. Concentrated immunoglobulin preparations from colostrum and milk of hyperimmunized cows showed efficacy in prevention of infections by bacteria, viruses and protozoa. A nutrition formula with milk-derived TGF- β 2 (Modulen IBD[®]) found application in treatment of pediatric Crohn's disease. In conclusion, the preparations containing milk-derived products are safe and effective measures in prevention and treatment of infections as well as autoimmune and neoplastic diseases.

Keywords:

α -lactalbumin • casein • glycomacropeptide • lactoperoxidase • lysozyme • proline-rich polypeptide • hyperimmune immunoglobulin concentrate • TGF- β • clinical trial

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Abbreviations: **A** – Ala (alanine); **ACE** – angiotensin-I-converting enzyme; **BAMLET** – bovine α -lactalbumin made lethal to tumor cells; **CLN** – Colostrinin; **E** – Glu (glutamic acid); **F** – Phe (phenylalanine); **G** – Gly (glycine); **GMP** – glycomacropeptide; **H** – His (histidine); **HAMLET** – human α -lactalbumin made lethal to tumor cells; **I** – Ile (isoleucine); **Ig** – immunoglobulins; **L** – Leu (leucine); **LA** – α -lactalbumin; **LCP** – lactoperoxidase; **LF** – lactoferrin; **LG** – β -lactoglobulin; **LNA** – large neutral amino acids; **M** – Met (methionine); **MFGM** – milk fat globule membrane proteins; **N** – Asn (asparagine); **OSCN/HOSCN** – hypothyocyanate/hypothyocyanous acid; **P** – Pro (proline); **PRP** – proline-rich polypeptide; **Q** – Gln (glutamine); **R** – Arg (arginine); **S** – Ser (serine); **SCN⁻** – thiocyanate; **TGF- β 2** – transforming growth factor beta 2; **V** – Val (valine); **W** – Trp (tryptophan); **Y** – Tyr (tyrosine)

INTRODUCTION

Milk is an essential diet for mammalian neonates and infants. It supplies vital nutritional ingredients for normal development and arms the neonate with immunoglobulins, proteins and peptides for innate immunity. Milk ingredients not only contribute to resistance to pathogens but also play a crucial role in promoting maturation of the gastrointestinal tract and immune system of the newborn (reviewed in [5]). Milk proteins are sometimes precursors of different biologically active peptides e.g. casein-derived glycomacropeptide and angiotensin-I-converting enzyme peptide inhibitors. Milk, and in particular colostrum, also contains a proline-rich polypeptide of important biological activities and therapeutic value. These peptides can be released by enzymatic hydrolysis either during gastrointestinal digestion or during food processing [26].

Biological properties of milk-contained proteins and peptides have been extensively studied in *in vitro* and animal models (reviewed in [19,44,53,91,119]). The proteins and peptides were also included in clinical studies showing a benefit in prevention and therapy of microelement deficiencies, cardiovascular diseases, oral cavity diseases, autoimmune and neoplastic diseases, inflammation, infection and recovery of the immune system function following chemotherapy. The results are very encouraging and have led to the broad application of milk constituents in the dairy and pharmaceutical industries.

The aim of this article is to review applications of the most important milk-derived proteins and peptides in clinical trials. The summary of the clinical effects of the investigated proteins and peptides is depicted in Table 1. Examples of bioactive peptides (peptide name, sequence and function) derived from milk proteins are presented in Table 2. Considering the high number of preclinical and clinical trials on lactoferrin, this will be a subject of another review (in preparation).

α -LACTALBUMIN AND GLYCOMACROPEPTIDE

The importance of α -lactalbumin (LA) in infant nutrition is obvious since this protein constitutes a large part of the whey and total protein in human milk. This is in contrast to bovine milk, where LA is only a minor part of the milk protein content (see Fig. 1). Nevertheless, the authors suggest that because of high amino acid homology (72%) between human and bovine LA [37], the bovine protein would be a valuable component of infant formulas as well

as patients' diet supplements. LA may be related to lysozyme because of similarities in their molecular structures and genetic features [71]. The protein is a component of lactose synthase and exhibits a number of biological activities, such as: ability to bind metal cations and fatty acids in an unfolded (apo) form, induction of apoptosis in tumors and immature cells, and bactericidal action by its peptidic fragments (reviewed in [7,37,80]). Glycomacropeptide (GMP) is a peptide derived from whey κ -casein (Table 2). It is released by chymosin and contains a high proportion of sialic acid, which may account for its biological activity (reviewed in [14,94]). In our study [117], GMP was shown to protect mice against endotoxemia and bacteremia with a higher efficacy than lactoferrin (protein with proved activity). LA and GMP are often used in a combination in nutritional preparations so therapeutic efficacy of these substances will be described together in one chapter.

LA or GMP-supplemented formulas were used in a study on infant *Rhesus* monkeys, as compared to control and breastfed infants, with no adverse effects on nutritional status [47]. Of note, GMP formula increased zinc absorption whereas LA supplementation resulted in a plasma amino acid pattern similar to that of breastfed monkey infants. Weight gain and safety of an experimental formula containing LA, as compared to a control one, in healthy 14-day-old or younger infants, fed for 12 weeks, were investigated [59]. Growth and adverse events data supported the safety of the experimental formula, which was better tolerated than the control formula. An LA-enriched formula administered for 120 days to infants was tested with regard to infant growth, protein markers and biochemical parameters [104]. The LA formula was safe, but no significant differences in the studied parameters were registered as compared to infants fed a standard formula or human milk. In another, multicentre, double-blind, randomized trial an LA-enriched and symbiotic-supplemented infant formula was tested for safety, tolerance and prevention of atopic dermatitis [84]. The infants fed the experimental formula exhibited less crying and agitation as well as less frequent manifestation of atopic dermatitis. Growth was similar in infants fed experimental and standard formula. Another study demonstrated that, compared to the standard formula-fed infants, the infants fed formula enriched with LA and GMP had weight gain similar to that of breastfed infants [87]. On the other hand, a study aimed at establishing an effect of LF and GMP-enriched formula on fecal microor-

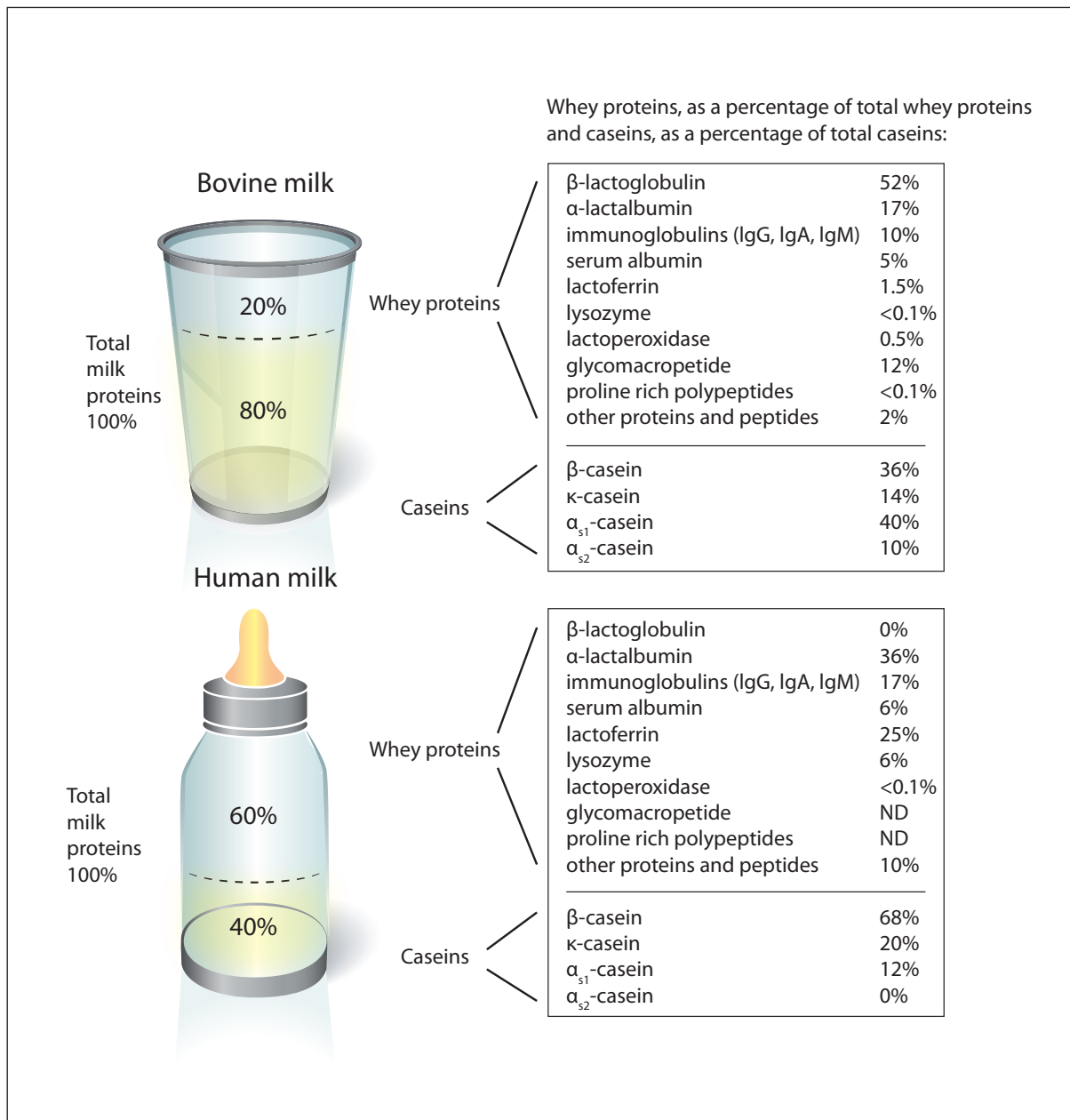


Fig. 1. Typical content of milk proteins in mature bovine and human milk. Shown are the major milk proteins, as well as selected additional proteins with relevance in health. The contents of proteins are expressed as percentage (%) of whey proteins and caseins in the total milk proteins and also whey proteins as a percentage of total whey proteins and caseins as a percentage of total caseins. The cited ratio of 60:40 of whey proteins to caseins in human milk is an approximation of the ratio during the normal course of lactation, but it varies from ~80:20 in early lactation (first days) to 50:50 in late lactation. The total protein content in breast milk is 14-16 g/L during early lactation, 8-10 g/L at 3-4 months of lactation and 7-8 g/L at 6 months and later [64]. The protein content in human milk is therefore 1.6-0.7% of the total milk ingredients. The protein content in bovine milk is 34-35 g/L (3.4-3.5% of the total milk ingredients). The data adapted from [53,61,81,91,107,118]. ND – no data

ganisms in healthy infants did not reveal any differences as compared to breastfed infants [15]. Likewise, LA- and LA-GMP-enriched formula had no effect on iron absorption in healthy term infants [98]. An LA-enriched and probiotic supplemented infant formula was also tested for effects on colic [23]. Although no differences were found with the control group (fed standard formula) regarding crying duration, the researchers registered lower “feeding-related” gastrointestinal side-effects in infants fed the experimental diet.

No significant effects of whey protein- and GMP-enriched diets on satiety in adults were found [56]. Likewise, no effects of GMP-enriched formula on cholecystokinin level (which may promote satiety), subjective feeling of satiety and food intake in 20 overweight males were found [49]. In a double-blind, randomized study in 127 obese volunteers meal replacement with GMP-enriched whey powder had no additional effect on the 12-month weight loss, but there were improvements in cardiovascular disease risk markers (total and LDL cholesterol, triacylglycerols, glu-

Table 1. Published clinical studies with milk proteins and peptides

Protein/peptide	Clinical effects, number of participants	References
α-Lactalbumin (LA)	Weight gain and safety (no adverse effects) in infants aged 14 days or less (n=193)	[59]
	Weight gain in infants fed experimental formula did not differ from the control group fed human milk (n=336)	[104]
	Prevention of atopic dermatitis, less crying and agitation in infants (n=97)	[84]
	Good weight and length gains in infants with colic, no differences in crying duration in infants, lower feeding-related side effects (n=66)	[23]
	Suppression of hunger, decreased fat balance, increased energy expenditure and protein balance (n=35)	[40]
	Increased loss of body fat and retention of lean muscle mass in obese subjects who drank Prolibra™, compared to supplementation with an isocaloric control that had a lower calcium and lower protein content (n=106)	[28]
	No effect on appetite, food intake or mood (n=18)	[8]
	Minimal effects on stress-induced mood (n=43)	[73]
	Improvement of abstract visual memory and impaired simple motor performance in depressed patients and healthy controls (n=43)	[12]
	Higher prolactin concentration, decreased cortisol levels and reduced depressive feelings under stress in stress-vulnerable subjects (n=58)	[69]
	Advantageous effects on cognitive performance in high and low stress-vulnerable subjects (n=52)	[68]
	Reduced sleepiness, improved brain sustained alertness in healthy subjects; increase in plasma Trp:LNAA ratio (n=28)	[67]
	Improved long-term memory for abstract figures in women with premenstrual complaints (n=16)	[90]
	Better weight gain, smaller number of rehydrations required in malnourished children with diarrhea (n=38)	[25]
Glycomacropeptide (GMP)	Equally effective in treatment of diarrhea in infants with control rehydration solution (standard glucose and maltodextrin plus sucrose)	[86]
	HAMLET effective in reducing volume and occurrence of cutaneous papillomas (n=40)	[33]
	Reduction by HAMLET of bladder cancer size (n=9)	[75]
	No significant effects on satiety in healthy adults (n=50)	[56]
	No effects on cholecystokinin level, subjective feeling of satiety and food intake in overweight/obese adults (n=20)	[49]
	No additional effect on 12-mo weight loss in obese adults, but improvement in cardiovascular disease risk markers (n=127)	[48]
	Well tolerated in phenylketonuria patients (n=15)	[55]
	No adverse reactions in phenylketonuria patients to GMP, decreased ureagenesis and increased plasma insulin as compared with a control diet, improved protein retention and phenylalanine utilization in phenylketonuria patients (n=11)	[106]
	Significantly lower postprandial ghrelin (appetite-stimulating hormone) concentration as compared to control diet (n=11)	[65]
	Decrease in gout flare symptoms in patients, improvements in pain and excretion of uric acid, improvement in tender joint count (n=120)	[18]
α-Lactalbumin (LA) + GMP	No difference in fecal microorganisms as compared to breast-fed infants (n=85)	[15]
	No effect on iron absorption in healthy term infants (n=40)	[98]
	Weight gain similar to breast-fed infants (n=96)	[87]
Lactoperoxidase (LCP)	Improvement in the number and severity of xerostomia symptoms in elderly patients using Biotène® products (toothpaste, mouthwash and gel) with lactoperoxidase enzyme system and LF (n=20)	[70]
	Improvement of symptoms in elderly patients with dry mouth using Biotène® mouthwash and Oral Balance® gel containing lactoperoxidase enzyme system, LF and lysozyme (n=20)	[31]
	Significant reduction in the symptoms scores in patients with burning mouth syndrome using Biotène® oral rinse (LCP and lysozyme), oral rinse with capsaicin or tablets with α-lipoic acid (n=56)	[66]
	No effects on accumulation and acidogenicity of dental plaques, salivary flow rate, peroxidase activity, thiocyanate levels and bacterial counts of lactoperoxidase enzyme system-containing Biotène® toothpaste applied in healthy volunteers with normal salivary flow rate (n=20)	[51]

	Lactoperoxidase enzyme system-containing Biotène toothpaste combined with Biotène mouthrinse (comprising also LF and lysozyme) relieved the symptoms of oral dryness in patients with xerostomia in Sjögren's syndrome, with no major effect in salivary microflora (n=20)	[50]
	Alleviation of dry mouth symptoms by Oral Balance gel (lactoperoxidase enzyme system, LF and lysozyme) for 4 weeks in secondary Sjögren's syndrome patients (n=21)	[4]
	Combined use of Biotène toothpaste with Oral Balance gel (both with lactoperoxidase enzyme system) for 4 weeks with good results in subjective relief of xerostomic symptoms; enhanced keratinization and decreased number of inflammatory cells in the buccal and mucosal smears	[88]
	Biotène toothpaste (lactoperoxidase enzyme system) applied in children with early childhood caries significantly reduced numbers of mutant streptococci and lactobacilli (n=30)	[46]
	Tablets containing LF and LCP reduced oral malodor and number of salivary bacteria in subjects with volatile sulfur compounds in mouth air above the olfactory threshold (n=15)	[93]
Lactoperoxidase (LCP)	Beneficial results in mouth scores in post-radiotherapy xerostomia in patients using Bioxtra ("whey extract") and Oral Balance (lactoperoxidase enzyme system, LF and lysozyme) mouth dry systems in the treatment of postradiotherapy xerostomia (n=20)	[92]
	Improved oral health, reduction of oral cariogenic bacteria in cancer patients after radiotherapy using lactoperoxidase enzyme system-containing Oral Balance gel (in bolus or slow-release form) for 4 weeks (n=22)	[72]
	Improved salivary flow and increased oral comfort, reduction of aerobic isolates, periodontal-associated bacteria and <i>Candida</i> species by Oral Balance gel and Biotène toothpaste in irradiated patients with oral cancer for 4 weeks following radiotherapy (n=36)	[76]
	Reduced rate of supragingival plaque formation and gingival inflammation by Biotène toothpaste (lactoperoxidase enzyme system) in radiation induced xerostomia during 52-day period (n=20)	[108]
	Improved gingival health, reduction of gingivitis by Biotène toothpaste (lactoperoxidase enzyme system) in cancer patients after radiotherapy (n=19)	[102]
	Subjective relief of radiation-induced xerostomic symptoms (e.g. oral discomfort, intraoral dryness, inability to eat normally) by combined use of Biotène products (mouthwash, toothpaste, chewing gum) and Oral Balance gel (n=28)	[110]
	Increase in hydrogen peroxide concentration in exhaled breath condensate after inhibition of LCP by dapson in nonsmoking asthmatic patients; suggested a potential role for LCP in scavenging of H ₂ O ₂ in asthmatic airway	[3]
	Mouthrinse used for 12 months in radiotherapy patients and Sjögren's syndrome patients with xerostomia; no significant differences in coronal caries between the test group and the control group (n=124)	[36]
	In adult patients with severe xerostomia a good moistening and lubrication effect with Dentacal mouth spray (n=38)	[35]
	Adults volunteers drank 200 ml of control milk or test milk with CPP-ACP for 3 weeks; all milk samples were effective in remineralization of enamel surface, but test milk produced greater remineralization (n=10)	[109]
Casein derivatives	GC Tooth Mousse applied on teeth of patients with dentin hypersensitivity caused by various factors; insufficient effectiveness and short-term therapeutic effect in soothing pain (n=13; 101 teeth)	[52]
	$\beta(1-25)$ -Fe labeled with iron 59, added to cow milk, was studied in young women (n=10) compared with control substance ferrous sulfate; although the absorption of $\beta(1-25)$ -Fe was not significantly higher than that of control, it displayed significantly higher tissue uptake.	[2]
	Single dose of casein hydrolysate (designed C12 peptide) either alone or combined with alginate in hypertensive subjects led to significant decrease of systolic and diastolic blood pressure (n=10)	[103]
	Antihypertensive effect in volunteers with high-normal pressure or mild hypertension taking casein hydrolysate containing ACE inhibitory tripeptides (VPP and IPP) in tablets for 6 weeks (n=131)	[74]
	Improvement of vascular endothelial functions and no change in systemic blood pressure in casein hydrolysate (containing ACE inhibitory tripeptides) treated mild-hypertensive subjects for one week (n=25)	[38]
	Casein hydrolysate tablets (with ACE inhibitory tripeptides) administered for 4 weeks caused mild improvement in hypertension without adverse effects (n=48)	[43]
	Casein hydrolysate (with ACE inhibitory tripeptides) given for 8 weeks to aged subjects with untreated stage-I hypertension led to reduction of systolic blood pressure (n=70)	[77]
	Nasogastric feeding with casein hydrolysate of elderly patients with ischemic stroke decreased IL-6 and increased glutathione serum levels (n=31)	[21]
	7-day treatment with casein hydrolysate (insuVida™) alone or with additional leucine significantly lowered plasma glucose as compared to placebo or intact casein (n=36)	[30]

cd. **Table 1.** Published clinical studies with milk proteins and peptides

Protein/peptide	Clinical effects, number of participants	References
Lysozyme	A beneficial effect of breast milk or infant formula enriched in lysozyme in feeding premature infants suffering from concomitant diseases; after 14-21 days of this feeding the following were observed: increase of body weight, more rapid sanation of inflammatory foci, normalization of the stool and stabilization of lysozyme levels in serum and stool (n=64)	[11]
	Preventive effect of lysozyme chloride given for 4-24 weeks before orthopedic surgery on post-transfusion hepatitis (n=260)	[89]
	A synergistic effect of lysozyme with antibiotics as compared with antibiotics alone in treatment of acute pneumonia (n=92) and pyelonephritis (n=83) children	[16]
	Antiviral and antimicrobial effects in healthy volunteers infected with non-pathogenic strains of measles virus and attenuated <i>Salmonella</i> Typhi (n=14)	[32]
	Effective in local treatment of crural ulcers refractory to previous treatment (n=20)	[29]
Proline-rich polypeptide (PRP)	Increase of immunological reactivity in patients with head and neck cancer treated for 6 months (n=16)	[42]
	100 µg Colostrin [®] tablets, every second day for 3 weeks, 2-week hiatus, 10 cycles, improved outcome in Alzheimer's patients with mild to moderate dementia (n=46)	[58]
	16-month trial (treatment as above), confirmation of the previous results (n=33)	[57]
Ig concentrate from hyperimmune bovine milk	15-week treatment in a multi-center trial, beneficial effects on cognitive symptoms and daily function Alzheimer's patients (n=105)	[9]
	Protective effect in healthy volunteers against <i>S. flexneri</i>	[99]
	Protection against diarrhea in volunteers infected with enterotoxigenic <i>E. coli</i> (n=25)	[27]
	No prophylactic effect against <i>E. coli</i> in healthy volunteers (n=20)	[100]
	Prophylaxis against rotavirus gastroenteritis in infants (n=10)	[24]
	Some, but not statistically significant improvement in treatment of acute rotaviral gastroenteritis in children (n=135)	[113]
TGF-β (Modulen IBD [®])	Protective effect (less diarrhea and reduction in oocyst excretion) in healthy volunteers infected with <i>C. parvum</i> (n=16)	[79]
	Amelioration of osteoarthritis symptoms (joint pain and stiffness and immobility) in adult patients (n=42)	[116]
	Remission in 80% of children with newly diagnosed CD and 58% with long-standing disease and improving nutritional status (n=27)	[20]
	Clinical and histological remission (improvement of degree of inflammation of the intestinal mucosa) in pediatric CD patients (n=14)	[78]
Milk fat globule membrane proteins (MFGM)	Improvement in disease severity score (clinical response), body mass index and erythrocyte sedimentation rate in CD children as compared with standard polymeric formula and standard nutrition (n=64)	[34]
	Clinical remission and mucosal healing (histological remission) and improvement in anthropometric measures in CD children (n=106)	[85]
Milk fat globule membrane proteins (MFGM)	Protective effect (lower incidence of diarrhea, severe diarrhea, prevalence and duration of diarrhea and numbers of children with persistent diarrhea) in Peruvian infants given complementary food with MFGM fraction for 6 months, as compared to control group given skim milk proteins (n=550)	[115]

Abbreviations used in Table: angiotensin-I-converting enzyme (ACE), casein phosphopeptide-amorphous calcium phosphate (CPP-ACP); Crohn's disease (CD); glycomacropeptide (GMP); human α-lactalbumin made lethal to tumor cells (HAMLET); immunoglobulins (Ig); α-lactalbumin (LA); lactoperoxidase (LCP); lactoferrin (LF); large neutral amino acids (LNAA); milk fat globule membrane proteins (MFGM).

Table 2. Bioactive peptides derived from bovine milk proteins (examples)

Peptides (name)	Origin	Fragments	Structure	Function	References
β-Lactotensin	LG	(146-149)	HIRL	Ileum contraction, hypertensive activity, memory consolidation, blood cholesterol reduction	[53,91]
Lactokinin	LG	(142-148)	ALPMHIR	ACE inhibitor	[19,26,91]
β-Lactosin	LG	(142-145)	ALPM	ACE inhibitor	[19]
β-Lactorphin	LG	(102-105)	YLLF	ACE inhibitor, opioid agonist, stimulatory effect on ileum	[44,53,91]
–	LG	(71-75)	IIAEK	Hypocholesterolemic effect	[19,105]

cd. **Table 2.** Bioactive peptides derived from bovine milk proteins (examples)

Peptides (name)	Origin	Fragments	Structure	Function	References
α-Lactorphin	LA	(50-53)	YGLF	ACE inhibitor, opioid agonist	[26,53,91]
LDT1	LA	(1-5)	EQLTK	Antibacterial activity	[19,105]
LDT2	LA	(17-31)S-5(109-114)	GYGGVSLPEWVCTTFALCSEK	Antibacterial activity	[19,105]
LDC	LA	(61-68)S-5(75-80)	CKDDQNPHSCKDF	Antibacterial activity	[19,105]
Casokinins	α _{s1} -casein	(23-24)	FF	ACE inhibitor	[19,26,105]
		(23-27)	FFVAP		
		(23-34)	FFVAPFPEVFGK		
		(146-147)	YP		
	α _{s2} -casein	(174-179)	FALPQY	ACE inhibitor	[19,26]
		(198-202)	TKVIP		
	β-casein	(74-76)	IPP	ACE inhibitor	[19,26,105]
		(84-86)	VPP		
(88-90)		LQP			
κ-casein	(193-202)	YQEPVLGPVR	ACE inhibitor	[19]	
	(108-110)	IPP			
Casoxins	κ-casein	(38-39)	YG	Opioid antagonist	[91,105]
		(35-42)	YPSYGLNY		
GMP	κ-casein	(106-169)	Various fragments	Antithrombotic effect, immunomodulatory effect, antibacterial activity	[53,91]
β-Casomorphins	β-casein	(60-66)	YFPFGPI	Opioid agonist	[19,44,105]
		(60-64)	YFPFG		
α-Casein exorphins (α-casomorphins)	α _{s1} -casein	(90-96)	RYLGYLE	Opioid agonist	[19,91]
		(90-95)	RYLGYL		
Isracidin	α _{s1} -casein	(1-23)	RPKHPIKHQGLPQEVLNENLLRF	Antibacterial activity, immunomodulatory effect	[19,105]
Casocidin-I	α _{s2} -casein	(165-203)	KKISQRYQKFKALPQYLKTVYQHQQKAM KPWIQPKTKVIPY	Antibacterial activity	[19,105]
Casopiastrin	κ-casein	(106-110)	MAIPP	Antithrombotic activity	[91,105]
–	α _{s1} -casein	(79-81)	KHI	Immunomodulatory effect	[19,105]
–	α _{s2} -casein	(1-32)	KNTMEHVSSSEESIISQETYKQEK NMAINPSK	Immunomodulatory effect	[19,105]
–	β-casein	(63-68)	PGPIP	Immunomodulatory effect	[19,105]
		(191-193)	LLY		
–	α _{s1} -casein	(107-108)	PQ	Antioxidant activity	[19]
Casein phosphopeptides	α _{s1} -casein	(43-58)	DIGSESTEDQAMEDIK	Mineral carries, anti- caries activity	[19,91,105]
		(66-74)	SSSEIVPN		
		(106-119)	VPQLEIVPNSAEER		
	α _{s2} -casein	(2-21)	NTMEHVSSSEESIISQETYK	Mineral carries, anti- caries activity	[19,91,105]
		(55-75)	GSSSESAEVATEEVKITVDD		
	β-casein	(126-136)	EQLSTSEENSK	Mineral carries, anti- caries activity	[13,19,91,105]
(1-25)		RELEELNVPGEIVSLSSSEESITR			
	(1-28)	RELEELNVPGEIVSLSSSEESITRINK			

Abbreviations used in table: glycomacropeptide (GMP); α-lactalbumin (LA); β-lactoglobulin (LG); CLN – Colostrinin; A – Ala (alanine); E – Glu (glutamic acid); F – Phe (phenylalanine); G – Gly (glycine); H – His (histidine); I – Ile (isoleucine); L – Leu (leucine); M – Met (methionine); N – Asn (asparagine); P – Pro (proline); Q – Gln (glutamine); R – Arg (arginine); S – Ser (serine); V – Val (valine); W – Trp (tryptophan); Y – Tyr (tyrosine). Amino acid sequences of functional protein chains (without signal peptides) of bovine proteins are presented. Protein chain and precursor sequences (that include signal peptides) are the following for: α_{s1}-casein – 214 residues includes the signal sequence 1-15, α_{s2}-casein – 222 residues with signal sequence 1-15, β-casein – 224 residues with signal sequence 1-15; κ-casein – 190 residues with signal sequence 1-21, LA – 142 residues with signal sequence 1-19 and LG – 178 residues with signal sequence 1-16 [105].

cose, insulin and blood pressure decreased and HDL cholesterol increased) [48]. However, whey protein- and whey protein-LA-enriched breakfast yoghurt drink increased energy expenditure and protein balance and decreased fat balance compared to control breakfast (whole milk). In addition, the LA-enriched yoghurt suppressed hunger more than the whey-enriched yoghurt [40]. In another double-blind 12-week study obese subjects drank the ProLibra™ beverage (Glanbia Nutritionals Inc.) which contains a combination of intact whey proteins and peptides and minerals purified from bovine milk. The supplement is high in leucine, bioactive peptides and calcium. The control group received an iso-caloric beverage containing maltodextrin, with a lower calcium and protein content. The supplementation with ProLibra™ increased the loss of body fat and the retention of lean muscle mass compared to supplementation with a control drink [28].

The formulas enriched in α -lactalbumin and GMP were also tested in various pathological conditions. A diet rich in LA hydrolysate was applied in malnourished children with diarrhea of various degree [25]. The results showed better weight gain and requirement of a smaller number of rehydrations during the first three weeks of hospitalization as compared to the control group. However, in a clinical trial involving children with diarrhea, where three different rehydration solutions were tested, no advantage of LA-containing formula over two other solutions in terms of stool output and duration of diarrhea was found [86]. Because of lack of phenylalanine, GMP is an attractive candidate for a diet of individuals suffering from phenylketonuria [55,106]. The authors compared a GMP-containing diet with phenylalanine-free amino acid formula in subjects with phenylketonuria and found that the experimental diet was well tolerated and improved protein retention and phenylalanine utilization as compared to the control diet. In addition, the GMP-containing diet lowered the plasma ghrelin (appetite-stimulating hormone) levels in such patients, which was associated with greater feelings of satiety [65]. In phenylketonuria patients complaints of persistent hunger are common. A skim milk powder enriched with GMP and milk fat extract was used in a randomized, double-blind 3-month trial for prevention of gout flares [18]. Control groups received lactose powder or skim milk powder. Although improvement in the studied parameters (pain, excretion of uric acid) was seen in all groups, only the GMP-enriched formula gave statistically significant results as compared with baseline conditions of patients.

A series of trials were also performed aimed at evaluation of effects of α -lactalbumin on mood, stress and memory. Brain serotonin, which is synthesized from tryptophan (W; Trp), influences food intake and mood [114] and Trp content is high in α -lactalbumin [37]. Trp uptake in the brain is dependent on the plasma ratio of Trp to the sum of other large neutral amino acids (LNAA). The effect of an LA supplement combined with regular diet on several parameters such as plasma Trp:LNAA ratio, serum prolactin as a marker of serotonin synthesis, food intake,

appetite, macronutrient preference and mood was studied in healthy males as compared to a regular diet [8]. Although the plasma Trp:LNAA ratio was significantly increased after the experimental diet, the authors could not demonstrate effects of that diet on appetite, food intake, macronutrient preference and mood. Other investigators [73] also were not able to show any effect of a one-day diet enriched in LA on stress-induced mood deterioration in depressed patients and healthy subjects, although the LA diet led to the expected rises in Trp:LNAA ratio. However, the same group of researchers [12] found in the category of recovered depressed patients that the LA-enriched diet improved abstract visual memory and impaired simple motor performance with no effect on mood. Other researchers focused predominantly on the effects of an LA-enriched diet on stress and sleepiness [67,68,69]. In subjects exposed to experimental stress after the intake of an LA-enriched diet the plasma Trp:LNAA ratio was higher by 48% than in subjects taking a control, sodium caseinate-enriched diet [69]. The stress-vulnerable subjects fed the experimental diet also had higher prolactin concentrations, decreased cortisol levels and reduced depressive feelings under stress. In the same category of patients the authors [68] demonstrated an advantageous effect of an LA diet on cognitive performance. In another study by the same group [67] healthy subjects with sleep complaints were given in the laboratory evening meals containing α -lactalbumin or tryptophan-low placebo. The experimental meal significantly increased the Trp:LNAA ratio, reduced sleepiness and improved brain-sustained alertness the next morning. A trial on women with premenstrual complaints showed that LA improved long-term memory for abstract figures but not for words [90].

α -Lactalbumin may be converted from its native regular state to a partially unfolded variant [96]. Such a variant of LA with a specific fatty acid (oleic acid C18:1) cofactor induces apoptosis of tumor and immature cells and is defined as HAMLET (human α -lactalbumin made lethal to tumor cells). The authors suggest that a similar complex may be formed in the stomach of a nursing child. In a clinical trial on patients with cutaneous papillomas HAMLET proved to be very effective in reducing both volume and occurrence of the lesions [33]. It also appeared that intravesical HAMLET application (25 mg/ml for 5 consecutive days) to bladder cancer patients before surgery resulted in increased shedding of dead tumor cells into the urine [75]. Upon surgery, the morphological changes and reduction in tumor size were confirmed. The adjacent healthy tissue showed no evidence of apoptosis or toxic response. The authors propose that local administration of HAMLET might be of value in treatment of bladder cancers. Bovine α -lactalbumin and oleic acid (BAMLET) also demonstrated anticancer properties, but, as yet, it was applied only in *in vitro* models [19].

LACTOPEROXIDASE

Lactoperoxidase (LCP) constitutes approximately 0.5% of whey proteins in bovine milk and only <0.1% in human

milk (Fig. 1). LCP has potent antibacterial and antifungal activities in the presence of hydrogen peroxide (H_2O_2) [111]. The enzyme catalyses the oxidation of thiocyanate (SCN^-) and produces intermediate products ($^{\circ}OSCN/HOSCN$) with antimicrobial activities. Such properties prompted investigators to apply lactoperoxidase-containing preparations in clinical trials in healthy subjects and patients. Results of these trials enabled pharmaceutical companies to launch cosmetics and oral hygiene products with LCP. To make LCP antimicrobial, its substrate (SCN^-) is added as potassium thiocyanate (KSCN), but H_2O_2 is generated *in situ* in the mouth by a glucose-glucose oxidase system added to these oral hygiene products. All components constitute together the "lactoperoxidase enzyme system" [101]. Other antimicrobial host proteins – lysozyme and lactoferrin (LF) – act in an additive and even synergistic way; therefore they are often combined with the lactoperoxidase enzyme system in oral health care products. A number of oral hygiene products/saliva substitutes have been developed during the last decades. They include not only toothpaste, but also non-alcoholic mouth-rinse, chewing gum, moisturizing gels and denture adhesives for patients with dry mouth problems. For commercial purposes bovine enzymes (LCP, LF and lysozyme) purified from colostrum or milk have been used because they are structurally and catalytically very close to human enzymes, and large-scale purification from human material is difficult and too expensive [reviewed in 101].

For example, the Biotène Oral Balance[®] gel (Lacledé Inc. Healthcare Products) is a saliva substitute product which contains the lactoperoxidase enzyme system and LF and proved to be effective in reducing symptoms of dry mouth (xerostomia) in the elderly [70]. Likewise, the clinical efficacy of the Biotène Oral Balance[®] gel and Biotène[®] mouthwash containing the lactoperoxidase enzyme system, LF and lysozyme in elderly subjects with xerostomia was also demonstrated [31]. Both hyposalivation and xerostomia increase with age and are often associated with frequent use of drugs. These symptoms may have a negative impact on the quality of life. They may lead to risk of oral infections, increased rate of dental caries, mucosal atrophy, burning and pain. In another clinical study on patients with burning mouth syndrome several preparations containing capsaicin (red pepper emulsion in water), α -lipoic acid in tablets (Tiobec[®]; Laborest Italia S.P.A.) or lysozyme-lactoperoxidase in oral rinse (Biotène[®]) as test drugs and boric acid as control were tested [66]. All of the treatments were more effective than boric acid. On the other hand, a double-blind crossover study with 20 healthy volunteers with normal salivary flow rate did not show any advantageous effect of 2-week use of lactoperoxidase enzyme system-containing toothpaste (Biotène[®]) on salivary flow rate, peroxidase activity, thiocyanate ($^{\circ}OSCN/HOSCN$, SCN^-) and total streptococci, mutans streptococci, lactobacilli and anaerobic flora in saliva and dental plaques [51]. In another study the same authors used hygiene products (Biotène[®] toothpaste and mouthrinse) consisting of lactoperoxidase enzyme system, lysozyme and LF in 20 patients with xerostomia in Sjögren's syn-

drome. They registered a relief from the symptoms of oral dryness, although no increase in salivary flow rate or its hypothiocyanate levels were found. No major changes occurred in salivary microflora either [50]. Biotène Oral Balance[®] gel containing lactoperoxidase enzyme system, LF and lysozyme also proved effective in 21 secondary Sjögren's syndrome patients with dry mouth complaints and hyposalivation, based on a 7-item questionnaire (dry mouth sensation and its effect on chewing, swallowing, taste, speech, burning sensation and denture retention) [4]. The lactoperoxidase enzyme system-containing products Biotène Oral Balance[®] gel in combination with Biotène[®] toothpaste were effective in symptomatic therapy in patients suffering from dry mouth syndrome with diseases of the oral mucosa [88]. Therefore, studies with patients with xerostomia/hyposalivation are of greater interest than those with healthy subjects with normal salivation.

Also promising results were obtained with the Biotène[®] toothpaste containing lactoperoxidase enzyme system (which has inhibitory action against cariogenic oral microflora) in children with early childhood caries [46]. It appeared that, in comparison to a control group (Colgate Active[®] toothpaste), the levels of thiocyanate ions were increased and the numbers of mutans streptococci and lactobacilli were significantly reduced during the experimental period. In a clinical trial with 15 subjects, tablets containing bovine LF and LCP (Morinaga Milk Industry) were effective in reducing oral malodor (volatile sulfur compounds in mouth air) and the number of salivary bacteria [93]. Radiotherapy of head and neck cancer is associated with xerostomia [17]. In a study on patients with post-radiotherapy xerostomia the efficacy of two lactoperoxidase-containing preparations, Bioextra[®] (Light-house Health Products Inc.) and Biotène Oral Balance[®] dry mouth systems, were investigated, with comparable, beneficial results on mouth scores [92]. Bioextra[®], which contains additional peptides from "whey extract" or colostrum (e.g. immunoglobulins, growth factors), was a little more effective in relieving the symptoms of xerostomia than Biotène Oral Balance[®]. In another clinical trial a comparison was made between Biotène Oral Balance[®] gel delivered to post radiotherapy-related xerostomia patients in two ways: by slow release *via* a novel intra-oral device or as an oral bolus [72]. The results showed that this gel, both in bolus and slow-release form, was equally effective in reduction of xerostomia symptoms and oral cariogenic microorganisms. Similar results were obtained in irradiated oral carcinoma patients with daily use of Biotène Oral Balance[®] gel and Biotène[®] toothpaste in combination, for 4 weeks after radiotherapy [76]. Control subjects received carboxymethylcellulose gel and Oral B[®] fluoride toothpaste. The treatment improved salivary flow and increased oral comfort in comparison to the control group. In addition, some aerobic isolates of microorganisms were eliminated, and periodontal-associated bacteria and candidal species were significantly lowered in the experimental group. Other studies with patients having radiation-induced hyposalivation/xer-

rostomia have shown the efficacy of Biotène® toothpaste containing lactoperoxidase enzyme system in control of gingival disease. The test toothpaste examined in 20 patients during a 52-day period reduced the rate of supragingival plaque formation and gingival inflammation compared with a control toothpaste [108]. A double-blind crossover study with 19 patients with irradiated head and neck cancer who had used the Biotène® toothpaste showed reduction of gingivitis [102]. Another study combined lactoperoxidase enzyme system-containing Biotène® products (toothpaste, mouthwash, chewing gum) and Biotène Oral Balance® with good results in subjective relief of radiation-induced xerostomic symptoms (e.g. oral discomfort, intraoral dryness, inability to eat normally). The lack of an appropriate control makes it, however, impossible to rule out the placebo effect [110]. In 2012 a randomized trial was completed applying Biotène Oral Balance® gel in 41 critically ill mechanically ventilated newborns [1]. Prolonged mechanical ventilation is a known risk factor strongly associated with ventilator associated pneumonia (VAP), which is caused by a profuse access of microflora in the oral cavity. Therefore, application of the antimicrobial gel may diminish the incidence of pulmonary inflammation in these children. The results of this trial are not available as yet (April 2013).

Hydrogen peroxide generated by neutrophils and eosinophils in asthma is known to damage the airway epithelium and to contribute to airway inflammation [39]. In an observational study by Al Obaidi et al. an attempt was undertaken to evaluate the contribution of lactoperoxidase in scavenging airway hydrogen peroxide and to propose a therapeutic approach for asthma [3]. Nonsmoking asthmatic patients enrolled in the study were administered dapsone (a lactoperoxidase inhibitor) for 8 weeks and hydrogen peroxide concentration in exhaled breath condensate was determined. Determination of hydrogen peroxide content revealed its significant increase when compared to the baseline. The results suggest a potential beneficial role for lactoperoxidase in scavenging of hydrogen peroxide in asthmatic airways.

CASEINS

Caseins constitute a large part (80%) of total proteins in bovine milk and a minor part (20-50%) of total proteins in human milk. The major constituents of the family of bovine caseins are β - and α_{s1} -caseins, and of human caseins – β - and κ -caseins (Fig. 1). Caseins not only provide adequate amounts of essential amino acids, but peptides derived from caseins have been shown to express various biological effects (Table 2). Bioactive peptides are produced by *in vitro* and *in vivo* enzymatic proteolysis of bovine and human caseins [19,91].

Casein derivatives were tested in treating dry mouth, dentin hypersensitivity and in caries prevention (reviewed in [6]). In a randomized clinical trial with subjects with salivary gland dysfunction (radiotherapy and Sjögren's syndrome patients) two mouthrinse solutions contain-

ing either casein derivatives with calcium phosphate or a 0.05% sodium fluoride (as control), used for 12 months, were compared [36]. No statistically significant differences between the two groups regarding coronal caries were found. A good moistening and lubrication effect of a phosphoprotein-calcium phosphate complex (Dentacal®; NSI Pty Ltd.), in the form of mouth spray, was obtained in a category of patients with severe xerostomia [35]. In a double-blind, crossover study 10 adult volunteers drank 200 ml of control milk or test milk with 2 or 5 grams of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), once a day for 3 weeks. The results demonstrated that all 3 milk samples remineralized enamel subsurface lesions, but milk with CPP-ACP produced greater remineralization than the control milk [109]. The aim of the next study was to evaluate the efficiency of GC Tooth Mousse® (GC Corporation), based on the Recaldent™ technology, with CPP-ACP in the treatment of patients with dentin hypersensitivity caused by various factors. The product was applied on surfaces of teeth for 3 minutes and pain intensity was tested. The study concluded that the efficacy and short-term therapeutic effect of CPP-ACP were insufficient [52].

The casein phosphopeptides (CPP) – strongly phosphorylated peptides obtained by enzymatic digestion of caseins – have a strong capacity to fix nutritionally interesting divalent cations such as calcium, iron or zinc, thus making them stable and soluble in different physico-chemical conditions, in particular of pH. Due to this property, CPP may be involved in intestinal absorption of minerals, in particular of calcium. The results of research are, however, contradictory (reviewed in [13]). CPP improve the absorption of calcium in the rat by limiting the precipitation of this mineral in the intestine and also the absorption of calcium and zinc in the human adult, but do not show a positive effect in other studies in the rat or pig. The phosphopeptide $\beta(1-25)$ derived from bovine β -casein that contains 4 phosphoserine residues in its sequence and can fix 4 to 5 iron atoms with a high affinity was studied in rats and humans [13]. The studies in rats demonstrated the efficiency of $\beta(1-25)$ on the absorption and bioavailability of iron, and the efficiency was confirmed in a first clinical study comparing Fe/ $\beta(1-25)$ and iron sulfate. The quantity of iron stored in the organs seven days after the ingestion of the preparations was higher in the group having received the Fe/ $\beta(1-25)$ complex [2]. These beneficial results may partly explain the high iron bioavailability of breast milk, which is rich in β -casein.

Casein hydrolysates were, on the other hand, applied in clinical trials of hypertensive subjects (reviewed in [26]). It appeared that proteolytic digest of casein by bacterial enzymes lowered blood pressure in spontaneously hypertensive rats due to release of angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) peptide inhibitors [112]. In a first small placebo-controlled crossover trial two single doses of bovine casein hydrolysate (designed C12 peptide), administered alone or in combination with alginate acid, significantly reduced systolic and diastolic blood

pressure in 10 hypertensive subjects [103]. In a larger single-blind, placebo-controlled study with 131 volunteers with high-normal blood pressure and mild hypertension, a casein hydrolysate, containing the major ACE inhibitory tripeptides VPP and IPP, was used in four different doses (in tablets) for 6 weeks. An antihypertensive effect was demonstrated, particularly in mildly hypertensive subjects [74]. In another study in humans with mild hypertension the casein hydrolysate containing the same antihypertensive tripeptides improved vascular endothelial functions independently of blood pressure-lowering effects [38]. It also appeared that a large excess (5 times more than the effective amount) of casein hydrolysate-containing tripeptides, given in tablets, reduced systolic blood pressure in subjects with mild hypertension, but not in normotensive subjects. In addition, neither an excessive reduction in blood pressure nor other adverse events were observed [43]. Lastly, casein hydrolysate containing the active tripeptides improved blood flow parameters in a double-blind, placebo-controlled trial in aged subjects with untreated stage-I-hypertension [77].

Casein or whey hydrolysate nutrition formulas, administered by nasogastric feeding, were used in elderly patients with acute ischemic stroke [21]. The mortality in both groups of patients was similar (33%). The parameters measured were: serum interleukin (IL)-6, C-reactive protein (CRP) and glutathione levels. Serum IL-6 concentration decreased and glutathione levels were significantly higher in whey-protein fed patients in comparison to the casein group. The authors conclude that whey protein enteral formula in ischemic stroke patients may decrease inflammation and increase antioxidant defense compared to casein-containing formula.

On the other hand, hydrolyzed casein was found effective in decreasing postprandial glucose concentrations in type 2 diabetes mellitus patients who continued their oral anti-diabetic medication [30]. In a double-blind trial single meal replacements with proprietary casein hydrolysate (insuVida™; DSM Nutritional Products) alone or with addition of leucine, unhydrolyzed casein or placebo were used. Postprandial serum glucose, insulin and glucagon concentrations were measured after 4 h. The addition of leucine to insuVida™ caused the biggest increase in insulin (by 51.8%). All three treatments increased glucagon concentration compared to placebo. In addition, a single dose of insuVida™ with or without leucine significantly lowered plasma glucose content compared to placebo and intact casein. The results suggest a benefit of the studied nutrients in management of type 2 diabetes.

Some peptides from caseins show pharmacological similarities to opium (morphine) and are called "opioid peptides". The major opioid peptides, called β -casomorphins and α -casomorphins, are fragments of β - and α -casein, respectively. Opioid peptides are opioid receptor agonists and they induce an analgesic and sedative effect due to their action on the nervous system. They also affect the gastrointestinal motility. However, all κ -casein fragments,

known as casoxin, behave as opioid antagonists. Opioid agonists and antagonists may be formed in the gut as a result of *in vivo* hydrolysis of milk caseins [19,91]. Several immunomodulating peptides can be released from α - and β -caseins, whereas the κ -casein fragment (casopiastrin) shows antithrombotic activity [91]. β -casein and its peptides, as well as α_s -casein, possess antioxidant and antimicrobial activity [19] (Table 2). As yet, however, none of the above-mentioned peptides has been clinically tested.

LYSOZYME

Lysozyme is present in human milk at relatively high concentrations (about 6% of the total whey proteins), while in bovine milk it is present at minor concentrations (<0.1% of the total whey proteins) (Fig. 1). This enzyme is known to degrade the outer cell walls of Gram-positive bacteria, but together with lactoferrin is able to kill also Gram-negative bacteria. Lysozyme is widely used as a food preservative, in infant feeding formulas and in pharmaceutical products for treatment of periodontitis, dry mouth syndrome and prevention of tooth decay (reviewed in [82]) but its application in clinical trials is limited.

Breast milk or dry adapted milk formula enriched in lysozyme (50 mg/L) was used in feeding premature infants suffering from concomitant diseases. The control group was fed breast milk or an artificial product without lysozyme. A beneficial effect was shown, as compared to that of control, taking into account increase in body weight, sanitation of the infectious inflammatory foci, normalization of the stool, and stabilization of lysozyme levels in the coprofiltrates and in the blood serum [11]. In a large randomized controlled trial a preventive effect of lysozyme on post-transfusion hepatitis in orthopedic diseases was studied. The patients who received blood transfusion during a period of 5 years (1970-1975) were given lysozyme chloride (60 to 170 mg/day) from 4 to 24 weeks. The incidence of post-transfusion hepatitis was 8.1% for the treated group in contrast to 20.4% for the control group [89]. Lysozyme was also used as an antimicrobial agent showing synergistic effects in combination with different classes of antibiotics such as benzylpenicillin, ampicillin, morphocycline, erythromycin and others, and aminoglycosides such as gentamycin, tobramycin, sisomicin and amikacin *in vitro* [16]. The authors confirmed these co-stimulatory, antibacterial effects in patients (children) with acute pneumonia and pyelonephritis of bacterial genesis. In case of pneumonia the combined action of lysozyme and conventional antibiotics resulted in more rapid elimination of fever, toxic and cardiorespiratory syndromes, cough and other signs of the disease. In the group of children suffering from pyelonephritis complete clinico-laboratory remission was observed in 81% of patients compared to 56.4% of cases treated with antibiotics alone. The antiviral and antimicrobial effects were also proved in healthy volunteers infected with non-pathogenic vaccine strains of living measles virus and of attenuated *Salmonella* Typhi [32]. A therapeutic action of local lysozyme treatment (solution in 0.9% NaCl, 1 mg/

mL) was also found in the case of crural ulcers [29]. That action was not due to effects of lysozyme on ulcer-associated bacterial flora since the cultured bacteria were insensitive *in vitro* to lysozyme concentrations applied *in vivo*. The authors suggested that the beneficial effect of lysozyme on wound healing could be due to its cationic influence on the cell membranes of the epithelium and to pH change in the ulcerations. It was also demonstrated that long-term treatment of head and neck cancer patients with lysozyme chloride during standard therapy led to a significant increase of the intradermal response to antigens phytohemagglutinin (PHA) and purified protein derivative (PPD) intradermal injections [42]. The patients had no local or systemic unfavorable effects due to lysozyme [11,29,42].

PROLINE-RICH POLYPEPTIDE (PRP)

Proline-rich polypeptide (PRP) is a uniform mixture of low-molecular-weight proline-rich polypeptide with immunoregulatory properties (reviewed by [117]). PRP was originally isolated from ovine colostrum in 1974 [45] and then named colostrinine (CLN) [41]. The immunoregulatory properties of PRP were, in part, also shared by its nonapeptide (VESYVPLFP) fragment [95]. PRP was subsequently shown to induce TNF- α , IFN- γ and IL-6 in human whole blood cultures and to exert psycho-stimulating effects in a pilot study in healthy volunteers [41,81]. It soon appeared evident that PRP is a very complex fraction, consisting of 32 peptides, and possessing homology to three protein precursors (annexin, β -casein and a hypothetical β -casein homolog) [54]. PRP shows immunomodulatory properties in mice, rats and chickens, inducing maturation and differentiation of thymocytes, induces neurite outgrowth of pheochromocytoma cells, extends the lifespan of fibroblast cells, and inhibits β -amyloid-induced apoptosis. Interestingly, the PRP complex was also shown to decrease hypersensitivity and allergic response to common allergens (house dust mites and ragweed pollen grains) in a mouse model [10]. The discovery that PRP promoted precognitive functions in experimental animal models, indicating prevention of pathological processes of the central nervous system, led to application of CRP in clinical trials with Alzheimer's disease (AD) patients.

In the first clinical trial PRP isolated from ovine colostrum (commercial name Colostrinin™; ReGen Therapeutics Ltd.) was applied as 100 μ g tablets in AD patients for a one-year period [58]. Tablets supplemented in selenium and placebo were used for control groups. The treatment was performed as cycles with a 2-week hiatus to avoid development of hyporeactivity. The results were assessed by psychiatrists blinded to the treatments assignment. It appeared that in 8 out of 15 patients the monitored parameters were improved whereas in the 7 others the disease was stabilized. No improvements in health status were registered in two other (controls) groups. The next 2 trials were performed according to the protocol described above. A long-term (16 months)

study confirmed the beneficial effect of Colostrinin™ treatment with very mild adverse side-effects [57]. The results of these studies were verified in six psychiatric centers in Poland [9]. The full analysis of this double-blind trial encompassed: Alzheimer's Disease Assessment Scale-cognitive portion (ADAS-cog), Clinical Global Impression of Change (CGIC), Instrumental Activities of Daily Living (IADL), Mini-Mental State Examination (MMSE) and ADAS-non cognitive test (ADAS-non cog) and overall Patient Response. The results were in favor of the CLN experimental group as compared to placebo (ADAS-cog, $p=0.02$; IADL, $p=0.02$; overall Patients Response, $p=0.03$). Thus, these observations indicate a beneficial effect of Colostrinin™ on cognitive symptoms and daily functions in AD patients. Colostrinin™, as a very promising preparation, has been used for several years to retard the development of AD.

In order to explain the mechanisms of action of CLN in the AD patients human neuronal SHSY-5Y cells were pretreated with CLN for 24 h [22]. The authors demonstrated that CLN reduced β -amyloid-inducible apoptosis of these cells. In another study the gene expression profile of human buccal mucosal cell line TR146, treated in culture with 100 μ g/ml of CLN, was investigated [97]. The results showed that CLN elicited complex and multiphasic changes in the cells' transcription process. Of note, CLN altered gene expression implicated in β -amyloid precursor protein synthesis, Tau phosphorylation and increased levels of β -amyloid-degrading enzymes. In addition, CLN enhanced the defense against oxidative stress and decreased expression of genes for inflammatory cytokines, which suggests suppression of the inflammatory processes preceding the Alzheimer's and other neurological diseases.

OTHER MILK-DERIVED PRODUCTS

Secretory IgA is the predominant immunoglobulin (Ig) in human milk. It is present in very high concentrations during early lactation and in lower but substantial concentrations throughout lactation. IgG is the predominant Ig in bovine milk (Fig. 1). The absorption of Ig from colostrum and milk provides passive immunity for newborns. High concentration of antibodies against a particular pathogen may be achieved by immunizing cows with the pathogen or its antigens. By combination of an appropriate strategy of vaccination and established production procedures in the dairy industry, large scale manufacture of various neutralizing IgGs is possible. On average, 500 g/liter of IgG can be harvested from each immunized cow immediately after calving.

Concentrates of Ig derived from milk or colostrum of hyperimmunized cows were found to be protective in prevention of some infections, e.g. with *Shigella flexneri* or virulent strains of *Escherichia coli*. In a randomized, double-blind trial, volunteers were given a hyperimmune Ig concentrate with a high titer of anti-*S. flexneri* 2a lipopolysaccharide (LPS) antibodies three times a day

for seven days [99]. The volunteers were subsequently challenged with 10^3 colony forming units (CFU) of *S. flexneri*. Interestingly, none of the subjects from the experimental group became ill, in contrast to the placebo group (45% ill persons). Thus, high-titered, orally administered bovine Ig concentrate protects against shigellosis and may be useful in preventing this disease among travelers, military personnel and individuals during *Shigella* outbreak. To find a preventive measure for diarrhea caused by enterotoxigenic *E. coli* (ETEC), a hyperimmune bovine milk antibody product was obtained containing antibodies against purified colonization factor antigens [27]. The product protected volunteers (14 out of 15) in comparison to a control, placebo group (3 out of 10) challenged orally with a virulent ETEC. Yet in another trial by these authors no effect of such a product on diarrhea in volunteers challenged with ETEC strain E24377A was found [100].

The hyperimmune milk products were also tested in prophylaxis and treatment of virus infections. A colostrum product, containing neutralizing antibody to different serotypes of human rotavirus, was used for two weeks in infants from a baby center, with advantageous results [24]. Rotavirus-associated diarrhea developed in 7 of the 10 infants in the control group and one of 6 infants in the experimental group (receiving the colostrum) had such symptoms. On the other hand, the efficacy of hyperimmune bovine colostrum from cows immunized with simian rotavirus SA11 in the treatment of rotaviral gastroenteritis was compared to ordinary colostrum or milk preparations in a large randomized, double-blind study involving 135 children aged 6-30 months [113]. Even though differences were observed in weight gain, shorter duration of diarrhea and fewer stools, they were statistically insignificant. The authors suggest that these differences are clinically unimportant in well-nourished immunocompetent children, but the tested hyperimmune colostrum had some effects and should be evaluated further.

A bovine hyperimmune anti-*Cryptosporidium* colostrum preparation was also effective in prophylaxis of volunteers challenged with the parasite *C. parvum* [79]. A trend toward lower incidence of diarrhea was observed in the experimental group, as well as a strong reduction in oocyst excretion in comparison with the respective control group. A concentrated form of hyperimmune milk product (milk protein concentrate, MPC) was also used in a double-blind, placebo-controlled clinical trial on adult patients suffering from osteoarthritis. This was a 6-week study having three different treatment protocols: MPC, glucosamine sulfate and placebo [116]. Osteoarthritis index scores (joint pain, joint stiffness, activities and total) were evaluated. The results showed that MPC ameliorated the symptoms of osteoarthritis as compared to the baseline evaluation in all scores.

Transforming growth factor beta (TGF- β) 1 and 2 are growth factors present in milk in low but physiological-

ly relevant concentrations (in human and bovine milk <1 mg/L) and have effects on several cellular processes, such as cell proliferation and differentiation. They affect T-cells, B-cells, NK-cells, dendritic cells, macrophages, epithelial, endothelial and hematopoietic cells [60]. TGF- β plays an important role in maturation of immature human intestinal epithelial cells and inhibits the inflammatory cytokine response [83]. TGF- β in milk can induce and maintain oral tolerance and therefore can be involved in prevention of allergy [63].

Taking advantage of the property of this cytokine, a new product was manufactured, with casein as its protein source, enriched in milk-derived TGF- β 2 (Modulen IBD[®]; Nestlé Nutrition), which found application in the treatment of pediatric Crohn's disease (CD). In a retrospective analysis, using this product for exclusive enteral feeding of children with Crohn's disease, remission in 80% of newly diagnosed CD and 58% with longstanding disease and improvement of nutritional status were registered [20]. In another study on pediatric CD patients fed Modulen IBD[®] for 8 weeks, clinical remission and improvement in the parameters characterizing inflammation of the intestinal mucosa (histological remission) were observed [78]. Other investigators reviewed charts of children with CD in a retrospective study [34]. 28 children received Modulen IBD[®], in addition to conventional treatment, as a supplement to their regular nutrition. These subjects were compared with 18 children supplemented with standard polymeric formula (Ensure Plus[®]; Abbot Laboratories) and 18 children with standard nutrition (without formula supplementation). A significant decline in disease severity score (clinical response) was associated with improvement in body mass index and inflammatory markers (erythrocyte sedimentation rate) and was observed only in the Modulen IBD[®] group. Children fed Ensure Plus[®] formula had only a decline in the disease severity score. In another, retrospective trial by Rubio et al. the 8-week application of Modulen IBD[®] as exclusive nutritional therapy, beside clinical remission of the disease (in 75% of orally and 85% of enterally treated patients) resulted in mucosal healing as evidenced in a follow-up endoscopy in a subgroup of patients. All patients also showed a significant improvement in anthropometric measures [85].

Mucins are another active protein component in milk (apart from whey proteins and caseins). Mucins specifically surround milk fat globules, forming a chemical barrier: milk fat globule membrane proteins (MFGM). Mucins constitute about 1-2% of total milk proteins. Human milk mucins can bind to rotaviruses, inhibit viral replication and prevent experimental gastroenteritis. They also inhibited binding of *E. coli* to buccal epithelial cells [62]. In a randomized, double-blind, placebo-controlled study with 550 infants (6-11 months old) the effect of MFGM-enriched protein fraction in a complementary food for 6 months was evaluated [115]. Nutritional status, anthropometry and diarrhea morbidity

were assessed. The consumption of the MFGM protein fraction reduced the incidence of diarrhea and severe diarrhea, its prevalence and duration and the number of children with persistent diarrhea as compared to the control infants who received skim milk proteins. This indicates that the addition of an MFGM fraction to the diet may reduce the incidence of diarrhea and improve health in vulnerable populations.

β -lactoglobulin (LG) is the most abundant whey protein in bovine milk, while it is absent in human milk (Fig. 1). As yet no clinical trials have been performed with use of this protein or the protein-derived peptides, but their activities were confirmed *in vitro* and in animal studies. LG is, among others, a source of peptides lowering bloodpressure via inhibition of ACE. Lactokinin (142-148), β -lactosin (142-145) and β -lactorphin (102-105) can serve as examples of such peptides. Conversely, β -lactotensin (146-149) exhibits hypertensive activity. β -lactorphin and β -lactotensin are also opioid receptor agonists. LG has also antioxidant, antitumor, antiviral and hypocholesterolemic effects [19,26,44,53,91] (Table 2).

CONCLUSIONS

Milk is a rich source of proteins and peptides of proven nutritional and immunotropic activities. Preparation of biologically active proteins and peptides usually requires enzymatic degradation or chemical modification or addition of specific cofactors. The milk-derived preparations have found broad application in the food industry, production of infant formulas, and hygiene products. The products have found application as preventive or therapeutic measures for a broad array of pathological states in neonates, infants, adults and the elderly, with no adverse effects. In conclusion, milk-derived proteins and peptides may represent a supplementary treatment to conventional therapy. Many of these proteins and peptides have been commercialized as ingredients of nutraceuticals (supplements of diet and functional food) and hygiene products. Sour milk Calpis® (Calpis Co., Japan) and fermented milk Evolus® (Valio, Finland) with IPP and VPP as active compounds, casein hydrolysate Casein DP® (Kanebo Ltd., Japan) with FFVAPFPEVFGK as the active compound and whey protein hydrolysate BioZate® (Davisco, U.S.) with whey peptides are examples of such commercial products [26].

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