Peptides of interest

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Comprehensive Review of Bioactive Peptides Encrypted in Bovine β-casein and Their Liberation by Lactic Acid Bacteria

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Introduction

Dairy products are foods prepared by fermentation from the raw material, milk. Examples are, sour cream, quark butter (milk), and cheese. (1). Milk is widely consumed around the world: according to the OECD–FAO[1] Agricultural Outlook 2019-2028, the world milk production (81% cow milk, 15% buffalo milk, and a total of 4% for goat, sheep and camel milk combined) reached 838 million tons in 2018 and is projected to reach 981 million tons by 2028 (2). Dairy products are tremendously diverse because of the versatile composition of milk and the types of microorganisms that can grow in milk. They contain many essential nutrients such as oleic acid, conjugated linoleic acid, omega-3 fatty acids, vitamins, minerals, high-quality proteins, peptides, and oligosaccharides (3). Besides offering these nutritional values, dairy product like yogurt and cheese also provide attractive organoleptic properties, i.e. texture and flavor, to enrich our daily diet (4) (Fig 1A).

Milk contains 2 major protein groups: caseins and whey proteins. Casein accounts for 80% of the proteins in bovine milk while whey proteins constitute the rest. Bovine casein is composed of four main subtypes according to the homology of their primary amino acid sequences: αs1-casein (αs1-CN), αs2-casein (αs2-CN), β-casein (β-CN), and κ-casein (κ-CN) (Fig 1B). Bovine whey protein is composed of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin (SA), immunoglobulins (IgGs), secretory component (SC), and lactoferrin (LF) (5).

Casein can be used in food application as the intact form present in mil; it can also be dissociated into caseinates, or hydrolyzed into peptides. In recent years there is an increasing interest in bioactive peptides derived from casein hydrolysates for bio-functional food applications (6). β-Casein is a precursor for a variety of bioactive peptides that play different biological functions and can positively affect the cardiovascular, immune, nervous, and/or human digestive system (7) (Fig 1C). These bioactive peptides generally comprise 2–20 amino acid (AA) residues (8). They are inactive within the sequence of the precursor protein and can be liberated via three possible mechanisms: (i) by gastrointestinal digestion (8); (ii) by proteolytic microorganisms (9); (iii) by proteolytic enzymes from plants or microorganisms (10).

1 OECD: Organization for Economic Co-operation and Development; FAO: Food and Agricultural Organization of United Nations.

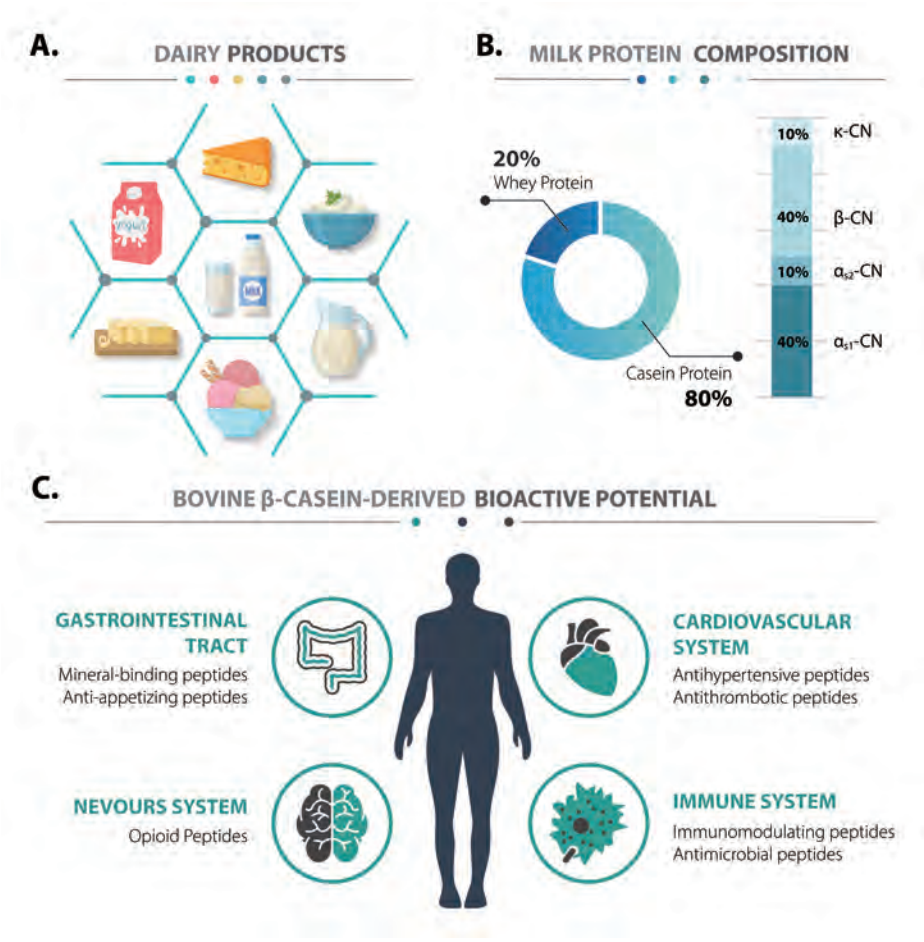

Figure 1. Dairy products and their bioactive effects on human body. A) Representative milk-derived (dairy) products. Clockwise, starting at noon: cheese, sour cream, soured or butter milk, ice cream, butter, yogurt. In the center: the raw starting material, milk. B) Pie chart showing the milk protein composition and percentage ratios; the stacked bar chart displays the casein composition and percentage distribution (11). C) Bovine β-casein-derived peptide bioactivities benefiting men.[1]

1 Standard licenses of the vector images used in Fig 1 were bought from shutterstock.com. The image IDs are 749418175, 1439669003, and 531099811, respectively.

Functionality of bioactive peptides derived from β-casein

Caseinophosphopeptides GI/IMMUNE System

Caseinophosphopeptides (CPPs) are phosphorylated casein-derived peptides that can bind to and solubilize minerals such as calcium and zinc (12). Phosphorous is bound to caseins via monoester linkages to serine residues. Bovine β-casein contains 5 serine residues which can be phosphorylated. Two CPPs have been identified in bovine β-casein, namely (numbering of precursor β-casein) fragment 16-40 (f(16-40)) RELEEL-NVPGEIVESLSSSEESITR and f(16-43) RELEELNVPGEIVESLSSSEESITRINK. The in vivo formation of f(16-40) was found to occur in the small intestinal tract of rats ingesting β-casein. This CPP increased the concentration of soluble calcium in the lumen of the distal ileum, enhancing the passive type of calcium absorption (13). Moreover, f(16-40) was also reported to affect the proliferation of mouse spleen cells and rabbit Peyer's patch cells(14). β-Casein f(16-43) was proven to have multiple immunoenhancing activities such as increasing immunoglobulin activity and cytokine production in human T, B and monocytic cell lines (15), and stimulate both proliferation and interleukin (IL)-6 expression of CD19+ cells (16).

β-casomorphins GI/NERVOUS/CARDIOVASCULAR/IMMUNE System

The opioid system plays a central role in modulating our mood and well-being; it consists of 3 G protein-coupled receptors (GPCRs): μ-, δ-, κ-. These receptors are located in the nervous, endocrine, immune systems, and in the intestinal tract of the mammalian organism (17, 18). They can interact with their endogenous as well as with exogenous opioids. β-casomorphins (β-CMs) are peptides with opioid activity and derive from bovine β-casein. β-CMs are μ-receptor agonists that possess many other biological activities (Table 1). The heptapeptide β-CM7, isolated from a bovine peptone digest, was the first exogenous opioid peptide described (19). Apart from its opioid activity, β-CM7 contributes significantly to mucin production in the gastrointestinal tract through the μ-opioid pathway (20). It was also shown to have a hypoglycemic effect on diabetic rats by increasing the level of plasma insulin (21). Moreover, β-CM7 exhibits angiotensin converting enzyme (ACE)-inhibitory activity with a half maximal inhibitory concentration (IC50) of 500 μM (22). β-CM5 is the strongest opioid agonist of the β-CMs identified so far and is particularly resistant to proteolytic digestion. It was shown to slow gastrointestinal motility in young rats (23). More recently, a rat colon in vitro study revealed that it increases mobility in the distal colon segment while it decreases mobility

of the whole large intestine (24). In addition, β-CM5 can stimulate neurite outgrowth of neuro-2a mouse neuroblastoma cells (25), and improves the disturbance in learning and memory resulting from cholinergic dysfunction (26).

Table 1

β-casomorphins (β-CMs) derived from bovine β-casein and their bioactivities

Name	Sequence [a]	Start [b]	End [b]	Bioactivity and Reference
β-CM4	YPFP	75	78	Opioid (27), Anticancer (28)
β-CM5	YPFPG	75	79	Opioid (29), Promotes neurite outgrowth (25), Modulates intestinal motility (24), Improves learning and memory (26), Induces inflammation (30)
β-CM6	YPFPGP	75	80	Opioid (31), DPP-IV-I (32)
β-CM7	YPFPGPI	75	81	Opioid (33, 34), Increases satiety (35), Antioxidative and Hypoglycemic (21, 36), Stimulates mucin secretion (20), Induces inflammation (30), Decreases anxiety and nociceptive sensitivity (37), Antiproliferative (38), ACE inhibitory (22)
β-CM8	YPFPGPIP	75	82	Opioid (39), ACE-I (40)
β-CM9	YPFPGPIPN	75	83	Opioid (33), ACE-I (41), DPP-IV-I (42)
β-CM11	YPFPGPIPNS	75	84	Opioid (43)
β-CM13	YPFPGPIPNSLPQ	75	87	Opioid (33)
β-CM21	YPFPG-PIPNSLPQNIP-PLTQT	75	95	Opioid (33)

[a] The peptide sequences are presented in the one letter amino acid code.
[b] The start and end positions of peptide from bovine β-casein sequence including signal peptide, sequence from UniProt (www.uniprot.org) with accession number P02666.

Dipeptidyl peptidase-4 inhibitory peptides [GI system]

Dipeptidyl peptidase-4 (DPP-IV) can hydrolyze incretin hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). These hormones enhance insulin secretion from pancreatic β cells in response to different nutrients. Inhibition of DPP-IV can prevent incretin degradation and, thus, DPP-IV inhibitory drugs are used to control high blood sugar in adults with type-2 diabetes (T2D) (44). To date, 21 DPP-IV inhibitory peptides have been identified from bovine β-casein (Table 2). They range in size from 3 to 9 AA residues with their DPP-IV IC50 values ranging from 46-1300 μM. Half of the peptides derive from bovine β-casein f(75-95). The majority of these peptides have a proline at the second position from the

N-terminal, indicating a possible structural pattern for DPP-IV- inhibitory peptides. Tripeptides with the sequence X[1]-Pro-X are competitive inhibitors of DPP-IV, e.g. LPL and LPQ, possess IC50 values of 82 µM and 241.4 µM, respectively. Small peptides are difficult to detect by mass spectrometry due to technical limits, but with the fast development of LCMS in recent years, more short peptides with DPP-IV-inhibitory activity might be uncovered that are possibly hidden in bovine β-casein since it contains 36 proline residues.

Table 2

DPP-IV inhibitory peptides derived from bovine β-casein

Sequence [a]	Start [b]	End[b]	DPP-IV IC50 value	Reference
VPGEIVE	23	29	224.5 ± 66.3 µM	(32)
YPFPGP	75	80	749.2 ± 122.3 µM	(32)
YPFPGPIPN	75	83	670 µM	(42)
FPGPIPN	77	83	260 µM	(42)
PGPIPNS	78	84	1000 µM	(42)
LPQ	85	87	82 µM	(42)
LPQNIPP	85	91	160 µM	(42)
LPQNIPPL	85	92	46 µM	(42)
LPQNIPPLT	85	93	205.2 ± 32.5 µM	(32)
PQNIPPL	86	92	1500 µM	(42)
IPPLTQT	89	95	465.1 ± 73.7 µM	(32)
IPPLTQTPV	89	97	1300 µM	(42)
FLQP	102	105	65.3 ± 3.5 µM	(45)
YPVEPF	129	134	138.0 ± 15.3 µM	(46)
LPL	150	152	241.4 ± 11.4 µM	(47)
LPLPL	150	154	325.0 ± 15.2 µM	(47)
LPLPLL	150	155	371.5 ± 60.6 µM	(32)
LPVP	186	189	87.0 ± 3.2 µM	(46)
LPVPQ	186	190	43.8 ± 8.8 µM	(32)
VLGP	212	215	580.4 ± 11.3 µM	(45)

[a, b] See legend to Table 1.

1 X indicates any amino acid residue

Antimicrobial peptides IMMUNE System

The continuous overuse and misuse of antibiotics has resulted in the worldwide emergence of multidrug-resistant bacterial strains (48). Therefore, it is important to find new (alternatives for) antibiotics in order to cope with the antibiotic-resistance crisis. Antimicrobial peptides (AMPs) are short and generally positively-charged peptides; they have been isolated from a wide range of sources (49). AMPs generated from food proteins have the advantage of being derived from harmless substances. Table 3 shows the AMPs derived from bovine β-casein. Their antibacterial effect has been examined against a selection of pathogenic bacteria (50) (51) as well as against probiotic bacteria (52) because an inhibitory effect on probiotic bacteria would thwart possible beneficial effects. Indeed, in the latter study, all the investigated β-casein-derived AMPs exhibited antimicrobial activity against the pathogens (*Staphylococcus carnosus*, *Escherichia coli*) as well as the probiotic bacteria (*Lactobacillus casei*, *Lactobacillus acidophilus*).

Table 3

Antimicrobial peptides (AMPs) derived from bovine β-casein

Sequence [a]	Start [b]	End [b]	Antimicrobial activity against [c]	Reference
RINK	40	43	*L. innocua, St. carnosus*	(50)
RINKK	40	44	*E. coli, Lb. innocua, St. carnosus*	(50)
NKKI	42	45	*E. coli, Lb. innocua, St. carnosus*	(50)
VKEAMAPK	113	120	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
EAMAPKHK	115	122	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
EAMAPK	115	120	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
HKEMPFPK	121	128	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
EMPFPK	123	128	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
VLPVPQK	185	191	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
VLPVPQKAVPYPQR	185	198	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
AVPYPQR	192	198	*E. coli, St. aureus, Lb. casei, Lb. acidophilus*	(52)
YQEPVLGPVRGPFPI	208	222	*E. coli*	(51)
YQEPVLGPVRGPFPIIV	208	224	*E. coli*	(51)

[a,b] See legend to Table 1.
[c] *L, Listeria, St, Staphylococcus, E, Escherichia, Lb, Lactobacillus*

Immunomodulatory peptides IMMUNE System

An immunomodulator, according to the Merriam-Webster's dictionary [1], is "a chemical agent that modifies the immune response or the functioning of the immune system". Immunomodulatory drugs such as cyclosporine and glucocorticoids are widely used to autoimmune disease and chronic inflammatory conditions like arthritis, respectively (53). However, adverse effects and high price restrict their application and most immunomodulatory drugs are not appropriate for long-term or preventive uses (54, 55). The discovery of increasingly more novel immunomodulatory peptides from food sources provides a promising strategy for dietary modulation of the immune response (56, 57). A variety of immunopeptides derived from bovine β-casein are presented in Table 4. They can enhance immune cell functions such as lymphocyte proliferation and cytokine regulation. Moreover, they have also been shown to modulate inflammatory and humoral responses.

Table 4

Immunoregulatory peptides derived from bovine β-casein

Sequence [a]	Start [b]	End [b]	Immunomodulatory effect [c]	Reference
RELEELNVPGEIVESLSS-SEESITRINK	16	43	Increase proliferation and IL-6 expression of mouse CD19+ cells; Increases IgA, IL-5, IL-6 expression of mouse spleen cells; Increases the cytokines expression in intestinal epithelial cells	(16, 58, 59)
RELEELNVPGEIVESLSS-SEESITR	16	40	Increase proliferation of mouse spleen cells	(14)
SSS	32	34	Increase proliferation of B-lymphocytes and IgG/IgM/IgA expression of mouse spleen cells	(60)
INKKI	41	45	Antitumor	(61)
YPFPGPI	75	81	Induces pro-inflammatory effect, increases humoral response, infiltration of leucocytes in intestinal villi, and the expression of TLR-2 and TLR-4 in mice gut	(30)
YPFPG	75	79	Induces pro-inflammatory effect, increases humoral response, infiltration of leucocytes in intestinal villi, and the expression of TLR-2 and TLR-4 in mice gut	(30)

1 https://www.merriam-webster.com/dictionary/immunomodulator

Table 4

Immunoregulatory peptides derived from bovine β-casein

Sequence [a]	Start [b]	End [b]	Immunomodulatory effect [c]	Reference
PGPIPN	78	83	Inhibits proliferation of human ovarian cancer cell line SKOV3 and the primary ovarian cancer cells in vitro, decreases tumor growth rate in xenograft ovarian cancer model mice in a dose-dependent manner	(62)
LYQEPVLGPVRGPFPIIV	207	224	Increases proliferation in lymph node and spleen cells of rat	(63)
YQEPVLGPVRGPFPIIV	208	224	Enhances antimicrobial activity of microphases without pro-inflammatory effects for mice	(64)
QEPVL	209	213	Increases lymphocytes proliferation rates and cyclic AMP levels in vitro and in vivo (mice). Inhibits LPS-induced inflammation, cytokines, and TNF-α in vivo.	(65)
QEPV	209	212	Increases lymphocytes proliferation rates and cyclic AMP levels in vitro and in vivo (mice). Inhibits LPS-induced inflammation, cytokines, and TNF-α in vivo.	(65)

[a, b] See legend to Table 1.
[c] Key immunoregulatory effect of peptide. Abbreviations: Ig, Immunoglobulin; IL-6: Interleukin 6; TLR: Toll-like receptor; BCL2: B-cell lymphoma 2; cAMP: cyclic adenosine monophosphate;

ACE-inhibitory peptides CARDIO System

Hypertension is a "silent killer", because persons with hypertension are often asymptomatic for years before they suffer from the hypertension-associated diseases such as hypertensive heart disease, stroke, obesity, and dementia (66, 67). Angiotensin-I-Converting Enzyme (ACE) plays a major role in blood pressure regulation by converting the inactive decapeptide Angiotensin-I to the potent vasoconstrictor octapeptide Angiotensin-II. Consequently, the blood pressure rises (68). Therefore, ACE inhibitors are helpful agents in the therapeutic treatment of hypertension. Synthetic ACE inhibitors such as captopril and enalapril are used extensively but they have various adverse side effects such as rash, taste disturbance, an abnormally low concentration of neutrophils (neutropenia) and more (69).

A recent trend is to look for natural ACE inhibitor with milk being an adequate source for ACE inhibitory peptides. More than half of the bioactive peptides derived from bovine β-casein possess ACE-I activity (Fig 2A). After reviewing all literature on β-casein-derived

ACE-I peptides, only those recording an IC50 value were further examined here. Figs 2B and 2C represent the proportion and distribution of the ACE-I peptides' IC50 values, respectively. More than half (56%) of the peptides possess an IC50 less than 100 μM while less than 10% have an IC50 between 500-1000 μM. Fig 2D shows the location of each ACE-I peptide in the bovine β-casein precursor peptide. Peptide KVLILA is part of the signal peptide region (f(1-15). It was picked and synthesized after a virtual screening of an in-house database containing more than 2000 milk casein-derived peptides (70). Except for peptide RINKK, all other ACE-I peptides derive from β-casein f(61-224). Around one third of the peptides are embedded in f(61-92) while the rest is evenly spread over f(93-224). The most studied ACE-I tripeptides, VPP and IPP, were first reported by Nakamura et al. (71) in 1995. These authors used Saccharomyces cerevisiae and Lb. helveticus CP790 to ferment skim milk and the supernatant was used for ACE-I testing. Two peaks containing high ACE-I activity were further purified and, after a 4-step HPLC purification, the two peptides were identified and their IC50 were measured (9 μM for VPP and 5 μM for IPP). These two active peptides are commercially available in Calpis sour milk (Calpis Co., Japan) and Evolus® (Valio, Finland) (72). The most potent peptide is MAP, which has an IC50 of 0.8 μM. It was isolated from enzyme-modified cheese (EMC). The EMC was prepared from Danish skim-milk cheese, which was mixed with *Lactococcus* starter culture, Protease N, Umamizyme, and Flavourzyme 500L. The peptide MAP was identified and showed strong ACE-I activity and antihypertensive activity in spontaneously hypertensive rats (SHR) after a single oral administration at a dose of 3 mg/kg body weight (73).

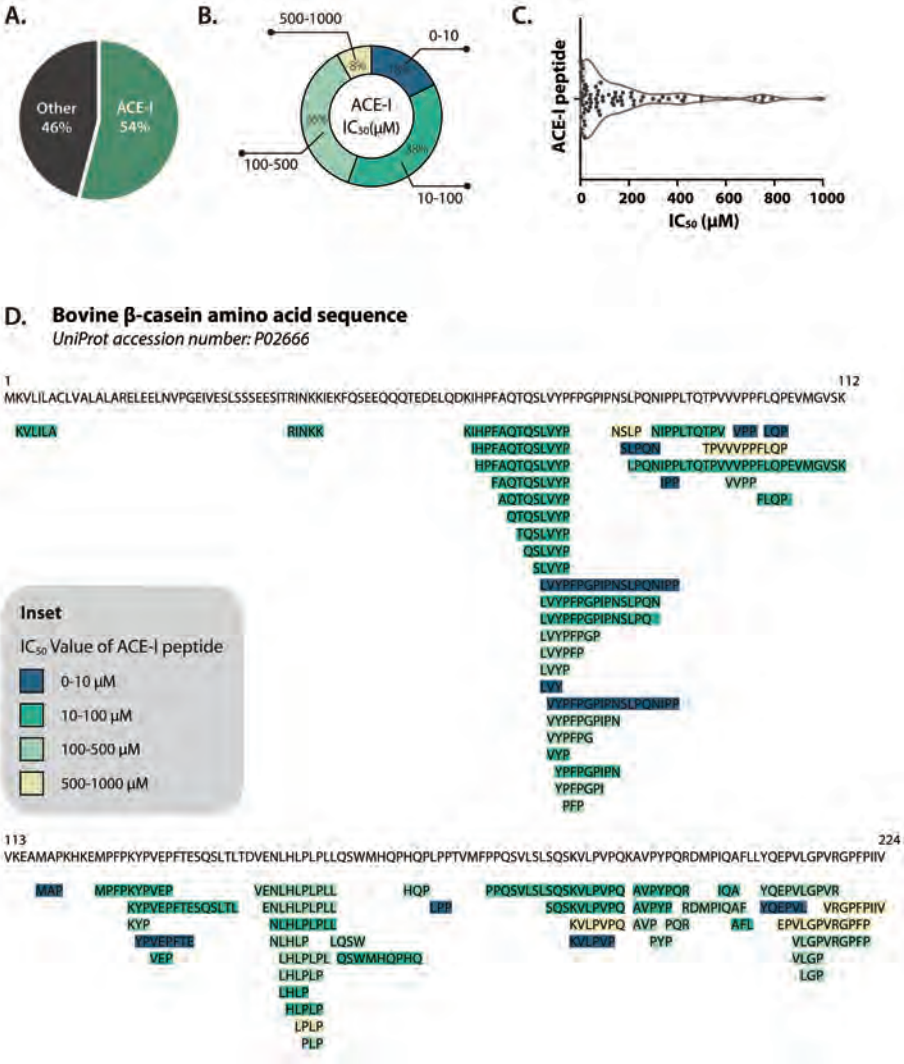

Figure 2 A) Pie chart showing the proportion of Angiotensin-I-Converting Enzyme inhibitory (ACE-I) peptides among all bovine β-casein-derived bioactive peptides. B) Doughnut chart visualizing the proportion of the half maximal inhibitory concentration (IC50) of all bovine β-casein-derived ACE-I peptides. C) Violin plot of the IC50 value distribution of all bovine β-casein-derived ACE-I peptides. D) Alignment all bovine β-casein-derived ACE-I peptides with bovine β-casein. Color of each peptide is based on its IC50 value, as indicated in the inset.

Other functional peptides

In addition to the functionalities mentioned above, bovine β-casein-derived peptides possess various other biological activities such as antioxidative (74), prolyl endopeptidase-inhibitory (75), and anticoagulant effects (76). We combined the bovine β-casein-derived bioactive peptides from three most popular milk-derived bioactive peptides databases: BIOPEP (77), MBPDB (78), and EROP-Moscow (79), excluding entries without reference or with wrong data. For instance, peptides PGP, PHQ, FPPQS, VLP, GPV are recorded only on BIOPEP but not in MBPDB and EROP-Moscow, and no reference literature can be found for those 5 peptides, thus we omitted them from our customized database. As for wrong data, peptide LVYPFPGPIP was recorded in MBPDB as having ACE-I activity, but in the reference literature that MBPDB provides, the ACE-I activity is actually for LVYPFPGPIH (the last amino acid residue in the C-terminal is not P but H). After excluding all those entries ultimately allowed selecting 136 of 176 bioactive peptides; they are all listed in Table 5.

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
1	KVLILA	2	7	ACE-I	(70)
2	RELEELNVPGEIVESLSS-SEESITRINK	16	43	Immuno-R	(15, 16, 57–60, 80–82)
3	RELEELNVPGEIVESLSS-SEESITR	16	40	Immuno-R	(12–14, 83)
4	LNVPGEIVE	21	29	ACE-I	(84)
5	VPGEIVE	23	29	DPP-IV-I	(32)
6	RINKK	40	44	ACE-I; Anti-M	(50, 85)
7	RINK	40	43	Anti-M	(50)
8	INKKI	41	45	Immuno-R; Anti-C	(61, 86)
9	NKKI	42	45	Anti-M	(50)
10	DELQDKIHPFAQTQSLVYP-FPGPIPNS	58	84	ACE-I	(87)
11	DKIHPF	62	67	ACE-I	(84)
12	KIHPFAQTQSLVYP	63	76	ACE-I	(88)
13	IHPFAQTQ	64	71	PEP-I	(89)
14	IHPFAQTQSLVYP	64	76	ACE-I	(88)

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
15	HPFAQTQSLVYP	65	76	ACE-I	(88)
16	FAQTQSLVYP	67	76	ACE-I	(88)
17	AQTQSLVYP	68	76	ACE-I	(88)
18	QTQSLVYP	69	76	ACE-I	(88)
19	TQSLVYP	70	76	ACE-I	(88)
20	QSLVYP	71	76	ACE-I	(88)
21	SLVYP	72	76	ACE-I	(88)
22	LVYPFPGPIPNSLPQNIPP	73	91	ACE-I	(90–92)
23	LVYPFPGPIPNSLPQN	73	88	ACE-I	(93)
24	LVYPFPGPIPNSLPQ	73	87	PEP-I	(75)
25	LVYPFPGP	73	80	ACE-I	(90)
26	LVYPFP	73	78	ACE-I	(94)
27	LVYP	73	76	ACE-I	(88)
28	LVY	73	75	ACE-I	(95)
29	VYPFPGPIP	74	82	PEP-I	(89)
30	VYPFPGPI	74	81	PEP-I	(89)
31	VYPFPGPIPN	74	83	ACE-I	(96)
32	VYPFPGPIPNSLPQNIPP	74	91	ACE-I	(91)
33	VYPFPG	74	79	ACE-I	(97)
34	VYP	74	76	ACE-I	(88, 97)
35	YPFPGPIP	75	82	Opioid; ACE-I	(39, 40)
36	YPFPGPIPNSL	75	85	Opioid	(43, 98)
37	YPFPGPI	75	81	Opioid; Satiety-Ic; Immuno-R; Anxiolytic; Anti-C	(19, 20, 99–102, 21, 27–30, 34, 35, 37)
38	YPFPG	75	79	Opioid; PNO; IM-Ic; Immuno-R; L&M-Ip	(19, 24–26, 29, 30)
39	YPFPGP	75	80	Opioid; DPP-IV-I	(19, 27, 31, 32)
40	YPFP	75	78	Opioid; Anti-C	(28, 31, 103, 104)
41	YPFPGPIPN	75	83	ACE-I; DPP-IV-I; Opioid	(33, 41, 42, 96)
42	YPFPGPIPNSLPQ	75	87	Opioid	(33)
43	YPFPGPIPNSLPQNIPPLTQT	75	95	Opioid	(33)

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
44	PFPGPI	76	81	Cathepsin B-I	(105)
45	FPGPIPN	77	83	DPP-IV-I	(42)
46	PGPIPN	78	83	Immuno-R; Anti-C	(62, 106–113)
47	NSLP	83	86	ACE-I	(114)
48	SLPQN	84	88	ACE-I	(85)
49	LPQNIPPL	85	92	DPP-IV-I	(42)
50	LPQNIPPLT	85	93	DPP-IV-I	(32)
51	LPQNIPP	85	91	DPP-IV-I	(42)
52	LPQNIPPLTQTPVVVP-PFLQPEVMGVSK	85	112	ACE-I	(87)
53	LPQ	85	87	DPP-IV-I	(42)
54	PQNIPPL	86	92	DPP-IV-I	(42)
55	NIPPLTQTPV	88	97	ACE-I	(84)
56	IPPLTQT	89	95	DPP-IV-I	(32)
57	IPP	89	91	ACE-I	(71)
58	LTQTPVVVPPF	92	102	ACE-I	(92, 115)
59	TQTPVVVPPFLQPE	93	106	Anti-O	(74)
60	TPVVVPPFLQP	95	105	ACE-I	(97)
61	VVVPPF	97	102	ACE-I	(92)
62	VVPP	98	101	ACE-I	(70)
63	VPP	99	101	ACE-I; Anti-Infla; Boneloss-R	(71, 85, 124, 116–123)
64	FLQP	102	105	ACE-I; DPP-IV-I	(45, 114)
65	LQP	103	105	ACE-I	(73)
66	GVSKVKEAMAPKHKEMPF-PKYPVEPFTESQ	109	138	Opioid; MUC-Ic	(125–128)
67	VKEAMAPK	113	120	Anti-O; Anti-M	(52, 129)
68	EAMAPKHK	115	122	Anti-M	(52)
69	EAMAPK	115	120	Anti-M	(52)
70	MAP	117	119	ACE-I	(73, 114)
71	HKEMPFPK	121	128	Anti-M	(52, 130, 131)
72	EMPFPK	123	128	MUC-Ic; Anti-M; Anti-M; Brad-P	(52, 126, 132, 133)

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
73	MPFPKYPVEP	124	133	ACE-I	(134)
74	KYPVEPFTESQSLTL	128	142	ACE-I	(87)
75	KYP	128	130	ACE-I	(114)
76	YPVEPF	129	134	Opioid; DPP-IV-I; MUC-Ic	(32, 33, 46, 126)
77	YPVEPFTE	129	136	ACE-I; Brad-P	(133)
78	VEP	131	133	ACE-I	(114)
79	HLPLP	140	144	ACE-I	(88, 135, 136)
80	LPLP	143	146	ACE-I	(88)
81	VENLHLPLPLL	145	155	ACE-I	(137)
82	ENLHLPLPLL	146	155	ACE-I	(137)
83	NLHLP	147	151	ACE-I	(88)
84	NLHLPLPLL	147	155	ACE-I	(137)
85	LHLP	148	151	ACE-I	(88)
86	LHLPLPL	148	154	ACE-I	(92)
87	LHLPLP	148	153	ACE-I	(92)
88	LPLPLL	150	155	DPP-IV-I	(32)
89	LPLPL	150	154	DPP-IV-I	(32, 47)
90	LPL	150	152	DPP-IV-I	(47)
91	LQSW	155	158	ACE-I	(138)
92	QSWMHQPHQ	156	164	ACE-I	(139)
93	HQP	163	165	ACE-I	(114)
94	PLP	165	167	ACE-I	(88)
95	LPP	166	168	ACE-I	(114)
96	PPQSVLSLSQSKVLPVPQ	173	190	ACE-I	(87)
97	SQSKVLPVPQ	181	190	ACE-I	(134)
98	SKVLPVPQ	183	190	ACE-I	(87)
99	KVLPVPQK	184	191	Anti-O	(129)
100	KVLPVPQ	184	190	ACE-I	(138)
101	KVLPVP	184	189	ACE-I	(138, 140)
102	VLPVPQK	185	191	Anti-O; Anti-M	(52, 129)

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
103	VLPVPQKAVPYPQR	185	198	Anti-M	(52)
104	LPVPQ	186	190	DPP-IV-I	(32)
105	LPVP	186	189	DPP-IV-I	(46)
106	AVPYPQR	192	198	ACE-I; Anti-O; Anti-M	(52, 129, 132, 141, 142)
107	AVPYP	192	196	ACE-I	(143)
108	AVP	192	194	ACE-I	(143)
109	VPYPQ	193	197	Anti-O	(144)
110	PYPQ	194	197	Anti-O	(145)
111	PYP	194	196	ACE-I	(143)
112	PQR	196	198	ACE-I	(146)
113	RDMPIQAF	198	205	ACE-I	(87)
114	DMPIQAFLLYQEPVLGPVR	199	217	Anti-Infla	(147)
115	IQA	202	204	ACE-I	(114)
116	AFL	204	206	ACE-I	(148)
117	LLYQEPVLGPVRGPFPIIV	206	224	ACE-I	(87)
118	LLY	206	208	Immuno-R	(149)
119	LYQEPVLGPVRGPFPIIV	207	224	Immuno-R	(63)
120	YQEPVLGPVRGPFPI	208	222	Anti-M	(51)
121	YQEPVLGPVRGPFPIIV	208	224	ACE-I; Anti-M; Immuno-R	(51, 64, 87, 150)
122	YQEPVLGPVR	208	217	ACE-I; Anti-O; Anti-Infla; Anti-Co	(76, 151, 152)
123	YQEPVL	208	213	ACE-I	(85, 132)
124	QEPVL	209	213	Immuno-R	(65)
125	QEPV	209	212	Immuno-R	(65)
126	QEPVLGPVRGPFPIIV	209	224	ACE-I	(151)
127	EPVLGPVRGP	210	219	Cyto-M	(153)
128	EPVLGPVRGPFP	210	221	ACE-I	(134)
129	VLGP	212	215	ACE-I; DPP-IV-I	(45, 114)
130	VLGPVRGPFP	212	221	ACE-I	(92)
131	LGP	213	215	ACE-I	(154, 155)

Table 5

Selection of 136 certified bioactive peptides derived from bovine β-casein

NO	Sequence [a]	Start [b]	End [b]	Bioactivity [c]	Reference
132	VRGPFPIIV	216	224	ACE-I	(92)
133	VRGPFP	216	221	ACE-I	(156)
134	GPFPIIV	218	224	ACE-I	(157, 158)
135	GPFPI	218	222	Cathepsin B-I	(105)
136	PFP	219	221	ACE-I	(159)

a,b See legend to Table 1.
C Bioactivities of indicated peptide. Abbreviations: ACE-I, angiotensin-converting-enzyme inhibitory; Anti-M, antimicrobial; Anti-O, antioxidative; DPP-IV-I, dipeptidyl peptidase 4 inhibitory; Immuno-R, immunoregulatory; Anti-C, anticancer; PEP-I, prolyl endopeptidase-inhibitory; Satiety-Ic, Satiety Increasement; IM-Ic, Intestinal Mobility Increasement; PNO, Promoting Neurite Outgrowth; L&M-Ip, Learning and Memory Improvement; Cathepsin B-I, Cathepsin B inhibitory; Inflam-P, Inflammation Promotion; Boneloss-R, Bone loss reduction; Anti-Infla, Anti-inflammatory; MUC-Ic, MUC expression increasement; Brad-P, Bradykinin-Potentiating; Anti-Co, Anticoagulant; Cyto-M, Cytomodulatory.

Lactic acid bacteria: promising bioactive peptide producers

Lactic acid bacteria (LAB) are a group of Gram-positive, low GC-content bacteria that are widely used in fermented foods such as derived from milk, meat, fish and vegetables and are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (160, 161). Since the first industrial use of LAB as starter culture for the production of cheese and sour milk in 1890s, their economic importance has gradually and steadily increased and, as a consequence, they have been studied comprehensively (162). Besides their food preservative action by the lactic acid they produce from sugars (lactose in the case of the dairy strains) they add texture and flavor to dairy products. LAB have also been used as cell factories, producing chemicals such as lactic acid or mannitol (163), and are considered as delivery vehicles for therapeutic treatment (164). Fermentation, however was and still is the dominating application for LAB. Not only do these organisms provide nutritional value to the fermented products, they also have great potential as production organisms for bioactive peptides, such as antihypertensive peptides, antimicrobial peptides, opioid peptides (165).

LAB proteolytic system for casein degradation

Dairy LAB utilize a proteolytic system to degrade casein to obtain amino acids that are essential for cell growth. At the same time, the liberated amino acids and small peptides

contribute to the organoleptic quality of the fermented milk. The proteolytic system of *Lactococcus lactis*, the most studied of the LAB, has been extensively scrutinized (160, 166): Casein degradation is initiated by a cell-envelope proteinase, which hydrolyses the extracellular protein (β-casein in this case) into oligopeptides of varying length. Oligopeptides are subsequently taken up by the cells via an oligopeptide transporter system and further degraded in the cytoplasm by a series of peptidases into shorter peptides and amino acids. Unused amino acids and peptides are released into the external environment during the growth and after the cells have autolyzed (11). Some of the peptides (might) possess biological activities.

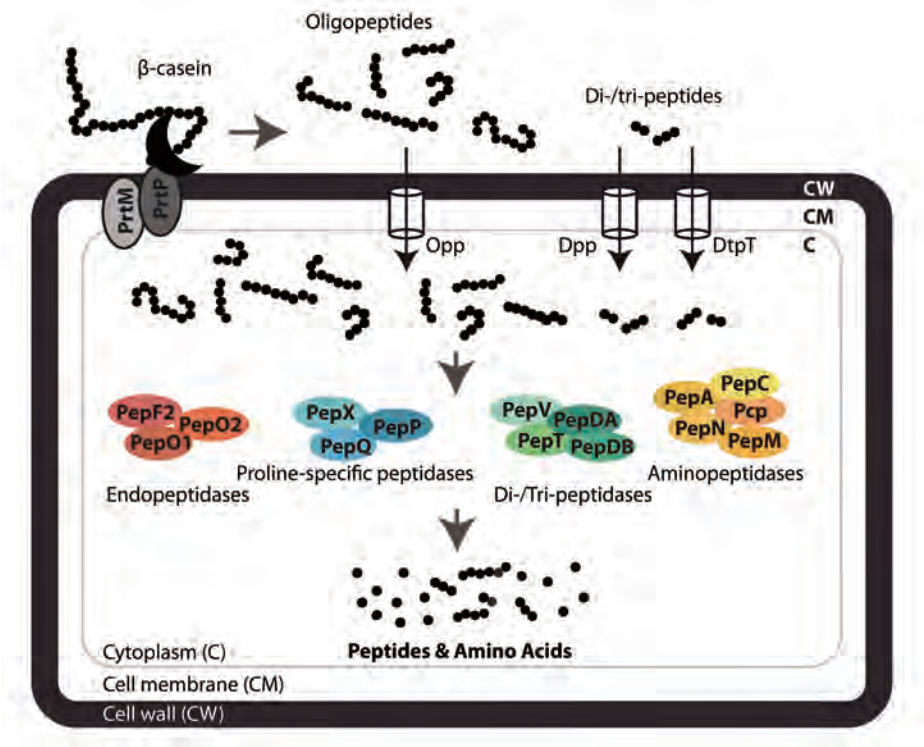

Figure 3 Schematic representation of the proteolytic system of *Lc. lactis* MG1363 carrying the cell envelope-associated proteinase/maturase genes *prtP/M* on a plasmid. β-casein hydrolysis is initiated by PrtP, after its autoproteolytic activation with the aid of PrtM (167). Subsequently, oligopeptides are transported into the cell by the oligopeptide permease Opp while di-/tripeptides are internalized by the Dpp or DtpT transport system (168). The peptides are then degraded into smaller peptides and free amino acids by the concerted action of 15 peptidases, which are classified and colored by their indicated cleavage specificity (169).

Bioactive peptides produced by LAB

Certain species of LAB are able to release bioactive peptide from food protein, particularly from milk caseins. The hydrolytic ability and the bioactivity obtained depends on the proteolytic enzymes of the strains and, of course, on the amino acid sequence of different type of caseins. *Lb. helveticus* PR4 can liberate an antimicrobial peptide from human β-casein that is active against a large spectrum of Gram-positive and Gram-negative bacteria, such as *Enterococcus faecium, Bacillus megaterium, E. coli, L. innocua, Salmonella spp., Yersinia enterocolitica,* and *St. aureus* (90). Antioxidative activity was found in *Bifidobacterium bifidum* MF 20/5 (isolated from Bion-3® Japan) fermented milk; the antioxidative peptide was identified to be VLPVPQK (94). More antioxidative peptides were identified in a Gouda-type cheese: using liquid chromatography, four peptides, all with X-Pro at their N terminal end, showed IC50 values of < 200 μM. The peptide LPQNIPPL showed the highest antioxidative activity (42). LAB can also liberate immunomodulatory peptides. Immunomodulating effect was found in *Lb. helveticus* LH-2 fermented milk. The cell-free fermented milk and its fractions were tested in vitro for immunomodulating activity using murine macrophages. The fraction stimulating the macrophages the most was further analyzed and four novel peptides were identified (170). Furthermore, *Enterococcus faecalis* CECT5727 and BCS27, *Lb. delbrueckii, Lb. helveticus, Sa. cerevisiae, Lb. delbrueckii* subsp. *bulgaricus SS1, Lb. delbrueckii* subsp. *lactis*, and *Lc. lactis* subsp. *cremoris* FT4 can modulate blood pressure by producing ACE-I peptides from milk proteins (Table 5) (72, 84).

Casein-derived bioactive peptides can be liberated by LAB proteolysis; they may also be further degraded into smaller, non-functional peptides or free amino acids if the fermentation process continues. Thus, a proper balance is required between the initial proteolysis liberating certain bioactive peptides and their further degradation. On the other hand, initially liberated non-bioactive peptides may become functional upon subsequent peptidolytic activity. To liberate a desired bioactive peptide, a certain combination of peptidolytic activities must be overexpressed, inhibited, or removed. To be able to do this, in-depth knowledge of the proteinase and intracellular peptidase activities and genes is needed. In fact, such knowhow has been obtained in the extensively studied LAB *Lc. lactis* (see above). Based on this knowledge, directed engineering of LAB proteolytic system would be the next step. Various genome engineering tools for LAB has been developed over the past decades (171): Regulated (over)expression of genes in *Lc. lactis* can be achieved using the NIsin-Controlled Expression (NICE) system, which

is the most widely used inducible gene expression system in *Lc. lactis*. The NICE system is based on the quorum sensor two-component system composed by NisR and NisK. The system responds to the presence of the inducer nisin, an antimicrobial peptide produced by certain strains of Lc. lactis (172). Gene knockout can be accomplished in *Lc. lactis* by the double crossover method using the non/replicative pORI integration vector (173) or plasmid pCS1966 (174). Besides these traditional methods, novel genome engineering technologies have also been established in LAB, such as the bacteriophage-derived recombinase enzymes for single-stranded DNA recombineering in *Lb. reuteri* and *Lc. lactis* (175), and the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated system (Cas) CRISPR/Cas-based method, which probably the most powerful and popular gene editing tool in the past decade, especially for eukaryotic organisms for which other (targeted) approaches are lacking (176).

LAB Engineering using CRISPR/Cas

CRISPR/Cas is an RNA-mediated adaptive immune system found in bacteria and archaea. It protects host cells from invasive genetic elements such as bacteriophages and plasmids (177). In-depth knowledge of the mechanism of action of various of these systems has allowed creating valuable tools for gene editing, both in prokaryotic and in (higher) eukaryotic organisms. The type-II CRISPR/Cas engineering system uses Cas9 from *Streptococcus pyogenes*. Cas9 is an endonuclease that is guided by a single-guide RNA (sgRNA) that specifically hybridizes to its homologous target sequence in DNA and induces a double-strand DNA break (DSB). The binding specificity is determined by the sgRNA-DNA base pairing and the protospacer adjacent motif (PAM) sequence (in *S. pyogenes* this is NGG) (178). Using an engineered nuclease-deficient Cas9, i.e. dCas9, enables repurposing of the system: dCas9 still forms a complex with the sgRNA and binds to its specific DNA target, but instead of making a DSB, it works as a "road block" preventing access or passage of other DNA binding proteins. Positioned at the proper position on DNA, it could lead to gene silencing (CRISPRi) by preventing access of RNA polymerase to its promoter (occluding transcription initiation) or by precluding transcript elongation (179) (Fig 4).

The CRISPR/Cas9 system has been applied in various bacterial genera, such as *Bacillus, Escherichia, Clostridium,* and *Streptococcus* (180–183). As for LAB, Cas9-induced DSB was applied in an CRISPR/Cas9-assisted ssDNA recombineering system in *Lb. reuteri* (184).

Berlec et al. constructed an inducible dual-promoter plasmid in *Lc. lactis*. Based on the NICE system (see above), this plasmid contains the dCas9 and sgRNA genes under the nisin-inducible promoter PnisA. It was successfully employed for transcriptional repression of a target gene, htrA (185). *Lc. lactis* genome editing mainly relies on the RecA-dependent homologous double-crossover events using non-replicative or conditionally replicative plasmids (174, 186). The protocols for gene knockout or gene insertion are generally performed in two steps (vector integration and subsequent co-integrate resolution) and usually consume 3 weeks to generate one mutant. The marker-free strain obtained can then be used in a next round such that multiple genes can be mutated in the same strain. A drawback is the extended time required to obtain a multiple mutant strain.

Understanding and utilizing the powerful CRISPR/Cas based tool to engineer the proteolytic system of *Lc. lactis* could in principle save both time and labor. Deploying CRISPR/dCas9 (instead of CRISPR/Cas9) is more suitable for *Lc. lactis* because it cannot repair double-strand breaks in DNA due to the lack of a non-homologous end joining (NHEJ) pathway. Although knocking down multiple genes by CRISPR/dCas9 can be fast, the extent to which the gene(s) have been silenced cannot be a priori ensured. Thus, clean multiple knockouts should also be processed in parallel, in order to obtain stable strains and to evaluate and compare the efficiency and effectiveness of both methods.

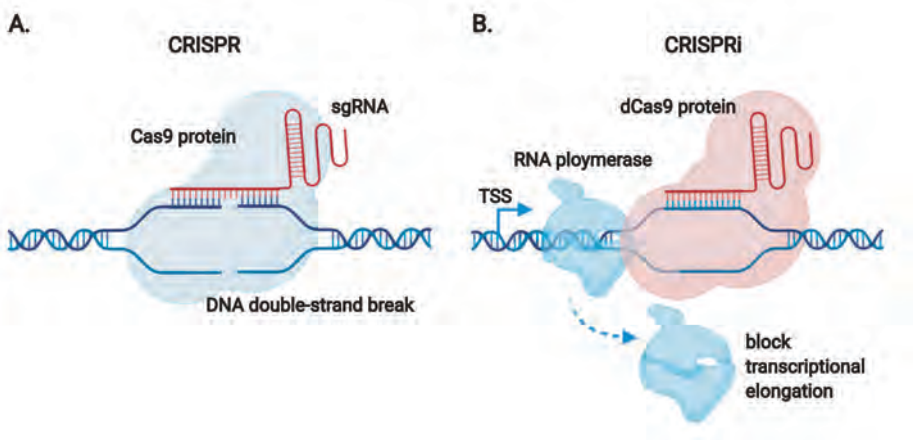

Figure 4 Gene editing (knockout) versus gene inactivation (knockdown) using *S. pyogenes* Cas9 and dCas9 (adapted from Qi et al. (179)). A) The *S. pyogenes* Cas9 enzyme binds to single-guide RNA (sgRNA) molecule and forms a protein-RNA complex. The complex is targeted to specific DNA sequences by direct pairing of the sgRNA with the target DNA. These interactions result in the cleavage of the double-strand target DNA. B) The S. pyogenes dCas9 protein contains mutations in

its RuvC1 (D10A) and HNH (H841A) domains, which inactivate its nuclease function (179). dCas9 is still able to form a complex with the sgRNA and bind to its specific DNA target. When dCas9-sgRNA complex binds downstream of the transcription start site (TSS), it can block transcription elongation by blocking RNA polymerase and transcript elongation. [1]

Thesis outline

This thesis focuses on *Lc. lactis* proteolytic system engineering and its application in milk-derived bioactive peptide discovery. In Chapter 1, we give a comprehensive review of functionalities of bovine β-casein-derived bioactive peptides, the ability that LAB have in producing bioactive peptides during the fermentation process, and the possibility of deploying CRISPR/Cas-based technology in LAB genome engineering. In Chapter 2, we designed and prepared a gene knockout plasmid library for 16 intracellular peptidases and constructed a variety of single- and multiple-peptidase knockout mutants. The two major goals were: A) to knockout as many peptidase genes as possible; B) to knockout peptidases from the same specificity group, i.e., all endopeptidases, aminopeptidases, di-/tripeptidases, or all proline-specific peptidases. Ultimately, we obtained two peptidase-mutants in which 7 peptidase genes were removed and 4 mutants in which all genes of one of the above-mentioned groups of peptidases were deleted, respectively. We then developed an analytical pipeline for large-scale intracellular peptidomics of *Lc. lactis*. The six multiple-peptidase mutants that we obtained were examined using this pipeline, and a strain-dependent accumulation of bioactive peptides was observed. This work suggests that both the number of different bioactive peptides and the bioactivity diversity can be increased by genetically editing the proteolytic system of *Lc. lactis*.

On the way of constructing the di-/tripeptidase mutant, an interesting observation was made: the dipeptidase mutant MG*ΔpepV* does not grow overnight in SMGG growth medium . In Chapter 3, we explored the reason behind this phenomenon. The proteolytic system plays a vital role in *Lc. lactis* nitrogen metabolism with the dipeptidase PepV functioning in the last stages of proteolysis. A link between nitrogen metabolism and peptidoglycan (PG) biosynthesis was underlined by the phenotype (cell lysis and shape defects when grew in SMGG overnight) of MG*ΔpepV*. This phenotype was shown to be caused by a shortage of alanine because adding alanine can rescue the growth and restore the defects in cell shape. Moreover, strain MG*ΔpepV* is more resistant to vancomycin, an antibiotic targeting PG D-Ala–D-Ala ends, which confirmed that MG*ΔpepV* has an abnormal PG composition. A mutant of MG*ΔpepV* was obtained

1 The schematic figure was created with BioRender.com under Academic License.

in which growth inhibition and cell shape defects were alleviated. The strain was shown by genome sequencing to have a single point mutation, in the gene for the master regulator CodY, relative to its parent. Transcriptome sequencing (RNA-seq) was used to unravel the connections between PepV activity, CodY regulation, and PG synthesis in *Lc. lactis*.

As mentioned above, we constructed the several multi-peptidase knockout mutants. We used the traditional homologous double-crossover recombination method, which is quite labor intense and time consuming. If multiple peptidases can be knocked out or knocked down in a shorter time frame then more combinations of abrogated peptidase activities could be tested and further examined by the peptidomics pipeline we developed. Thus, in Chapter 4, we built both a chromosome-based and a plasmid-based CRISPR interference platform, useful for future applications in *Lc. lactis* genome engineering. We used the NICE system to drive the expression of dCas9 and the constitutive lactococcal protomer Pusp45 to control the production of several sgRNAs. Both in-fusion cloning and golden gate assembly methods can be used for fast replacement of the 20-base pair sgRNA targeting sequence in the vector. We designed sgRNA against genes which are involved in *Lc. Lactis* cell autolysis (*acmA*), cell division (*ftsZ*), or cell wall elongation (*pbp2b*). We observed all corresponding phenotypes (long chains that are also seen in deletion mutants of *acmA/ftsZ*, and spherical shapes for the *pbp2b* targeted cells). In Chapter 5, we deployed the CRISPRi system to help study the function of the enigmatic Usp45, the major secreted protein of *Lc. lactis*. The usp45 gene cannot be deleted via double crossover recombination as the gene is, apparently, essential. We used CRISPRi to gradually knockdown *usp45*. By examining the effect of knockdown and overexpression of *usp45* on *Lc. lactis* growth, phenotype and cell division we demonstrated that *Lc. lactis* Usp45 is crucial for proper cell division. The protein mediates cell separation probably by acting as a peptidoglycan hydrolase.

Chapter 6 summarizes and discusses the most important findings and future perspectives of the work described in this thesis.

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