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Summary, general discussion, and future perspective

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The goal of this thesis was to answer one question in particular: can one increase the quantity or the diversity (or both) of milk-derived bioactive peptides by engineering the *Lactococcus lactis* proteolytic system? The main research line explored that question itself. In addition, the three issues that derive from that main questions are separately tackled in this thesis: (i) bioactive peptides: How much do we know about bioactive peptides derived from milk-derived and, more specifically, beta-casein? (ii) *L. lactis* engineering: Which tools are there and are they good enough or can we develop new/better ones; (iii) 3. The *L. lactis* proteolytic system: what do we know about that system and, especially, what do we know about the in vivo (complementing) activities of the peptidases with respect to cellular growth and peptide degradation Chapter 1 comprehensively reviews the knowhow on β-casein-derived bioactive peptides and the potential of using lactic acid bacteria to produce such peptides; In Chapter 2, which is the founding chapter of the thesis, we engineered the *L. lactis* proteolytic system by making a large collection of various combinations of peptidase gene mutants and used those mutants to increase the quantity of different bioactive peptides and the diversity of different bioactivities which derived from β-casein; In Chapter 3, we broaden the knowledge about the *L. lactis* proteolytic system, and prove that the dipeptidase PepV plays an important role in peptidoglycan biosynthesis by acting as a link between nitrogen metabolism and cell wall synthesis. In Chapter 4, we expand the genetic tool box for *L. lactis* by developing plasmid- and genome-based CRISPRi systems that will allow rapidly e.g., editing biological pathways or characterizing essential genes, as was explored in Chapter 5.

THE ART OF BALANCING

Balancing is an importance aspect in biology. At a single-cell level, maintaining cell homeostasis requires balancing in every aspect: activation and inhibition of pathways, producing and consuming of chemicals or energy, etc. At the organismal level, for prokaryotes, proper cell division is vital for their survival, whereas programmed cell death is also essential: a balance between cell division and cell death is crucial for maintaining a glorious community for any species of microorganism. For animals, including human beings, the immune system helps defend against viral invasion: a balance of the immune response is key to upholding a proper health status. An excessive immune response may lead to allergy or to other medical problems such as a cytokine storm, whereas an insufficient immune response could lead to immunodeficiency.

Peptidase depletion and bioactive peptides production

When planning to generate an array of peptidase mutants, one might think that deletion of more peptidase genes would result in the generation of more bioactive peptides. Of course, one would need to carefully balance between leaving enough interesting peptides intact that might be bioactive and still "feeding" the bacteria with enough peptide-derived amino acids for proper growth. Indeed, in Chapter 2 we show that when deleting the genes of peptidases with the same function (*L. lactis* MGΔ*pepXPQ* and MGΔ*pepVD$_A$TD$_B$*), there are actually less peptides in the peptidomes than in that of their parent strain MG1363. This might be because they produce less peptides due to the lack of peptidase activity or because their growth was severely affected (unpublished data). If the latter is the case, this could be amended by adding amino acids that these mutants require (e.g. MGΔ*pepVD$_A$TD$_B$* will need alanine), or simply by adding all 20 amino acids to the media. One might in this way recover the growth of the two mutants, and expect a di-/tripeptides-rich or a proline-rich intracellular peptide pool, respectively.

From an application point of view, we are only in the screening stage. For some of the interesting mutants, e.g. endopeptidase mutant MGΔ*pepNXOTCF$_2$O$_2$*, we actually tried to test their angiotensin-converting-enzyme inhibitory (ACE-I) activity. The amount of intracellular peptidome obtained in Chapter 2 (starting with 50 ml of cell culture (OD600 = 0.7)), however, is way below the detecting limit of the ACE Activity Assay Kit (data not shown). We calculated that at least a 100-fold increase of cell culture would be needed for proper testing. Subsequently, examination in model animals (mouse, rabbit) of the peptidase-mutant-casein fermentation product and the synthesized peptides of interest should be performed before an industrial level application can be considered.

Competent or lysis: glycine and alanine competition during *L. lactis* peptidoglycan synthesis

Proper balancing of cell wall synthesis and repair/breakdown is crucial for bacteria in order to maintain cell shape and integrity as well as resist stresses. Glycine can weaken the cell wall of *L. lactis* by replacing the alanine residues in the peptidoglycan precursor, leading to improper cell wall synthesis. Addition of glycine to growing *L. lactis* cells is a commonly used procedure to make *L. lactis* electrocompetent cells. In Chapter 2, we observed an interesting phenotype during the sequential introduction of pep mutations for the construction of the di-tripeptidase mutant (MGΔ*pepVD$_A$TD$_B$*): the dipeptidase mutant MGΔ*pepV* does not grow overnight in SMGG medium, containing glycine, when inoculated directly from a glycerol stock. None of the other 14 single peptidase

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knockout mutants had that problem. The reason behind the phenomenon was further explored in Chapter 3. It appeared to be the fact that PepV is a main contributor to the intracellular alanine pool. Depletion of PepV helped glycine win the competition and take over the positions in peptidoglycan that are normally taken alanine. Apparently, none of the other peptidases in *L. lactis* MG1363, notably the aminopeptidases (PepN, PepC, PepA, Pcp) can take over that role. Although neither the dipeptidase PepV nor alanine is essential for *L. lactis* MG1363, adequate availability of either should ensure proper cell wall synthesis.

The interplay between biology and technology

New biological techniques often generate major biological discoveries, which, in turn, often lead again to new technological developments (1). Microbiology truly began with the discovery of microorganisms by Robert Hooke (in 1665) and Antoni van Leeuwenhoek (in 1670s) using microscopes. After a lapse of more than 350 years, microscopy became the backbone of microbiology and medical biology (2). Another example would be the arguably most important technique in molecular biology over the past half century: the polymerase chain reaction (PCR). None of us could image modern biology without PCR anymore. In addition, PCR has made its way into the world outside the laboratory: forensics, evolutionary studies, clinical applications, virus testing, etc. (3). The rapid development of high-performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS) has started and fueled the field of proteomics and peptidomics. For the work presented in this thesis, the deployment of HPLC-MS/MS has been the driver for the results presented in Chapter 2. The CRISPR (clustered regularly interspaced short palindromic repeats)-based gene technology is one of the most exciting recent discovery that is extremely rapidly advancing gene editing possibilities, especially in (higher) eukaryotic organisms that were until recently not amenable to specific and targeted mutagenesis strategies. This originally bacterial “foreign-DNA defense mechanism” has been explored in Chapters 4 and 5.

Peptidomics and bioactive peptide discovery

We engineered the proteolytic system of the *L. lactis* model strain MG1363 and describe a robust and comprehensive analytical framework of bacteria-casein incubation conditions, intracellular peptidome extraction, data analysis and visualization. This has allowed identifying casein-derived bioactive peptides produced by *L. lactis* MG1363 and

six of its isogenic peptidase mutants. This work was pioneered more than 2 decades ago, when Mierau et al. examined the HPLC chromatograms of intracellular amino acid and peptide fractions of *L. lactis* MG1363 and some of its peptidase mutants. However, the details of the chromatogram (e.g., which peptide sequences were present) were not further explored (4). Clearly, there were technical limitations in those days such as in complex sample separation and small peptide identification (5). Rapid developments in the technology of nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) and in algorithms for peptide identification have resulted in a dramatic upsurge in research in proteomics and its subfield, peptidomics (6, 7). Along with the increase in peptidomics studies in general, the discovery of bioactive peptides using peptidomic techniques has also expanded rapidly in the past 10 years. Especially, these technological advances have made the work described in this thesis (Chapter 2) achievable (Fig 1A). Overall, the further development of technology will allow identifying more novel bioactive peptides, while it may also support the exploration of more bioactivities in the already described bioactive peptides.

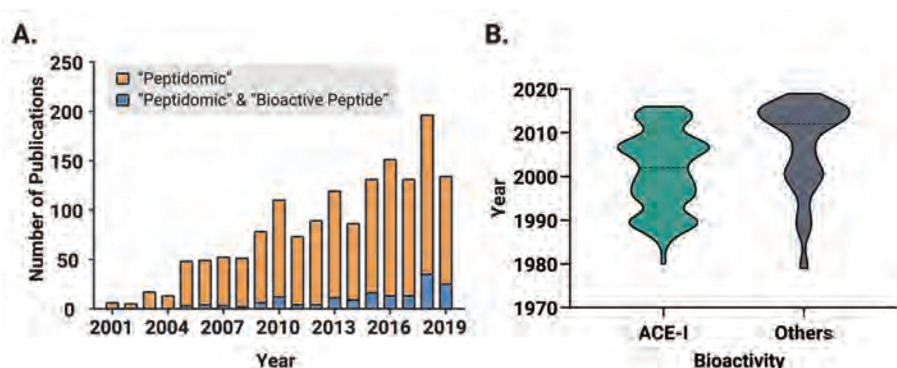

Figure 1. A) The number of publications obtained from PubMed with the search terms (yellow: peptidomics or peptidomic or peptidome) and (blue: peptidomics OR peptidomic) OR peptidome)) AND Bioactive peptide). B) Violin plots with mean values (dotted line) showing the publication years of papers on ACE-I peptides and "other peptides". All of these publications were taken from the database containing the 136 certified bioactive peptides (Table 5, Chapter 1).

Are angiotensin-converting-enzyme inhibitory (ACE-I) peptides really the most abundant bioactive peptides in milk?

Bioactive peptides that have been derived from bovine β-casein-derived are registered in three most popular milk-derived bioactive peptides databases (BIOPEP (4), MBPDB (5), and EROP-Moscow (6)). These data have been reviewed as part of the work in this

thesis (Chapter 1). Among all bioactivities described for peptides obtained from bovine β-casein, angiotensin-converting-enzyme inhibitory (ACE-I) action is most prevalent: more than half of the peptides present this activity. This naturally raises the following questions: are more ACE-I peptides identified because they are more abundant than peptides with other activities, or because they are easier to detect than others? ACE-I activity can be detected via a simple chemical reaction. As for antimicrobial peptides, their target(s) can be a single or a wide range of bacterial species, or even other microbes. This makes it time consuming to screen against the proper set of test strains. It is even more complex when trying to identify an immunomodulatory peptide due the complexity of the immune system and also because, often, animal experiments are involved. During the last 4 decades, the number of publications on ACE-I peptides per year is roughly the same while for other bioactive peptide activities, an obvious increase can be seen; more than half of them were published after 2010 (Fig 1B). For instance, in 1979 the heptapeptide YPFPGPI, also named β-CM7, was the first exogenous opioid peptide described (11). It was found to exhibit ACE-I activity in 2004 (12) and was shown to contribute significantly to mucin production in the gastrointestinal tract in 2006 (13). In 2012, it was identified as having a hypoglycemic effect on diabetic rats by increasing the level of plasma insulin (14).

Future perspective

Applying CRISPRi for future proteolytic system engineering

The major genome editing methods in *L. lactis* are based on RecA-dependent homologous double-crossover using non-replicative or conditionally replicating plasmids (15, 16). In fact, homologous double-crossover recombination has been used in Chapter 2 to make all the clean and stable peptidase knockout mutants. To make a gene knockout or gene insertion mutant usually consumes 3 weeks. The marker free strain obtained can then be used in next rounds of mutation so that multiple genes can be edited sequentially. To do this, multiple time slots are required. As shown in Chapter 2, the presence/absence of certain (groups of) peptidases might liberate interesting bioactivities from the β-casein molecule as is the case, for instance, for the mutants *L. lactis* MG*ΔpepOF$_2$O$_2$* and MG*ΔpepNXOTCF$_2$O$_2$*. However, specific mutations might also affect certain (essential) processes in such a way that the intracellular peptide pool is even poorer than in the wild type, as was observed in this study for the mutants *L. lactis*

MGΔ*pepXPQ* and MGΔ*pepVD*$_A$*TD*$_B$. One would know the results only after having spent a lot of time making and analyzing the specific mutants. Being able to apply CRISPRi for inhibiting certain groups of peptidase genes would allow fast and high-throughput screening for valuable combinations of peptidase knockdown mutants.

The NICE system employs *L. lactis* NZ9000 in which the *pepN* gene was disrupted to insert the *nisRK* genes (17). Therefore, we started the work presented in this thesis with *L. lactis* MG1363 and placed *nisRK* into another locus of its genome, *peudo10*. β-casein hydrolysis by *L. lactis* is initiated by the cell envelope-associated proteinase PrtP after its autoproteolytic activation with the aid of PrtM (18). In Chapter 2, these two genes were expressed using a plasmid with an erythromycin resistance marker (19), which conflicts with the sgRNA expression plasmid. We could use different vectors with different antibiotic markers to express *prtPM* and *sgRNAs*, respectively, or we could integrate the *prtPM* genes into another locus of the *L. lactis* genome (e.g., *pseudo70*) because they need to be always there. The *sgRNAs* should be expressed from an easily manipulatable plasmid (e.g., pTLR) since the *sgRNA* sequences will be frequently changed during the course of the work. The *dcas9-sfgfp* expression cassette could be integrated into the *pseudo29* locus that was used in Chapter 4. The next step would then be to construct *sgRNAs*-expression vectors with which multiple peptidase genes can be targeted at the same time. As detailed in Chapter 4, both in-fusion cloning and golden gate assembly methods would be good candidates for fast *sgRNA* replacements. After an array of *sgRNAs*-expressing plasmids have been prepared, and using the workflow developed in Chapter 2, the collection of peptidase knockdown strains could be quickly examined for bioactive peptide potential. Of course, an interesting combination of *pep* mutations could subsequently be hardwired by deleting these genes by DCO recombination to allow further and stable application.

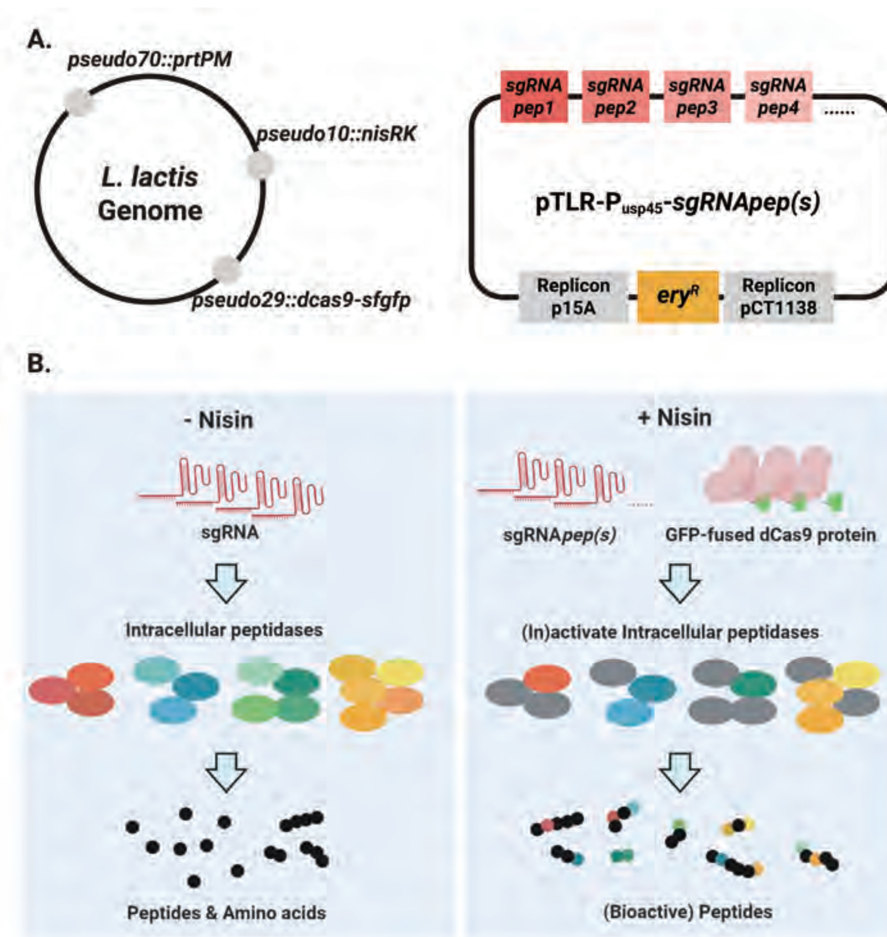

Figure 2. Applying CRISPRi in *L. lactis* for future bioactive-peptide discovery. A) left panel: Three genes are inserted into the *L. lactis* MG1363 genome: *nisR* and *nisK*, driven by their own promoter, are inserted into the *pseudo10* locus; the PnisA-*dcas9-sfgfp* cassette is inserted into the *pseudo29* locus; *prtP* and *prtM*, driven by their own promoters, are inserted into the *pseudo70* locus. Right panel: The sgRNAs targeting different peptidase genes (*sgRNApep*) are expressed in various combinations using a pTLR-based plasmid; they are controlled by P_{usp45} (20). Replicons p15A and pCT1138 are for replication in *E. coli* and *L. lactis*, respectively. ery^R, erythromycin resistance gene. B) Schematic representation of the CRISPRi-inhibited proteolytic system of *L. lactis* MG1363 derivatives. The figure is an adapted version of the combination of Chapter 1, Figure 3 and Chapter 4, Figure 1B. In the absence of nisin, sgRNA is expressed but dCas9-sfGFP is not. Consequently, the *L. lactis* proteolytic system works as normal and, after hydrolysis of β-casein by PrtP, the internalized oligopeptides will be degraded into free amino acids and/or small peptides. In the presence of nisin, sgRNA(s) and dCas9-sfGFP are expressed, dCas9-sfGFP is guided by sgRNA(s) to the target site(s), upon which the transcriptional elongation of the targeted peptidase gene(s) is blocked. As a result, certain peptides will stay intact and small (bioactive) peptides will accumulate intracellularly.

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