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Published in:
Applied and environmental microbiology

DOI:
10.1128/AEM.01319-20

Publication date:
2020

Editing of the proteolytic system of *Lactococcus lactis* increases its bioactive potential

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Keywords: *Lactococcus lactis*; Intracellular peptidomics; Proteolytic system; Bioactive peptides

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Running title: Editing *L. lactis* bioactive potential

14 **Abstract**

15 Large-scale mass spectrometry-based peptidomics for bioactive peptide discovery is
16 relatively unexplored because of challenges in intracellular peptide extraction and small
17 peptide identification. Here we present an analytical pipeline for large-scale intracellular
18 peptidomics of *Lactococcus lactis*. It entails an optimized sample preparation protocol for *L.*
19 *lactis*, used as an "enzyme complex" to digest β-casein, an extraction method for its
20 intracellular peptidome, and a peptidomics data analysis and visualization procedure. In
21 addition, we proofread the publicly available bioactive peptide databases and obtained an
22 optimized database of bioactive peptides derivable from bovine β-casein. We used the
23 pipeline to examine cultures of *L. lactis* MG1363 and a set of 6 isogenic multiple peptidase
24 mutants incubated with β-casein. We observed a clearly strain-dependent accumulation of
25 peptides with several bioactivities, such as ACE inhibitory, DPP-IV inhibitory,
26 immunoregulatory functions. The results suggest that both the number of different bioactive
27 peptides and the bioactivity diversity can be increased by editing the proteolytic system of *L.*
28 *lactis*. This comprehensive pipeline offers a model for bioactive peptide discovery in
29 combination with other proteins and might be applicable to other bacteria.

30 **Importance**

31 Lactic acid bacteria (LAB) are very important for the production of safe and healthy human
32 and animal fermented foods and feed and, increasingly more, in the functional food industry.
33 The intracellular peptidomes of LAB are promising reservoirs of bioactive peptides. We show
34 here that targeted genetic engineering of the peptide degradation pathway allows steering

35 the composition of the peptide pool of the LAB *Lactococcus lactis* and producing peptides
36 with interesting bioactivities. Our work could be used as a guideline for modifying proteolytic
37 systems in other LAB to further explore their potential as cell peptide factories.

38

Introduction

Members of the diverse group of lactic acid bacteria (LAB) have been associated with food fermentations since ancient times. LAB have been used as starter cultures for dairy production more than 100 years ago, which has given them their current industrial and economic importance (1). However, the value of those fermented products such as yogurt or cheese have remained restricted to providing basic nutrition, flavor, and texture. The potential of LAB as production organisms for functional foods is still largely unexplored. *Lactococcus lactis*, for instance, depends on the milk protein casein as the major source of nitrogen since it is auxotrophic for several amino acids. Casein degradation is accomplished in a three-step process. First, the extracellular cell envelope-associated proteinase (PrtP) degrades casein into oligopeptides. Second, (a selection of) these peptides are internalized via an oligopeptide transport system (Opp), while in the third and last step, multiple peptidases hydrolyze the oligopeptides into smaller peptides and, ultimately, into amino acids that are then available for *de novo* protein synthesis and other metabolic activities (2).

Functional food for health promotion or disease risk reduction has attracted the interest of food industries worldwide, with dairy products as one of the most popular categories. Milk proteins encode bioactive peptide sequences, which can be released by hydrolysis (3). These short peptides can display a spectrum of biological functions such as angiotensin-converting enzyme (ACE) inhibitory, dipeptidyl peptidase 4 inhibitory (DPP-IV-I), immunoregulatory, antioxidant, antimicrobial, and opioid activities. Bioactive peptides from milk proteins can be obtained via the action of microbial or non-microbial enzymes. Milk fermentation processes executed by LAB are preferable ways to release these peptides because of the food grade

safety status of these organisms. Moreover, proteolytic systems of LAB, especially that of *L. lactis*, have been comprehensively studied with respect to the genes and enzymes involved and their regulation (4, 5).

A lot of research has focused on the production of milk-derived bioactive peptides using LAB. Two major ways of bioactive peptide discovery can be discerned. First, casein proteins are either digested by purified digestive enzyme (trypsin) or LAB proteinase(s), after which the obtained products are identified (6–8). Second, an LAB cell culture is mixed with milk proteins and the supernatant is subsequently further characterized (9–11). These studies have identified numerous casein-derived bioactive peptides, most of which having ACE-inhibitory activity. From an application point of view, the costs of employing purified enzymes are too high for industrial-scale use. On the other hand, only utilizing the culture supernatant of proteolytically active cells does not exploit the full potential of the LAB as in that case only the proteinase specificity is being utilized while the activity of the more than 10 intracellular peptidases and possible hidden intracellular bioactive peptides are being ignored.

Our understanding of the intracellular peptide pool in LAB during growth in a milk medium, and the possible presence of bioactive variants, is limited to nearly absent due to the technical obstacles of preparing and separating the complex samples and the subsequent identification of the small peptides (12). Recent rapid developments in nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) technology and in algorithms for peptide identification have resulted in a dramatic increase in proteomics research and its sub-field, peptidomics (13, 14).

In this study, we engineered the proteolytic system of the *L. lactis* model strain MG1363 and describe a robust and comprehensive analytical framework of cell-casein incubation conditions, intracellular peptidome extraction, data analysis and visualization, and, ultimately, identification of casein-derived bioactive peptides produced by *L. lactis* MG1363 and six of its isogenic peptidase mutants. As proof-of-concept, this work offers a pipeline for the analysis and visualization of the intracellular peptidome of bacteria and explores the possibility of applying *L. lactis* or, for that matter, other bacteria as a cell factory to produce bioactive peptides.

Results

Engineering of *L. lactis* proteolytic system

The aim of this study is to build an analytical framework for the analysis of the intracellular peptidome of *L. lactis* and to discover (putative) bioactive peptides upon degradation of β-casein by the organism. To kick-start β-casein degradation by the *L. lactis* model strain MG1363, an extracellular cell wall-anchored proteinase PrtP (caseinase) is needed. The parent strain of *L. lactis* MG1363, *L. lactis* NCDO712, carries the 55-kb PrtP proteinase and lactose plasmid pLP712. This plasmid is too large to easily reintroduce in MG1363 and its peptidase knockout derivatives while it also contains one of the oligopeptidase genes, $pepF_1$ (15). Therefore, a new plasmid was constructed that encodes the proteinase PrtP and its maturase PrtM (16) from pLP712, named pCH020. *L. lactis* MG1363 possesses 15 intracellular peptidases that together degrade the PrtP-liberated casein-derived oligopeptides that are internalized by the oligopeptide permease Opp. The peptidase complement will ultimately result in the decomposition of the oligopeptides into shorter peptides and free amino acids. Undigested oligopeptides and peptidase-digested shorter versions of these peptides might possess bioactivities.

By removing different (groups of) peptidases, more and a greater variety of intracellular peptides should accumulate, increasing the chance of discovering (novel) bioactive peptides (Fig 1A). A total of thirty-seven single and multiple isogenic peptidase mutants were constructed from *L. lactis* MG1363 (Table 1) by employing sixteen peptidase gene replacement vectors based on the replication-deficient plasmid pCS1966 (17) (Fig 1B). Several

multi-peptidase deletion mutants were designed based on peptidase functional groups. Thus, four mutants were obtained in which all endopeptidases (MG*ΔpepOF*$_2$*O*$_2$), all aminopeptidases except PepM (MG*ΔpepANCpcp*; see below), all proline-specific peptidases (MG*ΔpepXPQ*) or the di-/tripeptidases (MG*ΔpepVD*$_A$*TD*$_B$) had been removed. In addition, in a strategy to delete as much peptidase genes as possible, half of them were deleted in strains MG*ΔpepNXOTCF*$_2$*O*$_2$ and MG*ΔpepNXOTCVD*$_A$. Note that *pepM* is an essential gene in MG1363 and is thus present in all peptidase mutants.

Each peptidase mutant that was examined with respect to its peptidome carried the plasmid pCH020. The strains carrying pCH020 were labeled PrtP$^+$, e.g. MG1363(PrtP$^+$) (See Table 1), but in the presentation of the results below, the addition PrtP$^+$ is omitted for reasons of simplicity.

Optimization of the intracellular peptidomics workflow

In order to obtain high-quality LC-MS data and convincing peptide identification results, three aspects were considered: the quality of *in vivo* β-casein degradation, *L. lactis* intracellular peptidome extraction, and the peptide identification algorithm. To optimize sample preparation for LC-MS-based intracellular peptidomics, each step of the workflow was considered (Fig 2). Our previous time-series RNA-seq results (18) revealed that the proteolytic system of *L. lactis* MG1363 is relatively highly active during the log-phase of growth, thus we chose to harvest cells in the mid-log phase ($OD_{600} \approx 1$) to start the *in vivo* β-casein degradation. Preliminary experiments employing different β-casein concentrations (1, 2, or 4 mg/ml) and incubation times (0.5, 1, 2, or 4h, or overnight) were tested and ultimately 4 mg/ml β-casein and 3.5 h incubation time were chosen to achieve a proper balance between sample quality

and time management (data not shown). Since Gram-positive bacteria such as *L. lactis* have a thick cell wall, obtaining the intracellular peptidome requires cell disruption using mechanical forces (19). Ultrasonication and the mini-beadbeater were tested, with both set-ups yielding similar results. However, when performing nanoLC-MS on the samples, it was observed that the sonicator probe introduced an overwhelming polyethylene glycol (PEG) contamination in the peptide fraction between 150-600 Da. This problem did not occur using glass-beads and the mini-beadbeater to break the cells (data not shown). Since the focus is to identify bioactive peptides, the intracellular proteome was enriched for small peptides by using the flow-through obtained after centrifuging the proteome sample over a 3-kDa cut-off filter, prior to analysis by nanoLC-MS/MS (see the M&M section). All peptidome samples obtained in this way were analyzed in biological triplicates. Excluding the β-casein *in vivo* degradation time, this optimized sample preparation protocol for rapid intracellular peptide extraction, from the breaking open of the cells to the filtering through the 3-kDa cut-off filter can be performed within one hour.

After obtaining the peptidome raw data, in order to find the most suitable search algorithm for our dataset, we tested 9 commonly used search engines for peptide identification. The PEAKS search engine (20) was used in combination with the commercial proteomics platform PEAKS studio (http://www.bioinfor.com/peaks-studio/), while for the other 8 SearchGUI was employed, an open-source interface configuring and running proteomics searches (21). All search engines were tested under the same setting using the raw data from MG1363 triplicates (see details in the M&M section). As Fig 2C shows, 5 out of 8 search engines in SearchGUI gave relatively the same level of unique peptide identifications: Tide (22) and

155 Comet (23) identified more peptides (both >400) than X! Tandem (24), MyriMatch (25), and
156 MS-GF+ (26) (all three >300), while MS Amanda (27), OMSSA (28) and Andromeda (29) did not
157 work properly for our data sets. Despite the fact that the MS-GF+ identification output is
158 lower (354) it has good reproducibility since the triplicates examined with MS-GF+ are
159 clustered in the heatmap shown in Fig 2D. As for PEAKS, it identified significantly more
160 unique peptides (947) than the other search engines and is also reproducible (Fig 2D). Thus,
161 we chose PEAKS as the search engine in this work. The identified peptides and proteins were
162 further analyzed and visualized by R and the web tool Peptigram (30).

163 **Peptidase deletion results in different intracellular peptidomes**

164 Fig 3 and Fig 4 give a gradually more detailed account of the results obtained for the 7 strains
165 tested, ranging from their peptidomics profiles to gene ontology analyses. Fig 3A shows the
166 numbers of identified peptide spectrum matches (PSMs), of unique peptides, and of unique
167 proteins in the various intracellular peptidomes. From this figure it is clear that the deletion of
168 all endopeptidase genes significantly increases the intracellular peptide pool. As for strain
169 MG*ΔpepOF*$_2$*O*$_2$, 3.2 k PSMs were detected while 2.6 k PSMs were obtained from the sample of
170 the parent strain MG1363. If in the strain lacking all endopeptidase activity, peptidases from
171 other functional groups, namely the aminopeptidases N and C, the proline-specific peptidase
172 PepX and the tri-peptidase PepT (MG*ΔpepNXOTCF*$_2$*O*$_2$) were removed, the number of PSMs
173 almost doubled compared to MG1363 (5.1 k vs. 2.6 k; see Fig 3A). Also, in strain
174 MG*ΔpepNXOTCVD*$_A$ a higher number PSMs (2.9 k) was detected than in MG1363 but it was
175 much lower than in MG*ΔpepNXOTCF*$_2$*O*$_2$. As these two multi-peptidase mutant strains have 5
176 peptidase gene deletions in common, it is highly likely that deletion of the endopeptidases

177 $PepF_2$ and $PepO_2$ is responsible for most of the increase in PSMs. The multi-aminopeptidase
178 mutant strain MG*ΔpepANCpcp* has slightly more PSMs detected than that of MG1363. All
179 these observations show that by eliminating (multiple) general peptidases, the intracellular
180 peptide pool will increase in both quantity and diversity. When peptidases with similar
181 specificities are removed, such as in strain *MGΔpepXPQ* lacking several proline-specific
182 peptidases, or in a strain deficient for peptidases playing important roles in the last stages of
183 peptide degradation (di-/tripeptidases mutant MG$\Delta pepVD_ATD_B$), a dramatic decrease in the
184 number of unique identified peptides is seen relative to strain MG1363. Strain MG$\Delta pepVD_ATD_B$
185 produce less than half the PSMs of MG1363, which might be due to the fact that deletion of
186 *pepV* affects cell wall synthesis, which ultimately disturbs other biological processes such as
187 nitrogen metabolism (31).

188 Hierarchical clustering of the identified peptides in the triplicate samples of each strain was
189 performed to assess the robustness of the developed methodology. The results presented in
190 Fig 3B show that the biological replicates of each strain delivered data of good quality and
191 reproducibility. The proteins identified in each mutant were compared with those of MG1363
192 on the basis of their functional grouping (Fig 3C). Approximately two thirds of the proteins
193 identified in the peptidome of *L. lactis* MG1363 can be detected in each of the peptidase
194 mutants. Notably, MG$\Delta pepNXOTCF_2O_2$ and MG$\Delta pepNXOTCVD_A$ are the top two strains with
195 respect to the number of unique proteins (168 and 97, respectively). Gene ontology (GO)
196 enrichment analyses were performed in order to investigate the functional profile of the
197 identified proteins from each strain and to evaluate the effects of peptidase deletions on the
198 peptidomes of the respective *pep* mutants. The cellular function grouping of the identified

199 proteins of MG1363 and its six isogenic *pep* mutants is shown in Fig 4. For well-characterized
200 proteins, the top three significantly enriched categories in all strains are translation (J), cell
201 cycle control (D), and replication (J). Around one quarter to one third of the proteins are
202 poorly characterized (R, S). Suppl. Fig 1 shows the details of the overlap in the peptidomes of
203 all 7 strains examined. Together with Fig 3 it can be seen that, although much more unique
204 proteins were identified in strain MG*ΔpepNXOTCF*$_2$*O*$_2$, the total number of biological function
205 groups did not increase.

206 **(Endo)Peptidase mutants accumulate β-casein peptides that differ in physicochemical**
207 **properties**

208 After having analyzed the intracellular peptidome profiles for the presence of peptides
209 derived from proteins expressed by those strains, we proceeded by examining the β-casein-
210 derived peptides therein. These peptides and their relative intensities were visualized using
211 the web tool Peptigram. In *L. lactis* strain MG1363, upon digestion of β-casein by the
212 extracellular proteinase PrtP and uptake of oligopeptides by the Opp system, peptides were
213 retrieved that cover the majority of the β-casein sequence (Fig 5A). Several regions in β-casein
214 that are not represented or retrieved in the intracellular peptide pool are shown as gaps. The
215 first 2 gaps represent fragment 1-15 (f1-15) and f30-40 of β-casein, which are observed in all
216 mutants. The first gap f1-15 is the signal peptide which exist in β-casein precursor (Uniprot
217 P02666) which contains 224 amino acids, while the β-casein (Cat #C6905, Sigma) we used
218 contains 209 amino acid residues (f16-224). The other 7 gaps seen in the MG1363-derived
219 peptide pattern are covered by the peptidome of one or more of the other mutant(s). It is
220 clear that all *pep* mutants produce different β-casein peptide profiles. In the intracellular

peptidome of the strain lacking all endopeptidase activity, MG*ΔpepNXOTCF*$_2$*O*$_2$, the highest relative intensity (dark green area in Fig. 5A) is seen around β-casein f180-200. This strain is also the most promising mutant with respect to possessing (more) β-casein-derived putative bioactive peptides, since the identified peptides from its intracellular peptidome cover almost all parts of the β-casein molecule and at the same time they have quite high intensities (Fig 5A). It has to be noted that in mass spectrometry, peptide intensity relies on peptide ionization capacity in addition to peptide abundance and, therefore, the observed intensities cannot directly be translated to peptide concentrations. However, for the same region of β-casein, e.g. f180-200, the peptide intensities obtained with MG*ΔpepNXOTCF*$_2$*O*$_2$ is dramatically higher than that obtained with the other strains. This implies that in the cytoplasm of this strain many more peptides from this region are present than in the cytoplasm of the other strains. Peptides identified in MG*ΔpepOF*$_2$*O*$_2$, MG*ΔpepNXOTCVD*$_A$, and MG*ΔpepANCpcp* also cover more of the β-casein molecule than seen in MG1363, which means that those *pep* mutants possess some β-casein-derived peptides that do not exist in the wildtype strain. Strains MG*ΔpepVD*$_A$*TD*$_B$ and MG*ΔpepXPQ* produce significantly less PSMs and peptides (Fig 3A) and, clearly, their peptidomes also contain less β-casein-derived peptides.

We then examined the physicochemical properties of the peptidomes of the various strains. For the β-casein-derived peptides, those obtained with MG1363 and its *pep* mutants have very different distributions in each physicochemical property. As expected, compared to the wildtype, the two endopeptidase mutants (MG*ΔpepNXOTCF*$_2$*O*$_2$ and MG*ΔpepOF*$_2$*O*$_2$) contained more longer β-casein-derived peptides, while shorter peptides are present in the exopeptidase mutants (see Fig 5B, Length). In agreement with its genetic make-up, the

mutant in which proline-specific peptidase genes have been deleted, strain MG*ΔpepXPQ*, produces much more proline-containing peptides than all other strains (see Fig 5B, Proline).

Optimization of β-casein-derived bioactive peptide database

L. lactis proteinase PrtP is a β-casein specific caseinase. To allow identifying bioactive peptides in our peptidome dataset, a comprehensive review of β-casein-derived bioactive peptides was performed of commonly used bioactive peptide databases. The most popular of those databases for milk-derived proteins are BIOPEP, MBPDB, and EROP-Moscow. There are pros and cons for each of these databases. BIOPEP (32) has more peptide entries but does not give proper literature references for each entry. Although EROP-Moscow (33) does have literature references, these have not been updated in recent years. Also, the query page of EROP-Moscow does not support multiple sequence searches. MBPDB (34) performs best in both searching and literature updating but it does not cover all peptides from the other two databases. For example, β-casein f(75-81) YPFPGPI is present and labeled in all 3 database as having opioid activity. BIOPEP does not provide a reference, while EROP-Moscow and MBPDB do. However, EROP-Moscow only provides the first research paper revealing the opioid activity (35), while there are 3 subsequent papers also proving the opioid activity of this peptide. In addition, 4 other bioactivities have been reported for this peptide over the past 2 decades in MBPDB: increased satiety (36), anxiety reducing (37), anticancer (38), and ACE-inhibitory (6).

We combined and curated these three databases by proofreading the data for each β-casein-derived bioactive peptide, excluding those for which: A) no reference was provided; B) a

264 reference was given but the activity was hypothetical or predicted only (39); C) data was
265 delivered but there is a discrepancy between the sequence from reference literature and the
266 database (40); and D) only a bitter taste was recorded (41). Thus, we obtained 176 unique
267 bioactive peptides of which, after excluding those falling under definitions A-D, 136 bioactive
268 peptides remained (Fig 4A). They were grouped by their bioactivities; it is clear that more than
269 half of the bioactive peptides have ACE-inhibitory activity (82/136 BPs). The second big
270 activity group contains around 20 peptides with immunomodulatory or dipeptidyl peptidase
271 IV inhibitory (DPP-IV-I) activity. The third, much smaller, group includes peptides with
272 antimicrobial, opioid, antioxidation, or prolyl-endopeptidase inhibitory activities. Note that 18
273 of the 136 peptides possess multiple bioactivities (Fig 6B).

274 **_L. lactis_ peptidase mutants produce more bioactive peptides than the wildtype strain**

275 Table 2 summarizes the bioactive peptides identified through nanoLC-MS/MS in the
276 intracellular peptidomes of each strain by comparing the identified β-casein-derived peptides
277 to our curated bioactive peptide database. As expected on the basis of the results presented
278 above, MG*ΔpepNXOTCF*$_2$*O*$_2$ is the most promising strain, as 18 bioactive peptides could be
279 identified in its intracellular peptidome. Most of the peptides have ACE-inhibitory activity
280 (11/18), while other bioactivities are also found, such as DPP-IV inhibitory, antioxidative,
281 antimicrobial and immunoregulatory activities. Strains MG*ΔpepNXOTCVD*$_A$ and
282 MG*ΔpepANCpcp* are second with respect to the number of bioactive peptides that are
283 obtained with these strains. Most of the β-casein-derived peptides identified in both strains
284 have the same sequence (7/9). All β-casein-derived bioactive peptides identified in the
285 peptidome of MG*ΔpepNXOTCVD*$_A$ are also present in that of MG*ΔpepNXOTCF*$_2$*O*$_2$. As for

286 MG*ΔpepANCpcp*, except AVPYPQR, the other 8 bioactive peptides are also observed in
287 MG*ΔpepNXOTCF*$_2$*O*$_2$. The β-casein-derived peptidome of MG*ΔpepOF*$_2$*O*$_2$, contains 6 bioactive
288 peptides; except for peptide VPVEPFTE, the other 5 peptides are also present in the samples
289 of MG*ΔpepOF*$_2$*O*$_2$. No bioactive peptides were observed when using MG*ΔpepVD*$_A$*TD*$_B$ to
290 degrade β-casein and only 2 were found when employing strain MG*ΔpepXPQ*. As explained
291 above, these might be caused by the disruption of other biological processes such as
292 peptidoglycan biosynthesis when deleting the dipeptidase PepV (31).
293

294 **Discussion**

295 In this study we present an analytical framework consisting of peptidome extraction, followed
296 by tandem mass spectrometric identification and bioinformatic analysis to untangle the
297 intracellular peptidome of *L. lactis* and to assess the potential of this organism as a cell factory
298 for the production of bioactive peptides. The developed protocol is reproducible and can be
299 performed in less than 2 h from peptidome extraction to mass spectrometric analysis.

300 The quality of peptide identification relies on a suitable searching algorithm. PEAKS studio
301 identified more peptides in our data sets than all the other search engines from SearchGUI
302 (Fig 2C) while it is also user-friendly because of its well-designed interface. However, when
303 this commercial tool is not accessible because of its price, SearchGUI could be a good
304 alternative because it includes the mainstream open-source search engines. MS-GF+ and
305 Andromeda are the top algorithms in the proteomics/peptidomics field. MS-GF+ delivered
306 relatively good and reproducible identification results. However, unlike PEAKS, it does not
307 provide the relative intensities of identified peptides, precluding a visualization of β-casein
308 digestion profiles as presented in Fig 5A. Thus, for data analysis consistency, we did not
309 combine the results from PEAKS and MS-GF+. Andromeda, the search engine of MaxQuant, is
310 designed for large mass-spectrometric data sets but is mostly geared towards human
311 proteomes/peptidomes and is unsuitable for our bacterial peptidomics data. Here we focused
312 on identifying bioactive peptides, which normally contain 2 to 20 amino acid residues (42).
313 Note that the oligopeptide transport system (Opp) of *L. lactis* possesses the capacity to
314 transport peptides from 4 up to at least 18 residues (43). We therefore set the mass
315 spectrometry detection window to 170-2000 Da, which generally covers peptides containing

316 2 to 18 amino acid residues considering that the average molecular weight of an amino acid is
317 110 Da. The gap regions in the β-casein peptide profile of each strain (Fig 5A) might be due to
318 the fact that some peptides have more than 18 amino acids residues thus beyond our
319 detection window.

320 The fact that the β-casein-derived bioactive peptides identified in the intracellular peptidome
321 of each strain treated under the same conditions differs, demonstrates the potential of these
322 *pep* mutants. The β-casein-derived peptides obtained with the wildtype strain MG1363 cover
323 the whole protein (Fig 5A) with bioactive peptides only originating from β-casein f(70-110)
324 (Table 2). Novel peptides are found when employing the *pep* mutant strains, which shows the
325 potential of β-casein as bioactive peptides source in combination with *L. lactis* serving as an
326 enzyme complex. The *L. lactis* peptidases have been classified in different groups on the basis
327 of their cleavage specificity. For example, enzymes in the endopeptidase group will cleave
328 internally in an oligopeptide and knocking them all out should result in the accumulation of
329 relatively longer peptides. Take peptide TQTPVVVPPFLQPEVM in Fig 7 as an example. It might
330 be that in MG1363, peptide TQTPVVVPPFLQPEVM was internalized and hydrolyzed into
331 TQTPVVVPPFLQPE/VM, TQTPVVVPPF/LQPEVM, and TQTPVVVPP/FLQPEVM. The longer N-
332 terminal parts in each case were detected while the C-terminals were not. This may be due to
333 the detection limit of the LC/MS equipment (VM), or the C-terminal peptides were degraded
334 further by other intracellular peptidases (LQPEVM, FLQPEVM). In mutant MG*ΔpepOF*$_2$*O*$_2$,
335 lacking the three endopeptidases, TQTPVVVPPFLQPEVM would not be degraded, leading to
336 its accumulation (Fig 7). In fact, the three peptides TQTPVVVPPFLQPEVM, TPVVVPPFLQPEVM,
337 and PVVVPPFLQPEVM were only detected in the mutants MG*ΔpepOF*$_2$*O*$_2$ and

338 MG*ΔpepNXOTCF*$_2$*O*$_2$, which both lack all endopeptidases. In some other mutants, smaller
339 derivative peptides identical to those in MG1363 can be observed, i.e. TQTPVVVPP and
340 PVVVPPF were also identified in strains MG*ΔpepNXOTCVD*$_A$ and MG*ΔpepANCpcp*, respectively,
341 while TPVVVPP was also identified in strain MG*ΔpepXPQ*. These observations indicate that one
342 or more of the endopeptidases prefers cutting C-terminal of glutamic acid, phenylalanine,
343 and proline residues, which coincide with a previous study showing that PepF can (likes to)
344 cut C-terminal proline and phenylalanine (44).

345 On the one hand, this presence/absence of certain (groups of) peptidases might liberate
346 interesting bioactivities from the β-casein molecule and on the other hand release enough
347 free amino acids so that *L. lactis* growth and functioning is not severely affected. When a
348 group of specialty peptidases is removed a problem might arise if they are not only
349 responsible for β-casein digestion but also are important in other metabolic pathway(s),
350 disrupting certain essential processes. A clear example is the role that PepV plays in
351 peptidoglycan synthesis (31).

352 Fermentation is an easy and cost-effective method to generate bioactive peptides in
353 fermented milk products. This study presents a comprehensive analysis of the *L. lactis*
354 intracellular peptidome after *in vivo* β-casein degradation. The work suggests that the
355 number of different bioactive peptides and the bioactivity diversity can be increased by
356 editing the proteolytic system of this LAB starter strain. *L. lactis* MGΔpep*NXOTCF*$_2$*O*$_2$ has the
357 best performance in producing peptides with high intensities of peptides that have a variety
358 of bioactivities. It might thus potentially be useful as bioactive peptide cell factory. The fact
359 that the peptides are intracellular should make them less sensitive to for instance digestive

enzymes. Our work could also be used as a guideline for modifying proteolytic systems in other LAB, and further analyzing and visualizing the intracellular proteome/peptidome data to explore their potential as peptide cell factories.

Acknowledgements

We thank Dr. Anne de Jong for bioinformatics support and Dr. Hjalmar Permentier for his nanoLC/MS/MS expertise. CH was supported by a scholarship from China Scholarship Council (CSC, file nr. 201505990303).

Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains used in this study are listed in Table 1. *Lactococcus lactis* MG1363 and its derivatives were cultivated in M17 medium (Cat # DF1856-17-4, BD Difco, Detroit, MI, USA)) containing 0.5% (w/v) glucose (GM17) at 30˚C. Erythromycin (Cat # E6376, Sigma-Aldrich, Santa Clara, CA, USA) was added at a final concentration of 5 µg/ml when required. Chemically defined SA medium with 0.5% (w/v) glucose and 20 µg/ml 5-fluoroorotic acid (5-FOA; Cat #F5013, Sigma-Aldrich, Santa Clara, CA, USA) as a sole pyrimidine source was used for the generation of chromosomal knockouts, as described previously (17). *Escherichia coli* DH5α was used for cloning purposes; it was cultivated aerobically at 37˚C in LB medium (Cat # LMM01, Formedium, Norfolk, UK) with erythromycin at a final concentration of 200 µg/ml when required.

Recombinant DNA techniques and oligonucleotides

Standard molecular cloning techniques were performed essentially as described (Sambrook and Russell, 2001). Chromosomal DNA from *L. lactis* was isolated using the GenElute™ Bacterial Genomic DNA Kit (Cat # NA2110-1KT, Sigma-Aldrich, Santa Clara, CA, USA). Plasmids and PCR products were isolated and purified using the NucleoSpin® Plasmid EasyPure kit (Cat # MN 740727.250, Macherey-Nagel, Leiden, the Netherlands) and NucleoSpin Gel & PCR Clean-up kit (Cat # MN 740609.250, Macherey-Nagel, Leiden, the Netherlands), respectively, according to the manufacturers' instructions. PCR reactions for cloning purpose were performed with Phusion High-Fidelity DNA Polymerase (Cat # F530L, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol; Enzymes were purchased from Fermentas (Thermo Fisher Scientific, MA, USA) and New England Biolabs (Ipswich, MA, USA). Colony PCR reactions were performed by homemade Pfu polymerase. Inserts and linearized vector were fused using the Quick-Fusion Cloning kit (Cat # B22612, BioConnect) according to the manufacturer with the modification that half of the recommended volume per reaction was used. Oligonucleotides employed in this study are listed in Supplemental Table 1 and were purchased from Biolegio BV (Nijmegen, The Netherlands). Competent E. coli cells were transformed using heat-shock (45), while electrocompetent L. lactis cells were transformed using electroporation (46) with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, CA, USA). All nucleotide sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands).

398 **Construction of integration plasmids for knocking out peptidase genes from *L. lactis***

399 **MG1363**

400 All plasmids that were used or constructed during this study are listed in Table 3. Relevant
401 regions of all plasmids were sequenced to confirm their nucleotide sequences. All integration
402 plasmids were constructed using the same workflow that is described here for only one,
403 pCH001, as an example: Linearized vector pCS1966 was amplified using primers
404 pCS1966_1FW/ pCS1966_1RV. Primers pairs pCH-0017/pCH-0018 and pCH-0019/pCH-0020
405 were used, respectively, to obtain upstream (UP_F2) and downstream (DOWN_F2) regions of
406 peptidase gene *pepF*$_2$. Primer pair pCH-0017/pCH0020 was used to perform an overlap PCR to
407 obtain the flanking region UP+DOWN_F2. Primers pCH-0017 and pCH-0020 contain 15
408 nucleotides at one end, overlapping with the sequence on the 5' end of the linearized vector,
409 followed by the flanking region of *pepF*$_2$ gene, and 15 nucleotides overlapping with the
410 sequence on the 3' end of the linearized vector. The fragment UP+DOWN_F2 was fused with
411 the linearized vector using Quick-Fusion, after which the reaction mixture was directly used to
412 transform competent *E. coli* DH5α. The resulting vector was designated pCH001. Primers pCH-
413 0083/0099/0100 were used for colony PCR and nucleotide sequencing confirmation.

414 **Construction of *L. lactis* (multi)peptidase knockout mutants**

415 All peptidase gene knockout strains were made using (a repetition of) the same workflow that
416 is only described here for the construction of the multiple endopeptidase mutant strain
417 CH018 as an example: Integration plasmid pCH011, a *pepO* knockout plasmid, was introduced
418 in MG1363 via electroporation. Knockout mutants were obtained by a two-step homologous

419 recombination strategy (17), First, plasmid chromosomal integrates were selected on
420 erythromycin containing GM17 plates. Subsequently, the marker-free knockout strain was
421 obtained through counterselection on 5-FOA in SA medium plates. The resulting strain,
422 CH011 (MG*ΔpepO*), underwent the same 2-step recombination protocol using pCH001 to
423 obtain the peptidase double mutant strain CH017 (MG*ΔpepOF*$_2$). Strain CH018
424 (MGΔpepOF2O2) was obtained using the strategy with plasmid pCH002 on strain CH017. All
425 relevant chromosomal regions of each deletion strain were confirmed by nucleotide
426 sequencing.

427 **Construction of plasmid pCH020 for expressing proteinase PrtP in *L. lactis* MG1363**

428 The flanking regions of the *prtPM* genes from plasmid pLP712 (47) were amplified together
429 using primers pCH-0173/pCH-0174. The fragment was ligated into plasmid pTLR employing
430 NcoI/XhoI restriction sites. The resulting plasmid was named pCH020.

431 **β-casein degradation *in vivo***

432 *In vivo* β-casein breakdown was examined using the method of Kunji et al. (49) with the
433 following modifications: an overnight culture was diluted to a starting optical density at 600
434 nm (OD600) of 0.05 in 50 ml of GM17 with 5 µg/ml erythromycin, when required. The culture
435 was grown at 30 °C and when the OD600 reached 0.7, which corresponds to the early
436 exponential growth phase, the cells were collected by centrifugation at 6000 g for 5 min. They
437 were washed twice with wash buffer (100 mM MES-KOH (pH6.5) with 2mM $CaCl_2$) to prevent
438 autoproteolysis and release of the proteinase PrtP. Cells were then concentrated to an OD600
439 of 14 and resuspended in 2 ml of 4 mg/ml β-casein (Cat # C6905, Sigma-Aldrich, Santa Clara,

CA, USA) in wash buffer with 0.5% (w/v) glucose. The suspension was incubated for 3 h at 30 °C with slow rotation (10 rpm) in a rotator incubator oven (Cat # G2545A, Agilent Technologies, Inc., CA, USA). Cells were then spun down at 12000 g for 3 min after which both the supernatant and the cells were saved at -80 °C until further use.

Cytoplasmic peptidome extract preparation

Frozen cells incubated with β-casein were thawed and resuspended in 2 ml 1 M LiCl in 50 mM Tris (pH 8.0) to extract proteins in the surface layer or anchored to the cell wall through non-covalent interactions (50). After incubation at 4 °C for 1 h, the cells were spun down at 4000 g for 10 min. The supernatant, named LiCl extract and saved at -80 °C. The cells were washed twice with MiliQ water (Milipore, MA, USA), and subsequently disrupted in a Mini-Beadbeater (Cat # 112011EUR, BioSpec, OK, USA) using 3 cycles of 1 min ON, 1 min OFF. Disrupted cells were spun down at 11000 xg at 4 °C for 10 min. The supernatant was collected and filtered through a 0.2 µm pore-size filter (Cat # 41055511, Boom BV, Meppel, the Netherlands), and then ultrafiltrated through Amicon 3-KDa molecular weight cut-off membranes (Cat # UFC500324, Millipore , MA, USA). The pool of peptides less than 3-KDa was collected and saved at -80 °C until further use.

nanoLC-MS/MS

All samples were analyzed at the Interfaculty Mass Spectrometry Center, University of Groningen, on a nanoLC-MS/MS consisting of an Ultimate 3000 LC system (Dionex, Amsterdam, the Netherlands) interfaced with a Q-Exactive plus mass spectrometer (Thermo Fisher Scientific, MA, USA). Peptide mixtures were loaded onto a 5 mm × 300 µm i.d. C18

PepMAP100 trapping column (Thermo Fisher Scientific, MA, USA) with 2% acetonitrile in 0.1% formic acid at 20 µL/min. After loading and washing for 3 min, peptides were eluted onto a 15 cm × 75 µm i.d. C18 PepMAP100 nanocolumn (Dionex, Amsterdam, the Netherlands). A mobile phase gradient at a flow rate of 300 nL/min and with a total run time of 75 min was used: 2% – 50% of solvent B in 60 min; 50% – 90% B in 1 min; 90% B during 13 min, and back to 2% B in 1 min (held for 15 min). Solvent A was 100:0 water/acetonitrile (v/v) with 0.1% formic acid, and solvent B was 0:100 water/acetonitrile (v/v) with 0.1% formic acid. In the nanospray source a stainless-steel emitter (Thermo Fisher Scientific, MA, USA) was used at a spray voltage of 1.8 kV with no sheath or auxiliary gas flow. The ion transfer tube temperature was 275 °C. Spectra were acquired in data-dependent mode with a survey scan at m/z 300 – 1650 at a resolution of 70 000 followed by MS/MS fragmentation of the top 10 precursor ions. Singly charged ions were excluded from MS/MS experiments and fragmented precursor ions were dynamically excluded for 20 s. PEAKS Studio version X software (Bioinformatics Solutions, Inc., Waterloo, Canada) was used to search the MS data against a protein sequence database of the L. lactis MG1363 proteome (UniProt database) to which the sequence of β-casein (Uniprot P02666) was added. Search parameters: no enzyme specificity; fixed modification: carbamidomethylation of cysteine; variable modifications: oxidation of methionine and phosphorylation of serine; precursor mass tolerance of 15 ppm; fragment mass tolerance of 0.02 Da. The false discovery rate was set at 0.1%.

Data analysis and visualization

The peptide spectrum matches (PSM), identified peptides, and identified protein number were obtained from PEAKS. The identified peptides were exported for further analysis in R by

using Pheatmap R package. Venn diagrams were made using the webtool Calculate and Draw Custom Venn Diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene ontology analysis was performed using Gene Set Enrichment Analysis (GSEA) provided by the GENOME2D software available at http://server.molgenrug.nl/index.php/gsea-pro. Circos plots were made using the Circlize R package (51). For further peptidomic analysis, peptide sequences of biological triplicate were combined, and only unique peptides present at least twice among triplicates were used. Thus, 7 datasets of all 21 samples were generated. The further data analysis mentioned below was performed on these combined datasets. Profiles of β-casein-derived peptides in the L. lactis intracellular peptidome were visualized by the web-based tool Peptigram (30). Data of the parameter relative intensity were generated from the average intensity of the replicates of each chosen peptide. Peptide physicochemical properties were computed using the aminoAcidProperties function of the R package "alakazam", version 0.2.8 (52). The proportion of proline in each peptide was manually calculated. The Upset plot of our optimized bioactive peptide database was generated using the Upset R package (53).

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700 **Table 1. Strains used in the peptidomic experiment.**

Strain	Species	Description	Short name	Ab^r	Reference
MG1363	*L. lactis*	Plasmid free derivative of NCDO712	MG1363		(54)
CH000	*L. lactis*	MG1363 with plasmid pCH020	MG1363(PrtP$^+$)	Ery	this study
IM14	*L. lactis*	MG1363Δ*pepNXOTC*	MGΔ*pepNXOTC*		(55)
CH001	*L. lactis*	MG1363Δ*pepF*$_2$	MGΔ*pepF*$_2$		this study
CH002	*L. lactis*	MG1363Δ*pepO*$_2$	MGΔ*pepO*$_2$		this study
CH003	*L. lactis*	MG1363Δ*pepA*	MGΔ*pepA*		this study
CH004	*L. lactis*	MG1363Δ*pepP*	MGΔ*pepP*		this study
CH005	*L. lactis*	MG1363Δ*pepV*	MGΔ*pepV*		this study
CH007	*L. lactis*	MG1363Δ*pcp*	MGΔ*pcp*		this study
CH008	*L. lactis*	MG1363Δ*pepQ*	MGΔ*pepQ*		this study
CH009	*L. lactis*	MG1363Δ*pepD*$_A$	MGΔ*pepD*$_A$		this study
CH010	*L. lactis*	MG1363 (pLP712Δ*pepF1*)	MGΔ*pepF*$_1$		this study
CH011	*L. lactis*	MG1363Δ*pepO*	MGΔ*pepO*		this study
CH012	*L. lactis*	MG1363Δ*pepC*	MGΔ*pepC*		this study
CH013	*L. lactis*	MG1363Δ*pepN*	MGΔ*pepN*		this study
CH014	*L. lactis*	MG1363Δ*pepX*	MGΔ*pepX*		this study
CH015	*L. lactis*	MG1363Δ*pepT*	MGΔ*pepT*		this study
CH016	*L. lactis*	MG1363Δ*pepDB*	MGΔ*pepD*$_B$		this study
CH017	*L. lactis*	MG1363Δ*pepOF*$_2$	MGΔ*pepOF*$_2$		this study
CH018	*L. lactis*	MG1363Δ*pepOF*$_2$*O*$_2$	MGΔ*pepOF*$_2$*O*$_2$		this study
CH019	*L. lactis*	MG1363Δ*pepVD*$_A$	MGΔ*pepVD*$_A$		this study
CH020	*L. lactis*	MG1363Δ*pepVD*$_A$*T*	MGΔ*pepVD*$_A$*T*		this study
CH021	*L. lactis*	MG1363Δ*pepVD*$_A$*TD*$_B$	MGΔ*pepVD*$_A$*TD*$_B$		this study
CH022	*L. lactis*	MG1363Δ*pepAN*	MGΔ*pepAN*		this study
CH023	*L. lactis*	MG1363Δ*pepANC*	MGΔ*pepANC*		this study
CH024	*L. lactis*	MG1363Δ*pepANCpcp*	MGΔ*pepANCpcp*		this study
CH025	*L. lactis*	MG1363Δ*pepXP*	MGΔ*pepXP*		this study
CH026	*L. lactis*	MG1363Δ*pepXPQ*	MGΔ*pepXPQ*		this study
CH027	*L. lactis*	MG1363Δpep*NXOTCF*$_2$	MGΔ*pepNXOTCF*$_2$		this study
CH028	*L. lactis*	MG1363Δ*pepNXOTCF*$_2$*O*$_2$	MGΔ*pepNXOTCF*$_2$*O*$_2$		this study
CH029	*L. lactis*	MG1363Δ*pepNXOTCV*	MGΔ*pepNXOTCV*		this study
CH030	*L. lactis*	MG1363Δ*pepNXOTCVD*$_A$	MGΔ*pepNXOTCVD*$_A$		this study
CH031	*L. lactis*	CH020 with plasmid pCH018	MGΔ*pepOF*$_2$*O*$_2$ (PrtP$^+$)	Ery	this study
CH032	*L. lactis*	CH021 with plasmid pCH020	MGΔ*pepVD*$_A$*TD*$_B$ (PrtP$^+$)	Ery	this study

Strain	Species	Description	Short name	Ab^r	Reference
CH033	*L. lactis*	CH024 with plasmid pCH020	MG*ΔpepANCpcp* (PrtP$^+$)	Ery	this study
CH034	*L. lactis*	CH026 with plasmid pCH020	MG*ΔpepXPQ* (PrtP$^+$)	Ery	this study
CH035	*L. lactis*	CH028 with plasmid pCH020	MG*ΔpepNXOTCF*$_2$*O*$_2$ (PrtP$^+$)	Ery	this study
CH036	*L. lactis*	CH030 with plasmid pCH020	MG*ΔpepNXOTCVD*$_A$ (PrtP$^+$)	Ery	this study
DH5α	*E. coli*	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*			(56)

701 Ab^r=Antibiotic resistance. Ery = erythromycin

702

703 **Table 2. Bovine β-casein-derived bioactive peptides identified in the intracellular**

704 **peptidome of *L. lactis* MG1363 and its peptidase knockout mutants.**

Peptide Sequence	β-CN fragment (start-end)	Theoretical Mass (Da)	Mass Error (ppm)	Bioactivity in Reference	Ref NO.
MG1363					
YPFPGPIPN	75-83	1000.5018	0.9 / 3.4 / -1.8	ACE-I; DPP-IV-I; Opioid	(57–59)
LPQNIPP	85-91	777.4385	3.3 / 4.9 / 3.6	DPP-IV-I	(58)
LPQNIPPL	85-92	890.5225	3.1 / - / -0.5	DPP-IV-I	(58)
TQTPVVVPPFLQPE	93-106	1550.8344	3.6 / 6.8 / -	Anti-O	(60)
MGΔpepOF$_2$O$_2$					
YPFPGPIPN	75-83	1000.5018	3.3 / 2.8 / 0	ACE-I; DPP-IV-I; Opioid	(57–59)
HKEMPFPK	121-128	1012.5164	0.3 / -2 / -1.3	Anti-M	(61)
YPVEPFTE	129-136	980.4491	9.7 / 8 / 4.3	ACE-I	(62)
SQSKVLPVPQ	181-190	1081.6132	6.7 / 6.9 / 5.4	ACE-I	(9)
RDMPIQAF	198-205	976.4800	- / 6.1 / 5	ACE-I	(40)
QEPVLGPVRGPFPIIV	209-224	1716.9926	4.8 / 6.1 / 2.5	ACE-I	(63)
MGΔpepNXOTCF$_2$O$_2$					
LNVPGEIVE	21-29	968.5178	2.5 / 3.8 / 2.5	ACE-I	(10)
VYPFPGPIPN	74-83	1099.5702	1.3 / 2 / 3.2	ACE-I; Anti-O	(64)
LVYPFPGPIPNSLPQ	73-87	1637.8817	3.6 / 3.8 / -	ACE-I; PEP-I	(65)
LPQNIPPL	85-92	890.5225	2.8 / 1.5 / 4	DPP-IV-I	(58)
PQNIPPL	86-92	777.4385	2.9 / 2 / 2.2	DPP-IV-I	(58)
NIPPLTQTPV	88-97	1078.6023	4.2 / 4.6 / 4/6	ACE-I	(10)
TQTPVVVPPFLQPE	93-106	1550.8344	2.5 / 2.5 / 2.5	Anti-O	(60)
VKEAMAPK	113-120	872.4789	-2.9 / -4.1 / -1.6	Anti-O; Anti-M	(66)
HKEMPFPK	121-128	1012.5164	5.2 / 2.9 / 4.1	Anti-M	(61)
LHLPLPL	148-154	801.5112	2.8 / 5.5 / 6.1	ACE-I	(11)
NLHLPLPLL	147-155	1028.6382	1.9 / 3.8 / 3.9	ACE-I	(67)
SQSKVLPVPQ	181-190	1081.6132	3.8 / 5.1 / 4.5	ACE-I	(9)
KVLPVPQK	184-191	907.5854	5.1 / 4.5 / 7	Anti-O	(66)
KVLPVPQ	184-190	779.4905	3.6 / 1.8 / 9.1	ACE-I	(68)
VLPVPQK	185-191	779.4905	4.7 / 6.9 / 7.6	Anti-M; Anti-O	(66)
RDMPIQAF	198-205	976.4800	4.7 / 3.8 / 4.9	ACE-I	(40)
YQEPVLGPVRGPFPIIV	208-224	1880.0559	4.3 / 4.3 / -	ACE-I; Anti-M; Immuno-R	(69–71)
QEPVLGPVRGPFPIIV	209-224	1716.9926	2.6 / 2.7 /2.2	ACE-I	(63)
MGΔpepXPQ					
YPFPGPIPN	85-83	1000.5018	-0.6 / -1.4 / -0.3	ACE-I; DPP-IV-I; Opioid	(57–59)
LPQNIPPL	85-92	890.5225	1.5 / 2.8 / -	DPP-IV-I	(58)

Peptide Sequence	β-CN fragment (start-end)	Theoretical Mass (Da)	Mass Error (ppm)	Bioactivity in Reference	Ref NO.
MGΔpepVD$_A$TD$_B$					
-					
MGΔpepNXOTCVD$_A$					
LNVPGEIVE	21-29	968.5178	- / 1.9 / 2	ACE-I	(10)
VYPFPGPIPN	74-83	1099.5702	- / 2.5 / 3.4	ACE-I; Anti-O	(64)
LPQNIPPL	85-92	890.5225	- / 3.9 / 4.9	DPP-IV-I	(58)
PQNIPPL	86-92	777.4385	- / 1.7 / 4.1	DPP-IV-I	(58)
NIPPLTQTPV	88-97	1078.6023	- / 3.9 / 5.5	ACE-I	(10)
TQTPVVVPPFLQPE	93-106	1550.8344	- / 3.6 / 4.7	Anti-O	(60)
LHLPLPL	148-154	801.5112	- / 5.8 / 3.5	ACE-I	(11)
SQSKVLPVPQ	181-190	1081.6132	- / 4.5 / 4.3	ACE-I	(9)
KVLPVPQ	184-190	779.4905	1.4 / 0.8 / 2.9	ACE-I	(68)
MGΔpepANCpcp					
LNVPGEIVE	21-29	968.5178	2.9 / 6.4 / 4.2	ACE-I	(10)
VYPFPGPIPN	74-83	1099.5702	5.4 / 5.5 / 4.8	ACE-I; Anti-O	(64)
NIPPLTQTPV	88-97	1078.6023	4.7 / 8 / 10	ACE-I	(10)
TQTPVVVPPFLQPE	93-106	1550.8344	6.2 / 8.2 / 6.7	Anti-O	(60)
HKEMPFPK	121-128	1012.5164	0.1 / 1.2 / -0.1	Anti-M	(61)
LHLPLPL	148-154	801.5112	5.2 / 9.4 / 6.7	ACE-I	(11)
SQSKVLPVPQ	181-190	1081.6132	4.7 / 4.9 / 6.5	ACE-I	(9)
KVLPVPQ	184-190	779.4905	4.1 / 1.8 / 3.9	ACE-I	(68)
AVPYPQR	192-198	829.4446	2.9 / 1.4 / -	ACE-I; Anti-M; Anti-O	(61, 66, 72)

β-CN, β-casein; ACE-I, angiotensin-converting-enzyme inhibitory; Anti-M, antimicrobial; Anti-O, antioxidative; DPP-IV-I, dipeptidyl peptidase 4 inhibitory; Immuno-R, immunoregulatory. The Mass Error is shown in parts per million (ppm) and is calculated as $10^6\times$ (observed mass - theoretical mass) / theoretical mass. The three numbers in the Mass Error column represent the biological triplicates; dash symbol (-) signifies that the peptide was not detected in one of the triplicates.

712 **Table 3. Plasmids used in this study.**

Plasmid	Host	Description	Ab^r	reference
pCS1966	*E. coli*	*L. lactis* integration vector	Ery	(17)
pTLR	*E. coli*	*L. lactis* expression shuttle vector	Ery	Lab collection
pLP712	*L. lactis*	Prt$^+$ Lac$^+$, 54kb proteinase/lactose plasmid of NCDO712		(54)
pCS1966	*E. coli*	*L. lactis* integration vector	Ery	this study
pCH001	*E. coli*	*L. lactis* integration vector, knockout *pepF*$_2$	Ery	this study
pCH002	*E. coli*	*L. lactis* integration vector, knockout *pepO*$_2$	Ery	this study
pCH003	*E. coli*	*L. lactis* integration vector, knockout *pepA*	Ery	this study
pCH004	*E. coli*	*L. lactis* integration vector, knockout *pepP*	Ery	this study
pCH005	*E. coli*	*L. lactis* integration vector, knockout *pepV*	Ery	this study
pCH006	*E. coli*	*L. lactis* integration vector, knockout *pepM*	Ery	this study
pCH007	*E. coli*	*L. lactis* integration vector, knockout *pcp*	Ery	this study
pCH008	*E. coli*	*L. lactis* integration vector, knockout *pepQ*	Ery	this study
pCH009	*E. coli*	*L. lactis* integration vector, knockout *pepD*$_A$	Ery	this study
pCH010	*E. coli*	*L. lactis* integration vector, knockout *pepF*$_1$ in pLP712	Ery	this study
pCH011	*E. coli*	*L. lactis* integration vector, knockout *pepO*	Ery	this study
pCH012	*E. coli*	*L. lactis* integration vector, knockout *pepC*	Ery	this study
pCH013	*E. coli*	*L. lactis* integration vector, knockout *pepN*	Ery	this study
pCH014	*E. coli*	*L. lactis* integration vector, knockout *pepX*	Ery	this study
pCH015	*E. coli*	*L. lactis* integration vector, knockout *pepT*	Ery	this study
pCH016	*E. coli*	*L. lactis* integration vector, knockout *pepD*$_B$	Ery	this study
pCH020	*E. coli* & *L. lactis*	pTLR-PrtPM, for expression of protease PrtP and PrtM under its own promoter	Ery	this study

713 Ab^r=Antibiotic resistance. Ery = erythromycin.

714

715 **Figure 1 Peptidomics and bioactive peptide discovery in *L. lactis*.** (A) Schematic
716 representation of the mutated proteolytic system of *L. lactis* MG1363 with the cell envelope-
717 associated proteinase (PrtP). β-casein hydrolysis is initiated by PrtP, after its autoproteolytic
718 activation with the aid of PrtM (16)). Subsequently, the oligopeptides are transported into the
719 cells by the Opp transport system while di-/tripeptides are internalized by the Dpp or DtpT
720 transport system. The peptides are then degraded by the concerted action of 15 peptidases,
721 which are classified and colored by their indicated cleavage specificity. When a specific
722 combination of peptidase genes is deleted, certain peptides will stay intact. Theoretically,
723 small (bioactive) peptides, instead of free amino acids, will accumulate intracellularly. (B)
724 General sketch of the peptidase gene knockout vector. The integration vector is based on
725 plasmid pCS1966 (17). It contains an erythromycin-resistance gene (*ery*), orotate transporter
726 gene (*oroP*), and a knockout cassette carrying two homology regions, one containing the
727 region upstream of the gene to be deleted (HRU), the other encompassing a region
728 downstream thereof (HRD), for double crossover integration at a certain peptidase gene locus.
729

730 **Figure 2 *L. lactis* intracellular peptidomic sample preparation and data analysis**
731 **optimization.** (A) Sample preparation workflow (for details, see M&M). Cells from
732 exponentially growing *L. lactis* MG1363 and its peptidase knock-out mutants were incubated
733 under slow rotation (180 rpm/min) in a β-casein solution (4 mg/ml) for 3.5 hours, after which
734 they were disrupted by minibead-beating. The intracellular proteome was extracted and
735 passed through a 3-kDa molecular weight cut-off filter. The filtrate, which is the intracellular
736 peptidome, was desalted using C18 Zip-tips and analyzed by nanoLC-MS/MS. (B)
737 Computational data analysis. Mass spectrum data (MS.raw) output and the proteome of *L.*
738 *lactis* MG1363 plus β-casein sequence (both obtained from Uniprot) were used as inputs for
739 PEAKS studio analysis. The output from PEAKS was further visualized through R programming
740 and the peptidomics visualization web server Peptigram. (C) Comparison of 9 commonly used
741 search engines on the *L. lactis* MG1363 peptidome. The bar chart shows the number of unique
742 peptides identified by each search engine. The number (white) is the mean value from
743 biological triplicates. Standard deviation is shown at the top of each bar. (D)
744 Presence/absence map of unique peptides from panel 2C, identified by the indicated 6 search
745 engines on the biological replicates (roman numbers).
746

747 **Figure 3 Overview of mass spectrum results from PEAKS studio of intracellular**
748 **peptidomes of *L. lactis* MG1363 and 6 of its peptidase mutants.** (A) Peptide spectrum
749 matches (PSMs), identified unique peptides and proteins identified in the peptidomes of the
750 indicated strains. The bar charts show the mean value, the exact value of which is shown in
751 the bar, and standard deviation from biological triplicates of each strain. (B) Presence/absence
752 map of identified unique peptides from panel 3A. (C) Venn diagram of identified unique
753 proteins from panel 3A (http://bioinformatics.psb.ugent.be/webtools/Venn/).
754

755 **Figure 4 Ontology analysis of unique proteins identified in the intracellular peptidomes**
756 **of *L. lactis* MG1363 and the indicated 6 isogenic *pep* mutants.** Correlation of each protein
757 (outer circle on the left side of each graph) and Clusters of Orthologous Groups (COG)
758 category is represented by lines. Abbreviations of COGs are listed in the inset. The figure was
759 generated by R package Circlize (51).

760

761 **Figure 5 Intracellular profile and physicochemical properties of peptides derived from**
762 **β-casein after its initial hydrolysis by extracellular PrtP in *L. lactis* MG1363 and its *pep***
763 **mutants.** (A) Intracellular peptides assigned to β-casein by the PEAKS X studio software in the
764 peptidomes of the indicated strains. The visualization tool Peptigram (30) was used to
765 generate the figure. The linear sequence of bovine β-casein (224 amino acid residues
766 including the signal sequence f1-15) is shown at the top. Each vertical green bar represents an
767 amino acid residue in β-casein, with the height denoting the count and the color intensity
768 corresponding to the sum of peptides overlapping at this position. (B) Violin plots with mean
769 value (black dots) showing the physicochemical properties of the intracellular peptides
770 assigned to β-casein in the peptidomes of *L. lactis* MG1363 and its peptidase mutants. The
771 inset shows the strain identity; strains are shown in the same order in each panel.

772

773 **Figure 6 Optimization of a β-casein bioactive-peptide database.** (A) Optimization strategy.
774 BP, bioactive peptide. (B) UpSetR plot (53) highlighting the intersection of peptides with
775 bioactivities, as indicated by the bullet points. Horizontal bars (Set size) indicate the number
776 of bioactive peptides for each bioactivity. The vertical bars (Intersection size) show the
777 number of peptides with a certain bioactivity activity. A peptide can have more than 1
778 bioactivity, as indicated by the connected bullet points. For example, the set size of PEP-I is
779 four of which 3 peptides have only PEP-I activity while 1 peptide has both PEP-I and ACE-I
780 activity. PEP-I: prolyl endopeptidase inhibitory, AntiO: antioxidation, AntiM: antimicrobial,
781 DPP-IV-I: dipeptidyl peptidase IV inhibitory, Immuno: immunomodulatory, ACE-I: angiotensin-
782 converting enzyme inhibitory.

783

784 **Fig 7. β-casein-derived peptides identified in the intracellular peptidomes of**

785 **MG*ΔpepOF*$_2$*O*$_2$ (peptide sequences in red) and MG1363 (peptide sequences in black).**

786

787

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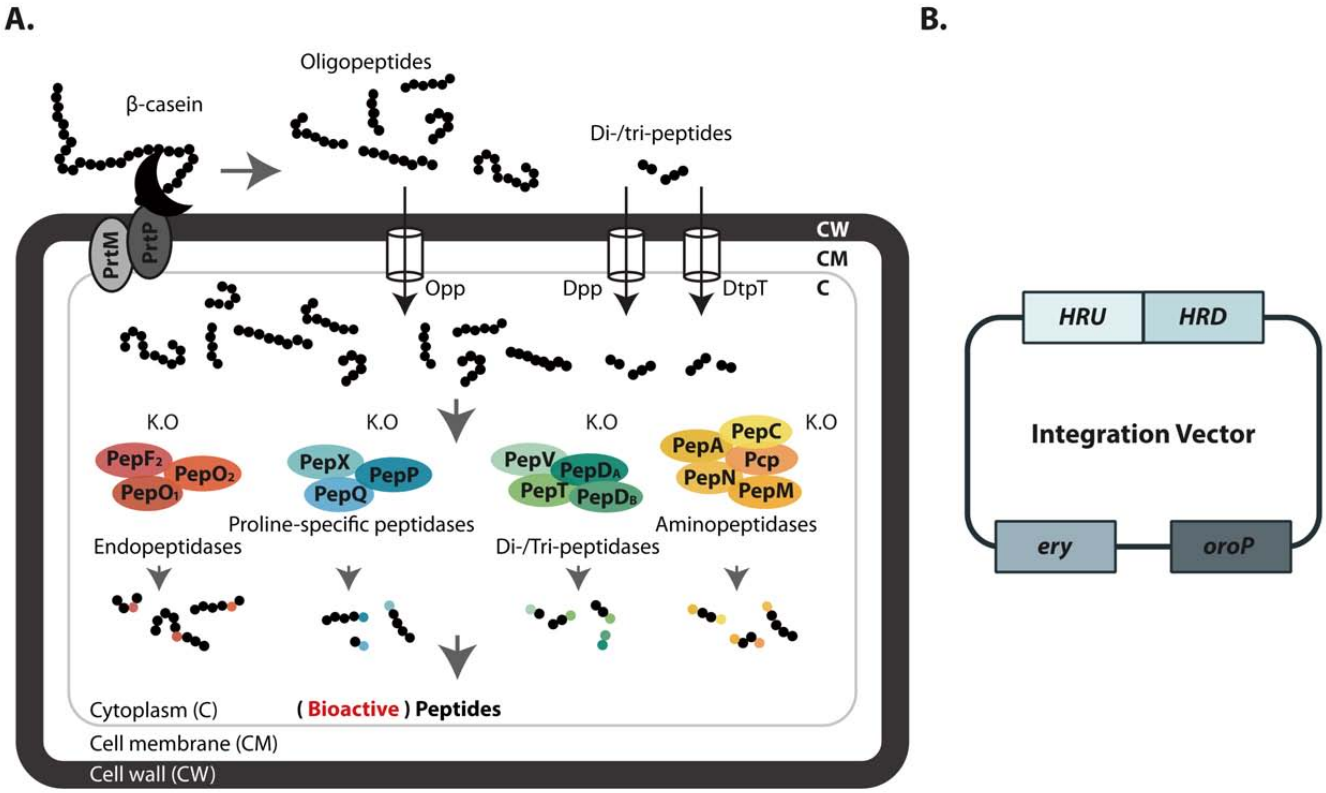
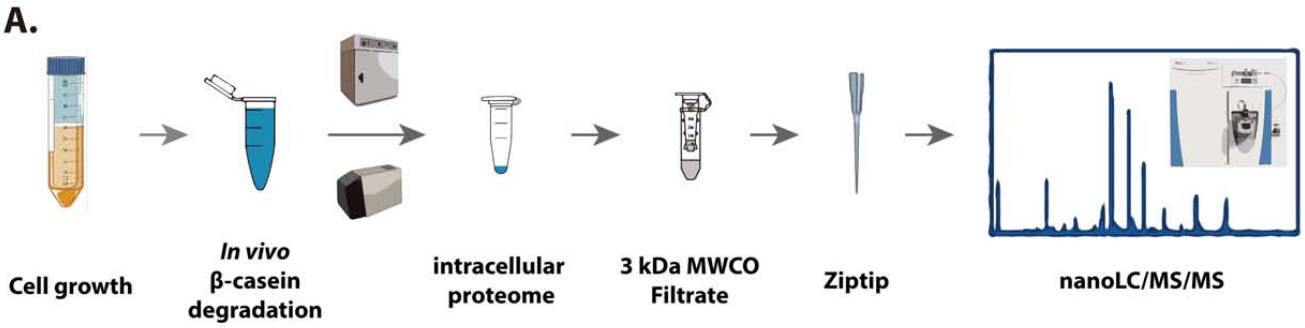
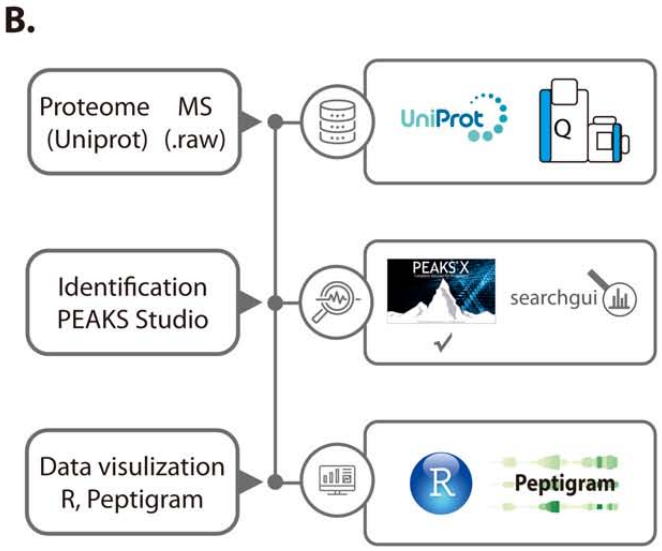
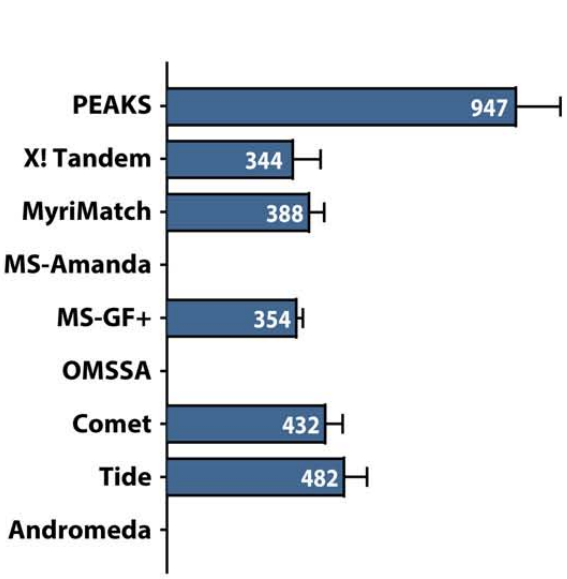
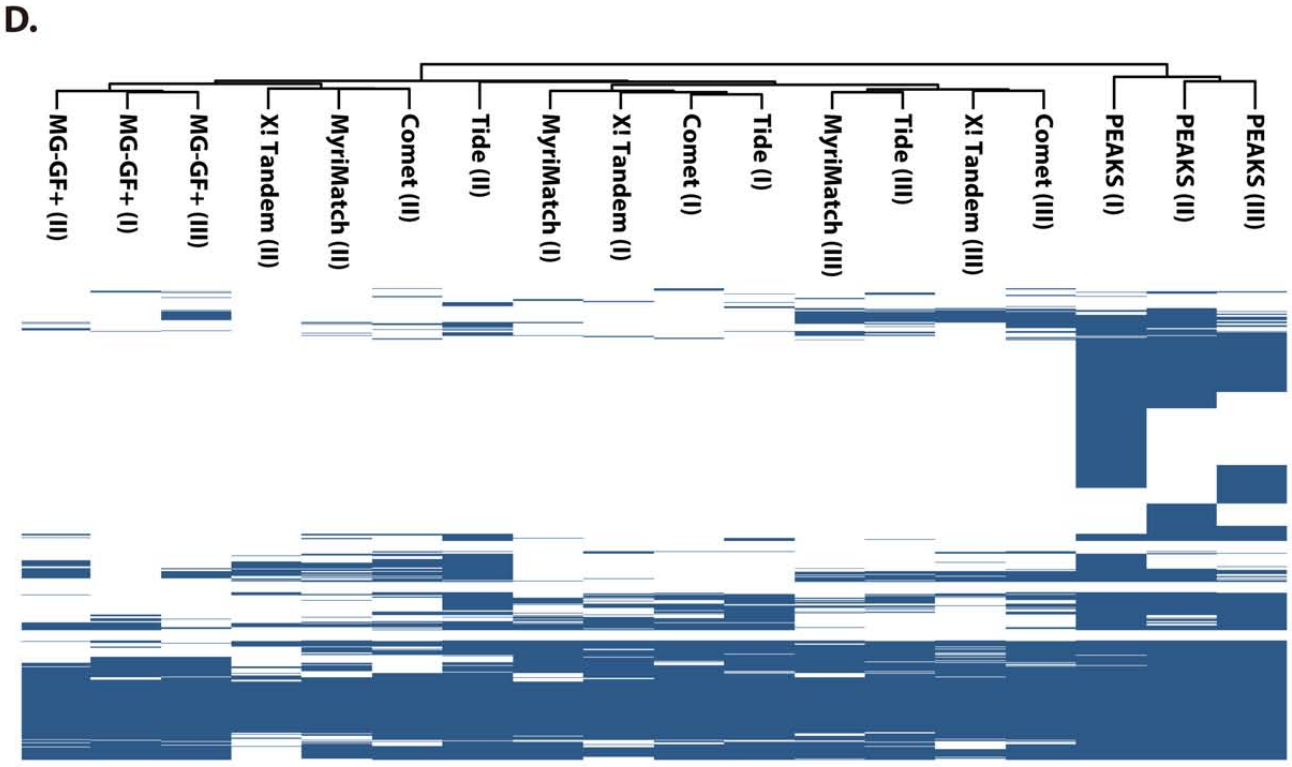
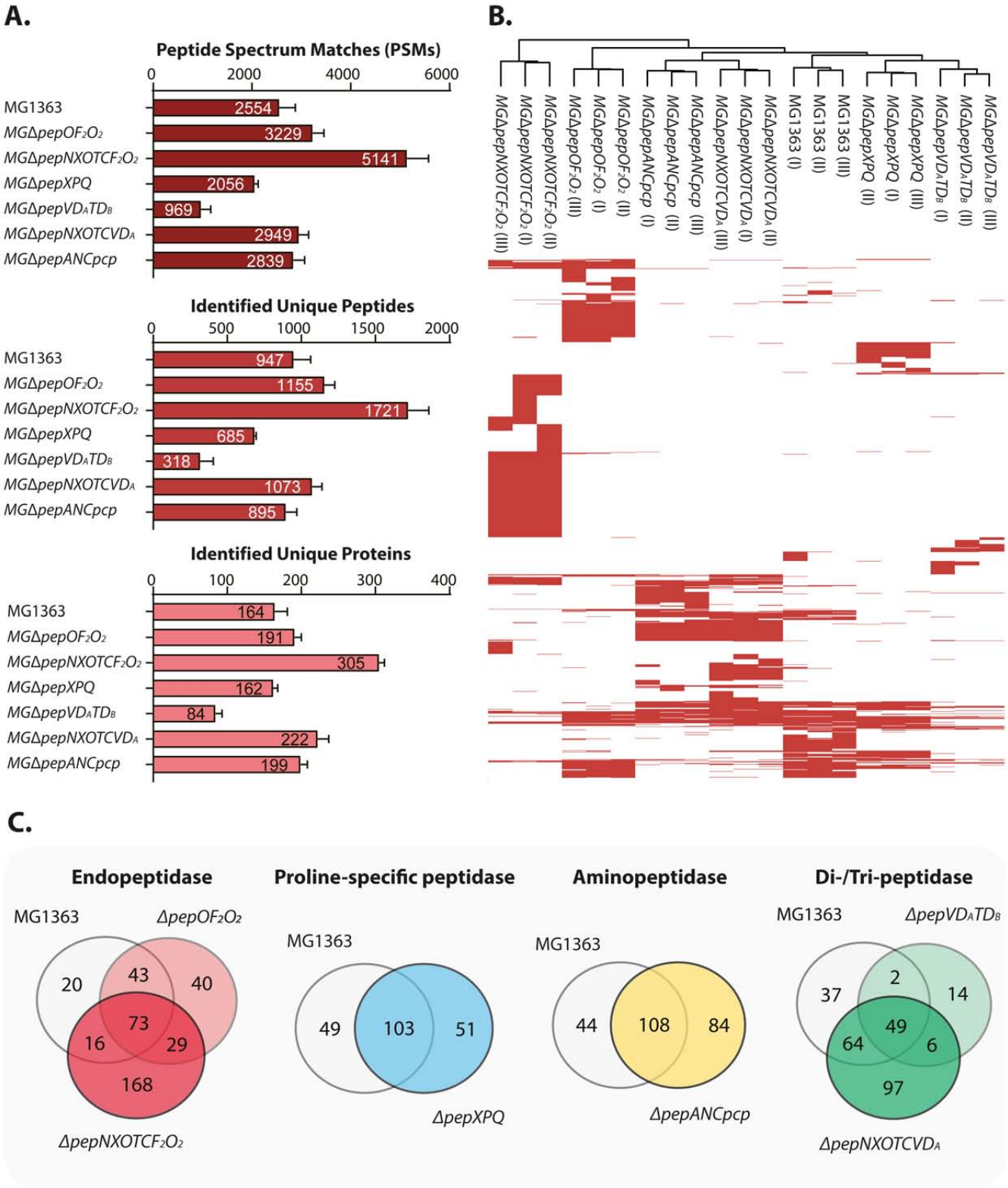


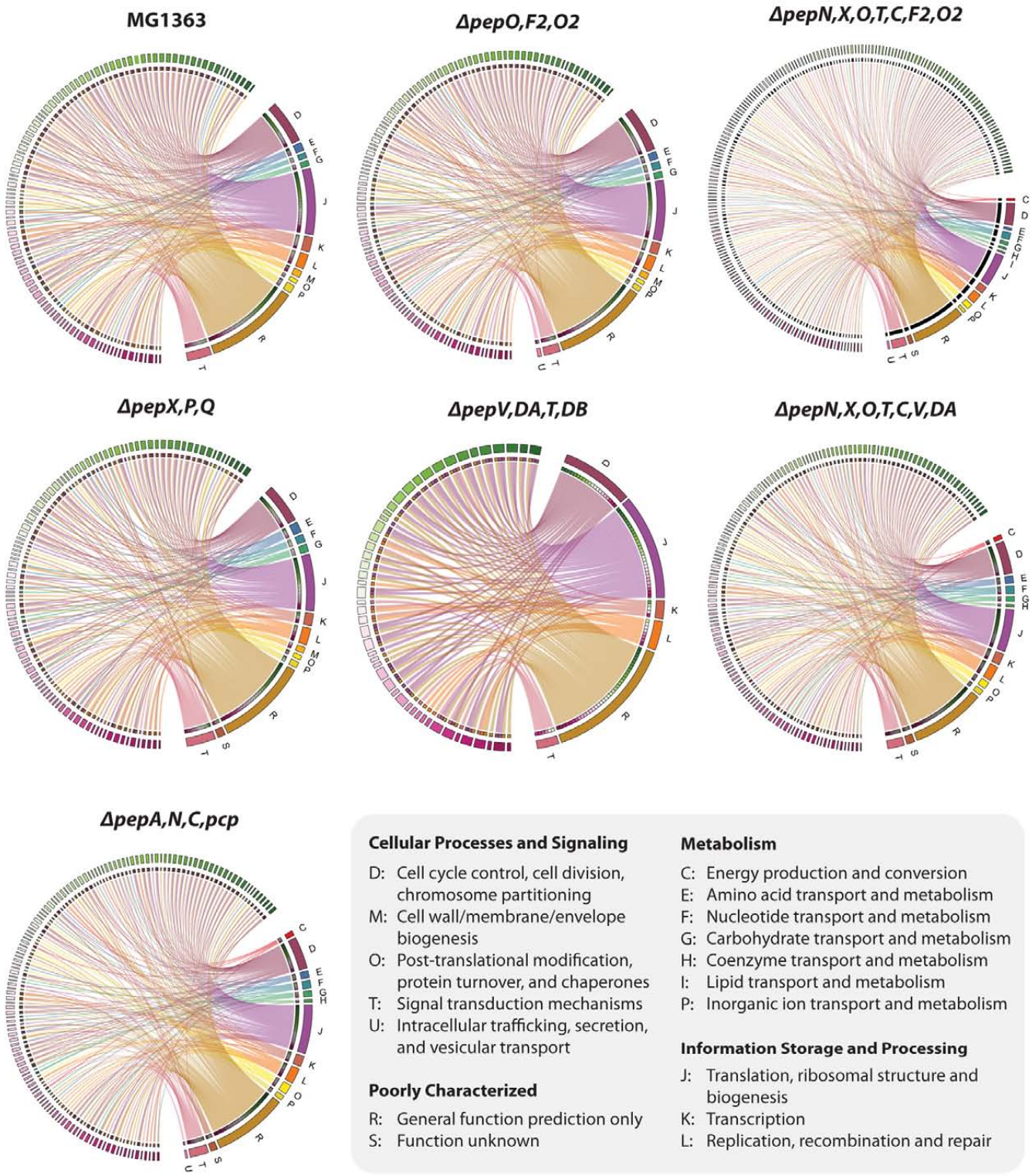












Cellular Processes and Signaling

- D: Cell cycle control, cell division, chromosome partitioning
- M: Cell wall/membrane/envelope biogenesis
- O: Post-translational modification, protein turnover, and chaperones
- T: Signal transduction mechanisms
- U: Intracellular trafficking, secretion, and vesicular transport

Poorly Characterized

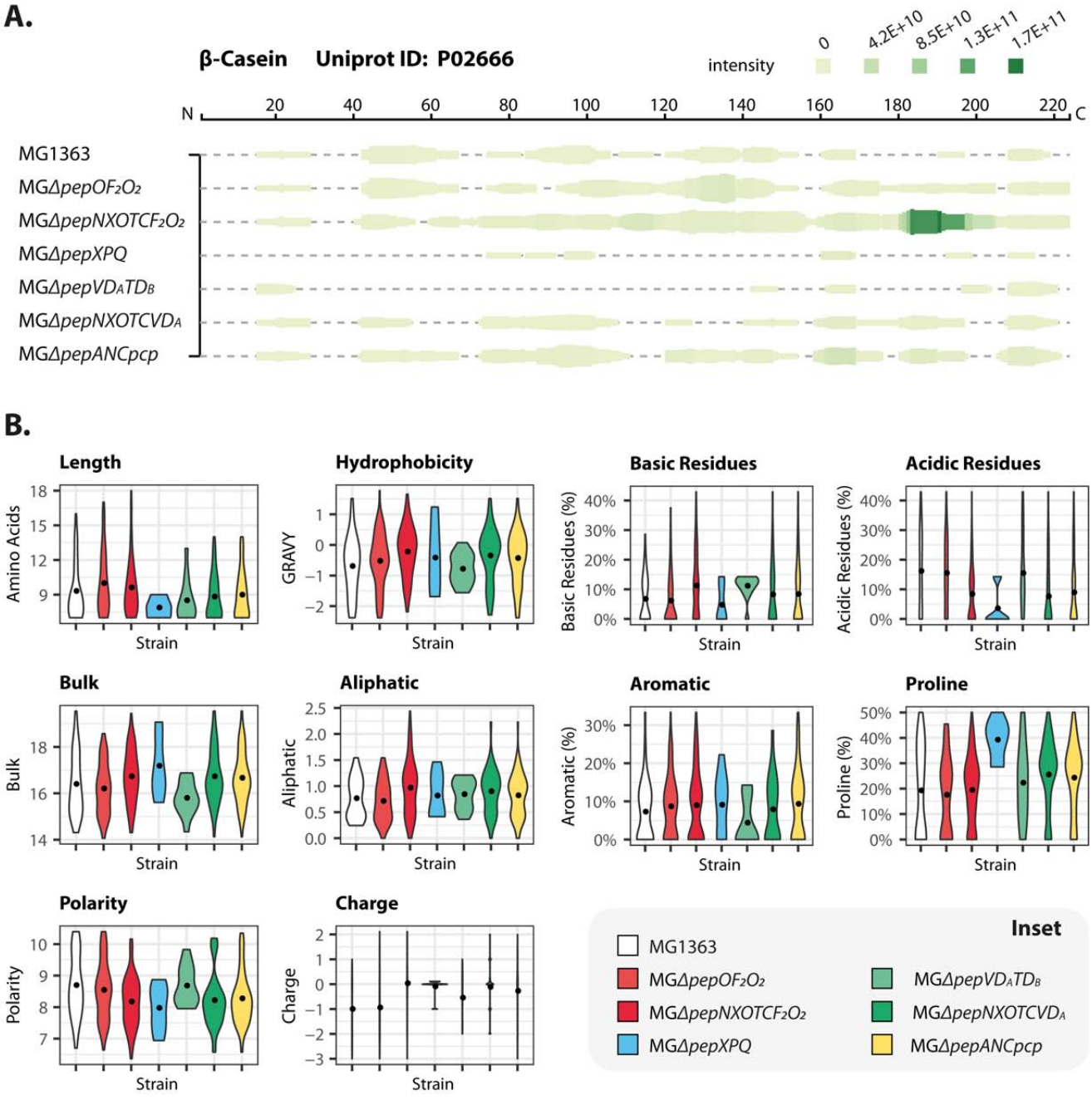
- R: General function prediction only
- S: Function unknown

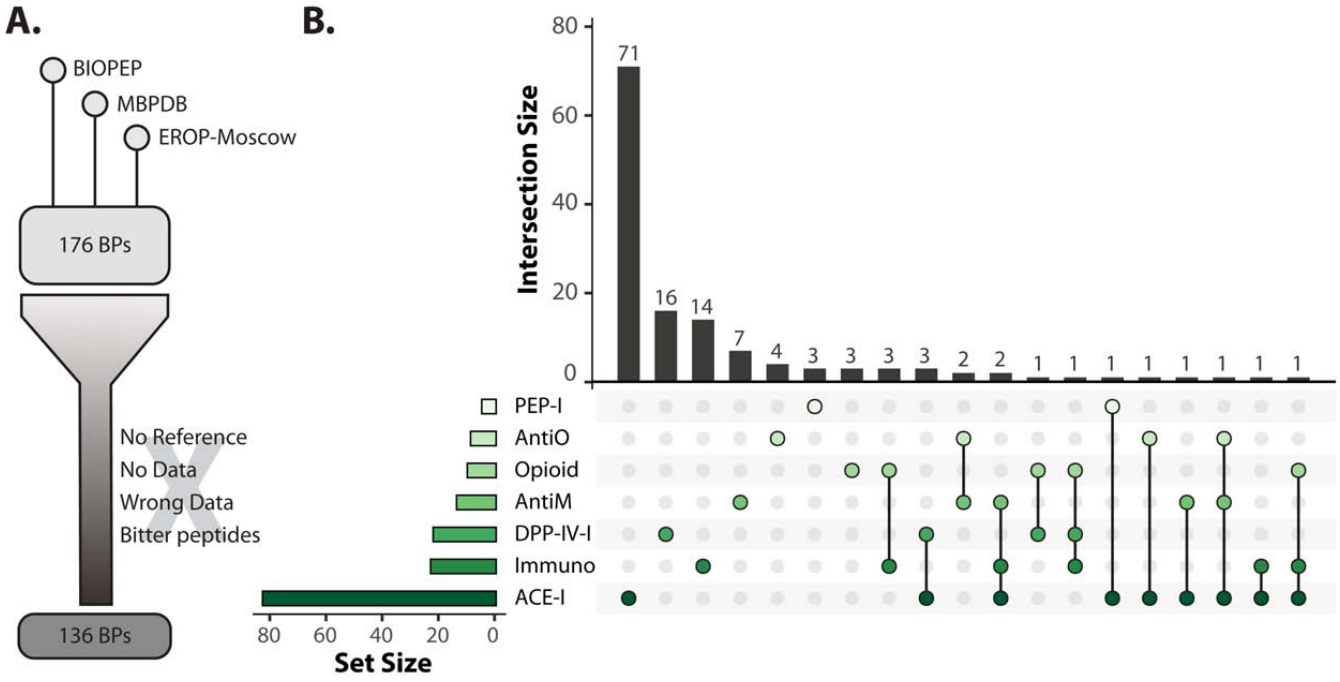
Metabolism

- C: Energy production and conversion
- E: Amino acid transport and metabolism
- F: Nucleotide transport and metabolism
- G: Carbohydrate transport and metabolism
- H: Coenzyme transport and metabolism
- I: Lipid transport and metabolism
- P: Inorganic ion transport and metabolism

Information Storage and Processing

- J: Translation, ribosomal structure and biogenesis
- K: Transcription
- L: Replication, recombination and repair





Strain	Identified peptides		
MG*ΔpepO*F_2O_2	TQTPVVVPPFLQPEVM	TPVVVPPFLQPEVM	PVVVPPFLQPEVM
MG1363	TQTPVVVPPFLQPE TQTPVVVPPF TQTPVVVPP	TPVVVPPF TPVVVPP	PVVVPPF