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Article

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On the Formation of Kokumi-Enhancing γ-Glutamyl Dipeptides in Parmesan Cheese by Means of γ-Glutamyltransferase Activity and Stable Isotope Double Labeling Studies

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ABSTRACT

Recently, γ-glutamyl dipeptides (γ-GPs) were found to be responsible for the attractive kokumi flavor of Parmesan cheese (PC). Quantitation of γ-GPs and their parent amino acids in 13, 24, and 30 months ripened PC samples by LC-MS/MS and stable isotope dilution analysis (SIDA), *in-cheese* ^{13}C-labelling studies, followed by analysis of the γ-glutamyl transferase (GGT) activity revealed γ-GPs to be generated most efficiently after 24 months of ripening by a GGT-catalyzed transfer of the γ-glutamyl moiety of L-glutamine onto various acceptor amino acids released upon casein proteolysis. Following the identification of milk as a potential GGT source in PC, the functionality of the milk's GGT to generate the target γ-GPs was validated by stable isotope double labeling (SIDL) experiments. Therefore, raw and heat-treated milk samples were incubated with L-glutamine-[U-^{13}C] and acceptor amino acids (X) and the hetero- (γ-Glu-[$^{13}C_5$]-X) and homotranspeptidation products (γ-Glu-Gln-[$^{13}C_{10}$]) were quantitated by LC-MS/MS-SIDA using γ-Glu-Ala-[$^{13}C_3$] as the internal standard. High GGT activity to generate the γ-GPs and preference for L-phenylalanine and L-methionine as acceptor amino acids was found in raw milk and milk samples heat-treated for 10 min up to a maximum of 65°C. In comparison, GGT activity and SIDL studies performed with inoculated *Lactobacillus* strains, including *L. harbinensis* and *L. casei* identified in PC by means of 16S rRNA gene sequencing, did not show any significant GGT activity and unequivocally demonstrated unpasteurized cow's milk, rather than microorganisms as a key factor in γ-glutamyl dipeptide generation in Parmesan cheese.

KEYWORDS:

Parmesan cheese, kokumi, γ-glutamyl peptides, GGT, γ-Glutamyl transferase, Lactobacillus, stable isotope labelling

INTRODUCTION

Besides their characteristic odors, cheeses are highly appreciated by consumers due to typical long-lasting taste profile. As the knowledge of the molecular blueprint of the cheeses' chemosensory signature is considered to be the prerequisite for a targeted tailoring of cheese manufacturing parameters, numerous studies have been performed in the last 20 years to identify the key molecules imparting the typical salty, sour, sweet, bitter, and umami taste of various cheeses.[1-9]

Taste reengineering and omission experiments revealed that besides basic taste compounds such as, e.g. amino acids, organic acids, minerals, a group of γ-L-glutamyl dipeptides are key to create the desirable long-lasting mouthfulness, complexity, and continuity of taste, coined kokumi taste,[9-13] perceived for matured cheeses such as, e.g. Gouda, Parmesan, as well as blue-veined cheeses like Blue Shropshire.[14] Intriguingly, this kokumi enhancing activity of the γ-L-glutamyl dipeptides is strongly structure dependent as the corresponding α-L-glutamyl dipeptides were found to be inactive.[9,14] Quantitation of γ-glutamyl peptides in Comté, Parmesan cheese, Gouda, Goat, Milner, Camembert, Mouton, Kernhem, Leerdamer, Swiss Gruyere, and Blue Shropshire cheese,[9,15-17] and analysis of enzyme activities proposed the γ-glutamyl transferase (GGT) to catalyze the generation of these taste enhancing peptides from the γ-glutamyl donor amino acid L-glutamine (**1**) via the covalent γ-glutamyl-enzyme conjugate (**2**) as the common key intermediate with another molecule L-glutamine to give the homotranspeptidation product γ-Glu-Gln (**3**) and any other L-amino acid to afford the heterotranspeptidation products γ-Glu-X (**4**), respectively (**Figure 1**).[17] Alternatively, hydrolytic cleavage of intermediate **2** can give rise to the free amino acid L-glutamate (**5**).

The γ-glutamyl transferase (GGT) has been reported to be present in mammalian tissues, some bacteria, and mold,[18-20] and, therefore, the source as well as the substrate specificity of the GGT is likely to differ depending on the cheese manufacturing parameters, as well as the microorganisms used as starter and ripening cultures. Well in line with the high amounts of γ-L-glutamyl dipeptides (~3.5 mmol/kg) reported in Blue Shropshire cheese, *Penicillium roquefortii* has been found to secrete the enzyme GTT and to be responsible for the γ-glutamyl dipeptide production in blue-mold cheese.[17] However, Parmesan cheese was very recently reported to contain γ-L-glutamyl dipeptides in by far higher amounts of ~20 mmol/kg,[9] thus opening the question as to how the peptides are formed in the absence of mold strains.

In order to determine the factors affecting γ-glutamyl dipeptide production in Parmesan cheese, the first objectives of the present investigation was to quantitate the kokumi-active γ-L-glutamyl dipeptides, their free precursor amino acids, as well as their sensorially inactive, isomeric α-L-glutamyl dipeptides in Parmesan cheeses differing in the ripening stage (13, 24, and 30 month). In order to locate the source of GGT activity in Parmesan cheese, the cheeses's microflora should be analyzed and the GGT activity of the bacterial strains identified as well as the GGT activity of raw and thermally treated milk samples should be determined. Finally, stable isotope double labeling (SIDL) experiments should be performed in cheese and milk samples using the stable-isotope labeled donor amino acid L-glutamine-[U-^{13}C] and non-labelled acceptor amino acids, and the *de-novo* generation of ^{13}C-labeled γ-glutamyl dipeptides should be quantitatively monitored by means of LC-MS/MS using γ-Glu-Ala-[$^{13}C_3$] as the internal standard.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), respectively. α- and γ-Glutamyl dipeptides as well as Gly-Gly were obtained from Bachem (Weil am Rhein, Germany), and L-glutamine-[U-^{13}C] was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). γ-Glu-Ala-[$^{13}C_3$] was synthesized as reported recently.[9] Solvents were of HPLC grade (Mallinckrodt Baker, Griesheim, Germany). Ultrapure water used for chromatography was purified by means of a MilliQ-water Gradient A 10 system (Millipore, Schwalbach, Germany). *Lactobacillus* strains DSM8744, DSM8745, DSM8746 (*L. rhamnosus*), DSM5622^T (*L. Paracasei ssp. paracasei*), DSM20258^T (*L. Paracasei ssp tolerans*) and TMW1.442 (*L. delbrueckii)* were obtained from Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising-Weihenstephan, Germany. Parmesan cheese samples ripened for 13 (PC-13), 24 (PC-24), and 30 (PC-30) months were obtained from a local Italian producer, delivered in 1 kg packages, and stored at -20 °C until use. Raw milk samples were obtained from a local farmer in Bavaria, Germany, kept at 4 °C, and used at the same day.

Preparation of the Water Soluble Extract (WSE). According to a previously published protocol,[8,9] a defined amount (50 g) of Parmesan cheese (PC-13, PC-24 and PC-30) was cut into small pieces, placed into a centrifuge beaker with deionized water (300 mL), homogenized for 5 min by means of an Ultra-Turrax T 25 digital (Ika Labortechnik, Staufen, Germany), and then centrifuged at 9000 rpm for 20 min at 4 °C by use of a Avanti J-E (Beckman- Coulter, Krefeld, Germany). The upper solid fat layer as well as the protein pellet was removed to afford the liquid layer including the cheese water solubles (pH 5.3). Protein pellet and fat layer were re-extracted with deionized

water (300 mL) as described above, the aqueous layers were pooled, and soluble casein was precipitated upon adjusting to pH 4.6 with traces of formic acid (1%, v/v; in water). After centrifugation (9000 rpm; 4 °C, 20 min), followed by paper filtration (Macherey-Nagel, 615-1/4) and freeze-drying (GAMMA 1/2-16LSC, Christ, Osterode, Germany), a casein-free water soluble extract (WSE) was obtained. WSE, protein pellet, and fat layer were stored at -20 °C until further analysis.

Quantitation of α- and γ-Glutamyl Dipeptides by Means of HPLC-MS/MS. Target peptides α- and γ-Glu-Gly, α- and γ-Glu-Ala, α- and γ-Glu-Val, α- and γ-Glu-Thr, α- and γ-Glu-Asp, α- and γ-Glu-Lys, α- and γ-Glu-Glu, α- and γ-Glu-Trp, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Gln, γ-Glu-Met, γ-Glu-His, γ-Glu-Phe, and γ-Glu-Tyr were quantitated in aliquots (1 g) of the cheese samples PC-13, PC-24, and PC-30 by means of a stable isotope dilution analysis using γ-Glu-Ala-[$^{13}C_3$] as the internal standard, following the protocol and using the mass spectrometric parameters reported recently.[9]

Quantitative Analysis of Free Amino Acids by means of LC-MS/MS. Free amino acids were quantified in samples of the lyophilized WSE (20 mg) prepared from cheese sample PC-13, PC-24 and PC-30 by means of HILIC-MS/MS and stable isotope dilution analysis (SIDA), following the analytical protocol and mass spectrometric parameters reported recently.[9]

Dry Matter Content. The dry matter content was determined in a vacuum drying oven kept at 65 °C as reported in the literature.[21]

Microflora Analysis. *Isolation of Bacteria from Cheese*. Cheese sample PC-13, PC-24, and PC-30 (10 g each), respectively, were weighted in a stomacher bag, peptone water (20 g/L, 90 mL) was added and, after homogenization in a stomacher (Stomacher 400, Colworth, London, GB) for 1 min, serial dilutions ranging from 10^{-2} to 10^{-5} were prepared from the single cheese suspensions. Aliquots (100 µL) of all dilutions were plated in duplicate on M17 medium, comprising 48 g/L of M17 and 15 g/L

of agar (for solid medium) as well as modified MRS medium (mMRS; pH 6.5) comprising 20 g/L of glucose, 10 g/L casein peptone, 5 g/L meat extract, 5 g/L yeast extract, 5 g/L sodium acetate, 2 g/L K_2HPO_4, 2 g/L diammonium citrate, 1 g/L Tween 80, 0.1 g/L $MgSO_4$ x $7H_2O$, 0.05 g/L $MnSO_4$ x H_2O, 0.2 mg/L each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin, and panthothenic acid, and 15 g/L of agar (for solid medium) and incubated for 5 days at room temperature, 30 and 37 °C, respectively.

Identification of Bacteria. From the obtained bacterial cultures, seven colonies (K1-K7) of a total of 334 were isolated and grown on solid mMRS medium. Cell morphology of two day incubated cultures was examined by normal light microscopy at x1200 magnification. Strains were grown in mMRS medium for 1 day at 30 °C and DNA was isolated by use of a bacterial DNA Kit E.Z.N.A. (Omega BioTek, Norcross, GA, USA) according to the manufacturer´s instructions. 16S rRNA gene sequencing was performed on the isolates in order to clarify their taxonomic position. The complete 16S rRNA gene was amplified with primers 616V (5´-AGAGTTTGATYMTGGCTCAG-3´) and 630R (5´-CAKAAAGGAGGTGATCC-3´). PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and eluted with elution buffer (60 µL). The sequences of the PCR products were identified via BLASTn (NCBI, Bethesda, MD, USA) search against the GenBank database (NCBI, Bethesda, MD, USA) and aligned by multiple-sequence comparison with the CLUSTAL W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/; EBI, Hinxton, UK).

Determination of the γ-Glutamyl Transferase (GGT) Activity. GGT activity in cheese and milk samples was determined by means of a photometric assay as reported earlier.[22] Raw milk and boiled milk (100°C, 60 s) were analyzed directly, or after heat treatment for 10 min at 55, 60, 65, 70, and 75 °C or for 60 min at 55 °C, respectively. Prior to analysis, milk samples were diluted 20-fold with sterilized milk. For photometric

analysis, a UV-2401 PC-type UV-Vis spectrophotometer (Shimadzu, Duisburg, Germany) was used. Data treatment was performed by use of the software UV Probe.

For determination of GGT activity in *L. casei* and *L. harbinensis*, isolated strains were grown in mMRS medium for 3 days at 30 °C, washed twice with tap water and, then, freeze dried. Preparation of harvested cells for the photometric measurement of GGT was done in two different ways. In a first experiment, the lyophilized cell material (10 mg) was extracted as described earlier.[17] Second, a defined amount (10 mg) of the obtained cell pellet was lysed with lysozyme (10 mg/mL,1 mL) in a water bath (37 °C, 1h), followed by sonication. Cell debris was removed from the cell-free extract by centrifugation (6000 g, 10 min) and an aliquot (200 µL) of the supernatant was used for measuring the GGT activity as reported earlier.[17]

Stable Isotope Double Labeling (SIDL) Study with *Lactobacillus* Strains. Isolated strains of *L. casei* and *L. harbinensis* as well as strains DSM8744, DSM8745, DSM8746 (*L. rhamnosus*), DSM5622^T (*L. paracasei ssp. paracasei*), DSM20258^T (*L. paracasei ssp tolerans*) and TMW1.442 (*L. delbrueckii*), were grown in mMRS medium for 3 days at 30 °C and washed twice with mMRS medium and incubation buffer (6.8 g/L KH_2PO_4, 0.1 g/L $MgSO_4$ x 7 H_2O, 0.05 g/L $MnSO_4$ x H_2O; adjusted to pH 6.5). To study the potential of these microorganisms to produce γ-glutamyl dipeptides, harvested cells were suspended in a mixture of incubation buffer (8 mL) and an aqueous solution (2 mL) of the γ-glutamyl donor amino acid L-glutamine-[U-^{13}C] (25 mmol/L) and the acceptor amino acids L-histidine, L-methionine, L-leucine, L-glutamic acid, and L-alanine (5 mmol/L, each). As a control, strains were suspended in incubation buffer lacking the amino acid mixture, respectively. All mixtures were incubated in sterile plastic tubes up to 21 days at room temperature. Aliquots (500 µL) were taken after 0, 1, 3, 7, 14, and 21 days, a defined amount of γ-Glu-Ala-[$^{13}C_3$] was added as internal standard for quantitation, membrane filtrated (Spartan, 13/0.45 µm RC, Schleicher & Schuell) and,

then, analyzed (5 µL) for the presence of the candidate heterotranspeptidation products γ-Glu-[$^{13}C_5$]-X and the ten-fold labeled homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] by means of HPLC-MS/MS as described below.

Stable Isotope Double Labeling (SIDL) Study in Parmesan Cheese. Cheese sample PC-24 was cut into small pieces and an aliquot (5 g) was weighted into a sterile glass vessel. An aqueous solution of the γ-glutamyl donor amino acid L-glutamine-[U-^{13}C] (20 mmol/L, 2.5 mL) was added and the mixture incubated at room temperature for 21 days whilst light shaking. As a control, a cheese sample was incubated with Millipore water (2.5 mL) and agitated under the same conditions. After incubation, a defined amount of γ-Glu-Ala-[$^{13}C_3$] was added as internal standard for quantitation and, then, the sample was analyzed for the presence of the heterotranspeptidation products γ-Glu-[$^{13}C_5$]-X and the ten-fold labeled homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] by means of HPLC-MS/MS as described below.

Stable Isotope Double Labeling (SIDL) Studies in Raw and Heat-Treated Milk. In a first experiment, aliquots (2 mL) of raw milk were placed in preheated reaction vessels, treated for 10 min at 55, 60, 65, 70, and 75 °C, respectively, and, then, cooled in an ice bath for 1 min. In addition, another raw milk sample was heated for 60 min at 55 °C and one sample was boiled at 100°C for 60 s. Aliquots (1 mL) of the raw milk and heat-treated milk samples were mixed with a solution (25 µL) of L-glutamine-[U-^{13}C] and L-glutamic acid (each 5 mmol/L) in boiled milk (expt. A). In a second set of experiments, raw milk samples (1 mL) were incubated with binary solutions (25 µL) containing L-glutamine-[U-^{13}C] (5 mmol/L in boiled milk) and L-glutamic acid, L-histidine, L-methionine, L-phenylalanine, L-lysine, L-leucine, L-aspartic acid or L-threonine (5 mmol/L in boiled milk, each), respectively (expt. B). In a third experiment, raw milk (1 mL) was incubated with a multi-component solution (25 µL) containing L-glutamine-[U-^{13}C] (40 mmol/L in boiled milk) and a mixture of L-glutamic acid, L-histidine, L-methionine, L-

phenylalanine, L-lysine, L-leucine, L-aspartic acid and L-threonine (5 mmol/L in boiled milk, each) (expt. C).

These treated milk samples (expt. A - C) were incubated for 30 min at 37 °C, then cooled to room temperature, an aliquot of each individual sample (500 µL) was transferred into an 1.5 mL Eppendorf cup, and the internal standard γ-Glu-Ala-[$^{13}C_3$] (3 mmol/L, 10 µL) was added. After vortexing for 30 s, acetonitrile (100 µL) was added, the sample vortexed again and, then, centrifuged (12000 rpm, 4 °C, 20 min). An aliquot (5 µL) of the supernatant was analyzed by means of LC-MS/MS for generated ^{13}C-labeled γ-glutamyl dipeptides as described below.

LC-MS/MS Analysis of Stable Isotope Labeled Peptides γ-Glu-[$^{13}C_5$]-X and γ-Glu-Gln-[$^{13}C_{10}$]. Using the multiple reaction monitoring (MRM) mode with the candidate mass transitions, given in **Table 1**, and by comparing of the retention times of the MS signals with those of the non-labeled reference compounds, the cheese and milk samples as well as the bacterial inoculations were screened for the 5-fold labelled heterotranspeptidation products γ-Glu-[$^{13}C_5$]-X and the ten-fold labeled homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] by means of HPLC-MS/MS. Accurate quantification of the $^{13}C_5$- and $^{13}C_{10}$-labeled γ-glutamyl dipeptides generated was performed using the internal standard γ-Glu-Ala-[$^{13}C_3$] as reported above for the non-labeled dipeptides, however, with a second set of calibration curves recorded from mixtures of the ^{13}C-labeled standard and all reference compounds in eight molar ratios from 0.005 to 3.0 keeping a constant concentration of the internal standard.

Liquid Chromatography/Mass Spectrometry (LC-MS/MS). LC-MS/MS measurements were acquired on an API 4000 Q-Trap LC-MS/MS system (Applied Biosystems Sciex Instruments, Darmstadt, Germany) connected to a 1200 HPLC-system (Agilent, Waldbronn, Germany) running in the positive electrospray ionization

253 (ESI^+) mode. Nitrogen served as the nebulizer gas (45 psi) and as turbo gas for solvent
254 drying (55 psi) as well as the curtain (20 psi) and collision gas ($4.5x10^{-5}$ Torr). Ion spray
255 voltage was set at 5500 V and the source temperature at 400 °C. Quadrupoles were set
256 at unit resolution. ESI^+ mass and product ion spectra were acquired with direct flow
257 infusion via syringe pump. For instrumentation control and data acquisition Sciex
258 Analyst software v1.5 (Applied Biosystems, Darmstadt, Germany) was used.
259
260

261 ## RESULTS AND DISCUSSION

262

263 As γ-glutamyl peptides have been recently shown to be present in Parmesan
264 cheese in remarkably high concentrations,[9] α- and γ-glutamyl dipeptides should be
265 quantitated in Parmesan cheeses differing in the ripening status to gain a further insight
266 into the factors governing the generation of these peptides. To achieve this, Parmesan
267 cheese matured for 13 (PC-13), 24 (PC-24), and 30 (PC-30) months was analyzed for
268 α- and γ-glutamyl dipeptides by means of LC-MS/MS and stable isotope dilution
269 analysis as reported recently.[9]

270 **Influence of Ripening Stage on the Levels of α- and γ-Glutamyl Dipeptides**
271 **and Free Amino Acids in Parmesan Cheese.** A total of 8 α- and 15 γ-glutamyl
272 peptides were quantitated in Parmesan cheese samples PC-13, PC-24, and PC-30 and
273 results were calculated on the basis of dry matter (**Table 2**). Among the α-glutamyl
274 dipeptides, α-Glu-Lys and α-Glu-Glu were the most predominant representatives with
275 concentrations of 90.2 (PC-13), 243.1 (PC-24), 106.9 µmol/kg (PC-30) and 64.7 (PC-
276 13), 178.7 (PC-24), and 108.3 µmol/kg (PC-30), respectively, thus confirming literature
277 data for α-Glu-Glu.[17] As the amino acid sequence α-L-glutamyl-L-lysine is present in the

sequence of α- and ß-casein, the formation of this α-glutamyl peptide is well in line with a proteolytic breakdown of these proteins. With increasing ripening degree from 13 to 24 months, the total amount of α-glutamyl dipeptides increased from 239 (PC-13) to 582 µmol/kg (PC-24) with α-Glu-Glu, α-Glu-Lys, and α-Glu-Thr showing the strongest increase, followed by a slight decrease to 339 µmol/kg (PC-30) with prolonging the ripening time to 30 months (**Table 2**), most likely due to their further enzymatic breakdown to give free amino acids.[23]

Also the γ-glutamyl dipeptide generation went through a maximum after a maturation time of 24 months (**Table 2**). γ-Glu-His was the most abundant representative showing an increasing concentration from 2807.8 (PC-13) to 8486.9 µmol/kg (PC-24), followed by a slight decrease to 6075.0 µmol/kg after 30 months (PC-30). The same trend was observed for the other γ-glutamyl dipeptides like the quantitatively predominating γ-Glu-Glu (1677.0 - 4564.9 µmol/kg) and γ-Glu-Thr (1026.8 - 3533.0 µmol/kg) exceeding a level of 3000 µmol/kg after 24 months. Among the γ-glutamyl peptides, γ-Glu-Lys, γ-Glu-Asp, γ-Glu-Val, γ-Glu-Thr, γ-Glu-Ile, γ-Glu-His, and γ-Glu-Phe showed the highest increase between 13 and 24 months, reflected by the high concentration ratio (PC-24/13) of 4.1-3.0 (**Table 2**). An exception was found for γ-Glu-Gln, which was present in its highest concentration after 24 months and, then, decreased by more than 50% with prolonging the ripening time to 30 months (**Table 2**).

The amount of all γ-glutamyl dipeptides totaled 10039 (PC-13), 28249 (PC-24), and 19724 µmol/kg (PC-30), thus confirming the extraordinarily high concentrations of γ-glutamyl dipeptides in Parmesan cheese when compared to other cheeses such as, e.g. Blue Shropshire (3590.0 µmol/kg), ripened goat cheese (2621.2 µmol/kg), Swiss Gruyere (2007.3 µmol/kg), Camembert (157.1 µmol/kg) matured Gouda cheese (89.5 µmol/kg), respectively.[17] Although both, α- and γ-glutamyl peptides, showed a decrease

in concentration from 24 to 30 months, the ratio of γ/α-glutamyl peptides increased strongly from 42.0 (PC-13) over 48.5 (PC-24) to 58.2 (PC-30), thus indicating a high transpeptidation activity of the enzyme GGT up to 24 months of ripening and an increased hydrolytic activity of the enzyme at a later maturation stage.[18]

As free amino acids, released upon proteolysis of milk proteins,[23] are considered as γ-glutamyl peptide precursors,[19] they were quantitated in PC-13, PC-24 and PC-30 by means of LC-MS/MS and stable isotope dilution analysis (**Table 3**). The amino acid concentrations increased from PC-13 to PC-24, followed by a slight decrease with extending the maturation to 30 months, which is well in line with their transformation into further reaction products including aroma active compounds.[24] In comparison to all other amino acids, the concentration of L-glutamine showed a significant decrease from 11.3 (PC-13) over 7.4 (PC-24) to 3.5 mmol/kg (PC-30), thus confirming its consumption as γ-glutamyl donor amino acid during GGT-catalyzed isopeptide generation. Confirming literature data,[25,26] glutamic acid, lysine, valine, leucine and isoleucine were formed as the most abundant free amino acids independent of the ripening stage (**Table 3**). This corroborates well with the likewise high concentrations of the α-glutamyl dipeptides γ-Glu-Glu, γ-Glu-Lys, γ-Glu-Val, and γ-Glu-Leu (**Table 2**), thus suggesting a high concentration of free acceptor amino acids to facilitate the generation of the corresponding γ-glutamyl peptide. Interestingly, the acceptor amino acids histidine and threonine, required for the generation of the two predominant γ-glutamyl dipeptides γ-Glu-His and γ-Glu-Thr, could only be found in low to moderate concentration ranges, e.g. 31.1 (PC-13), 47.8 (PC-24), and 40.1 mmol/kg (PC-30) for histidine and 49.4 (PC-13), 73.1 (PC-24), and 68.1 mmol/kg (PC-30) for threonine. However, histidine and threonine showed a high relative concentration increase as indicated by a relatively high PC-24/PC-13 ratio of 1.5 (**Table 2**). This dependency of peptide formation and release

of the corresponding amino acid could also be found for γ-Glu-Asp and aspartic acid, which both show one of the highest PC-24/PC-13 ratios of 3.8 and 1.6 respectively, as well as for γ-Glu-Trp (PC-24/PC-13: 3.2) and tryptophan (PC-24/PC-13: 1.5). The opposite was found for phenylalanine and its corresponding γ-glutamyl peptide, e.g. the phenylalanine concentration was only moderate and increased only slightly with prolonging maturation from 13 to 24 months (PC-24/13 ratio: 1.3), whereas γ-Glu-Phe was found amongst the most increasing peptides (PC-24/13 ratio: 3.0). This finding might indicate phenylalanine as a preferred acceptor amino acid for the GGT in Parmesan cheese.

Next to the availability of free precursor amino acids, the GGT's activity and substrate specificity seem to play a crucial role in γ-glutamyl dipeptide generation. Therefore, the following experiments targeted the identification of the GGT source in Parmesan cheese and the characterization of GGT's substrate specificity.

Characterization of the GGT Activity in Parmesan Cheese. In order to quantitate the GGT activity of Parmesan cheese, the samples PC-13, PC-24, and PC-30 were analyzed by a photometric measurement of *p*-nitroaniline liberated from the donor substrate γ-glutamyl-*p*-nitroanilide upon GGT-catalysed transfer of the γ-glutamyl moiety onto the acceptor substrate glycylglycine yielding γ-glutamylglycylglycine.[22] Independent of the maturation stage, very high GGT activities of 14.6, 15.3, and 14.7 U/g (dry matter) were found for the Parmesan cheese samples PC-13, PC-24, and PC-30 (**Table 4**), thus exceeding by far the GGT activity reported for other cheeses like Blue Shropshire (0.5 U/g), Gouda cheese (3.9 U/g), and Swiss Gruyere (2.5 U/g), respectively.[17] As milk cream has been reported to show higher GGT activity than compared to whole milk,[27] this high GGT activity in Parmesan cheese might be due to the higher proportion of milk cream used in Parmesan cheese manufacturing.

To further confirm the findings of the photometric GGT activity measurement and gain a further insight into the enzyme's substrate specificity, a stable isotope double labeling (SIDL) study was performed with the cheese sample P-24. The cheese sample was spiked with an aqueous solution of the stable isotope labelled γ-glutamyl donor amino acid L-glutamine-[U-^{13}C] and, after incubation for three weeks at r.t., a defined amount of γ-Glu-Ala-[$^{13}C_3$] was added as internal standard for quantitation and, then, the sample was analyzed for the presence of the heterotranspeptidation products γ-Glu-[$^{13}C_5$]-X and the ten-fold labeled homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] by means of HPLC-MS/MS. In a control experiment, cheese sample PC-24 was spiked with a small volume of water (blank). Mass transitions for the^{13}C-labeled γ-glutamyl dipeptides were calculated on basis of the optimized mass transitions of their corresponding unlabeled twin molecules, whereas the characteristic y_1 and b_1-CO_2 fragment ions were used for detection (**Figure 1**). As L-glutamine-[U-^{13}C] itself could serve as an acceptor amino acid, the calculated mass transitions of the five-fold labeled heterotranspeptidation products (γ-Glu-[$^{13}C_5$]-X) and the ten-fold labeled homotranspeptidation product (γ-Glu-Gln-[$^{13}C_{10}$]) were selected for MS-screening. Using these calculated mass transitions (**Table 1**), comparing the retention times of the MS-peaks recorded for γ-Glu-[$^{13}C_5$]-X and γ-Glu-Gln-[$^{13}C_{10}$] with those of the unlabeled γ-glutamyl dipeptides, followed by co-chromatography revealed a total of 14 ^{13}C-labeled γ-glutamyl dipeptides, namely γ-Glu-[$^{13}C_5$]-Lys, γ-Glu-[$^{13}C_5$]-His, γ-Glu-[$^{13}C_5$]-Gln, γ-Glu-[$^{13}C_5$]-Phe, γ-Glu-[$^{13}C_5$]-Glu, γ-Glu-[$^{13}C_5$]-Met, γ-Glu-[$^{13}C_5$]-Thr, γ-Glu-[$^{13}C_5$]-Gly, γ-Glu-[$^{13}C_5$]-Val, γ-Glu-[$^{13}C_5$]-Leu, γ-Glu-[$^{13}C_5$]-Asp, γ-Glu-[$^{13}C_5$]-Trp, γ-Glu-[$^{13}C_5$]-Tyr, and γ-Glu-Gln-[$^{13}C_{10}$] in the cheese sample spiked with L-glutamine-[U-^{13}C] prior to incubation, while no peaks could be found in the blank sample. As an example, the MS-signals of six *de-novo* generated $^{13}C_5$-labeled γ-glutamyl dipeptides and their corresponding

unlabeled isomers are shown in **Figure 2**. Interestingly, γ-Glu-[$^{13}C_5$]-Ala and γ-Glu-[$^{13}C_5$]-Ile were not detectable in the cheese spiked with L-glutamine-[U-^{13}C], thus indicating a lower specificity for the acceptor amino acids L-alanine and L-isoleucine. Quantitation of the *de-novo* generated peptides γ-Glu-[$^{13}C_5$]-X and γ-Glu-Gln-[$^{13}C_{10}$] by means of LC-MS/MS and stable isotope dilution analysis using γ-Glu-Ala-[$^{13}C_3$] as the labelled internal standard revealed γ-Glu-[$^{13}C_5$]-Lys (37.35 µmol/kg), γ-Glu-[$^{13}C_5$]-His (10.30 µmol/kg), γ-Glu-[$^{13}C_5$]-Phe (3.36 µmol/kg), and γ-Glu-[$^{13}C_5$]-Glu (2.04 µmol/kg) as the major peptides (**Table 5**). As these data clearly demonstrate significant GGT activity to generate γ-glutamyl dipeptides, the following experiments were targeting the origin of the enzyme GGT and should answer the question as to whether the GGT is derived from the cheese's microflora or from the milk used for cheese manufacturing.

Microflora Analysis in Parmesan Cheese and GGT Avtivity of *Lactobacillus* Strains. To analyze the cheese's microflora, peptone extracts were prepared from samples PC-13, PC-24 and PC-30, and serial dilutions of all three extracts were plated on M17 and mMRS solid medium, respectively. After 5 days incubation at room temperature, 30 and 37 °C, respectively, microbial growth could only be observed when the PC-13 extract was incubated at 30 °C and 37 °C, respectively. The number of colony forming units (CFU) was 80 (30 °C) and 91 (37 °C) on M17 medium and 98 (30 °C) and 65 (37 °C) when grown on mMRS medium. Seven colonies were selected according to differences in colony morphology and RAPD pattern (data not shown) and, after 16S rRNA gene sequencing, *Lactobacillus harbinensis* (K1, K3, and K6) and *Lactobacillus casei* (K2, K4, K5, and K7) strains were identified. *Lactobacillus casei* is well-known to be present in Parmesan cheese in the early as well as advanced ripening stages, along with *L. paracasei ssp paracasei*, *L. paracasei ssp tolerans*, *L. rhamnosus* and *Pediococcus acidilactici*.[28,29] Interestingly, *L. harbinensis*, earlier reported in

fermented Chinese cabbage and Korean rice wine,[30,31] has been found for the first time as a constituent of the Parmesan cheese's microflora.

In order to investigate whether the microflora could serve as a GGT source in Parmesan cheese, the identified *Lactobacillus harbinensis* and *Lactobacillus casei* strains were analyzed in vitro for their GGT activity using the photometric assay. However, well in line with previous reports,[32] none of the inoculated strains did show any significant GGT activity. To further verify this observation and to extend the analysis of the GGT activity to other microorganisms earlier reported in Parmesan cheese, *L. delbrueckii*, known to be present in the starter cultures,[28] and *L. paracasei ssp. paracasei, L. paracasei ssp. tolerans*, and *L. rhamnosus,* found as microflora constituents in early ripening stages,[28] were used for the experiments next to *Lactobacillus harbinensis* and *Lactobacillus casei* identified above. After growth in MRS medium for three days, harvested cells of the *Lactobacillus* strains were incubated with a solution containing L-glutamine-[U-^{13}C] and a mixture of L-histidine, L-methionine, L-leucine, L-glutamic acid, and L-alanine for 0, 1, 3, 7, 14, and 21 days. However, HPLC-MS/MS did not show any trace amounts of γ-Glu-[$^{13}C_5$]-X and γ-Glu-Gln-[$^{13}C_{10}$], thus confirming that these *Lactobacillus* strains do not contribute to γ-glutamyl dipeptide generation in Parmesan cheese.

Analysis of the GGT Activity of Milk Samples. To investigate the GGT activity in milk samples, raw milk and thermally treated milk samples were analyzed by means of the photometric GGT assay. A high GGT activity of 5.3 U/mL was found in raw milk, whilst boiled milk (100°C, 60 s) did not show any GGT activity (**Table 4**), thus confirming earlier findings.[17,22,27] To monitor the influence of heat treatment on GGT activity, raw milk samples were heated for 10 min at 55, 60, 65, 70, and 75 °C, respectively, and an additional milk sample was heated for 60 min at 55 °C matching the parameters used in Parmesan cheese manufacturing. A constant decrease of GGT activity was found with

429 increasing temperature from 4.8 U/mL (55 °C, 10 min) to 0.2 U/mL (75 °C, 10 min),
430 while the GGT activity of the milk sample treated for 60 min at 55 °C (4.5 U/mL) was
431 very similar to that of raw milk (**Table 4**).

432 To gain some first insight into the substrate specificity of the milk's GGT, a series
433 of stable isotope double labeling studies have been performed with raw milk spiked with
434 L-glutamine-[U-^{13}C] and single or several L-amino acids. As the concentration of free
435 amino acids in milk is very low,[33] first, a raw milk sample was incubated with the donor
436 amino acid L-glutamine-[U-^{13}C] and the acceptor amino acid L-glutamic acid for 30 min
437 at 37 °C, followed by quantitative LC-MS/MS of ^{13}C-labeled γ-glutamyl peptides using γ-
438 Glu-Ala-[$^{13}C_3$] as internal standard. As given in **Figure 3**, the homotranspeptidation
439 product γ-Glu-Gln-[$^{13}C_{10}$] and the heterotranspeptidation product γ-Glu-[$^{13}C_5$]-Glu were
440 found in precursor-spiked raw milk after incubation, whereas LC-MS/MS analysis the
441 non-spiked milk sample (control) did not show any traces of the labeled peptides.

442 In order to answer the question as to how heat treatment of the milk is affecting
443 GGT activity, raw and heat-treated milk samples were incubated with L-glutamine-
444 [U-^{13}C] and L-glutamic acid, then, the internal standard γ-Glu-Ala-[$^{13}C_3$] was added, and
445 γ-Glu-[$^{13}C_5$]-Glu and γ-Glu-Gln-[$^{13}C_{10}$] were analyzed by means of LC-MS/MS-SIDA.
446 The concentrations of γ-Glu-[$^{13}C_5$]-Glu and γ-Glu-Gln-[$^{13}C_{10}$] remained constant up to a
447 temperature of 65°C, thereafter a rapid decline of the target peptides was observed and
448 diminished completely when the milk was heated for 1 min at 75°C, nicely running in
449 parallel to the GGT activity analyzed by means of the photometric assay (**Figure 4**).
450 Interestingly, γ-Glu-Gln-[$^{13}C_{10}$] was always found in higher concentrations than γ-Glu-
451 [$^{13}C_5$]-Glu, thus indicating L-glutamine as the better acceptor amino acid in comparison
452 to L-glutamic acid, which is in line with earlier data.[17,19,20,34]

To gain some more insight into the GGT's substrate specificity, milk samples were incubated with binary mixtures of the donor amino acid L-glutamine-[U-^{13}C] and the acceptor amino acid L-methionine, L-phenylalanine, L-lysine, L-histidine, L-leucine, L-aspartic acid, L-threonine or L-glutamic acid, respectively, and the corresponding heterotranspeptidation products γ-Glu-[$^{13}C_5$]-X as well as the homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] were quantitated by means of LC-MS/MS and stable isotope dilution analysis using γ-Glu-Ala-[$^{13}C_3$] as the internal standard (**Figure 5**). Among the amino acids tested, L-methionine, L-phenylalanine, L-lysine, and L-histidine were transformed more efficiently into the corresponding heterotranspeptidation products, whereas incubation of L-glutamine-[U-^{13}C] with L-leucine, L-aspartic acid, L-threonine, or L-glutamic acid resulted in a preferred generation of the homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$] (**Figure 5**). These data clearly indicate a higher substrate specificity of the milk's GGT for L-methionine, L-lysine, L-histidine, and L-phenylalanine, the latter of which is in contrast to the poor acceptor specificity reported in literature.[35] Interestingly, the acceptor amino acid also seemed to have an influence on the generation of the homotranspeptidation product γ-Glu-Gln-[$^{13}C_{10}$]. For example, in the incubation mixture of L-glutamine-[U-^{13}C] and L-methionine, γ-Glu-[$^{13}C_5$]-Met and γ-Glu-Gln-[$^{13}C_{10}$] were found in concentrations of 1437.4 and 201.8 nmol/mL. Incubation of raw milk with L-glutamine-[U-^{13}C] and L-phenylalanine generated the heterotranspeptidation product in a similar amount (1433.1 nmol/mL), while γ-Glu-Gln-[$^{13}C_{10}$] was formed only at very low levels of 45.1 nmol/mL (**Figure 5**). In comparison, incubation of raw milk with L-glutamine-[U-^{13}C] and L-histidine delivered only low concentrations of γ-Glu-Gln-[$^{13}C_{10}$] (80.8 nmol/mL), which is nearly twice as much as in the L-phenylalanine-containing experiment, and a relatively low amount of γ-Glu-[$^{13}C_5$]-His (132.1 nmol/mL). Incubating raw milk with L-glutamine-[U-^{13}C] and L-threonine γ-Glu-Gln-[$^{13}C_{10}$] revealed γ-Glu-Gln-

[$^{13}C_{10}$] also in a concentration of ~80 nmol/mL, but the heterotranspeptidation product (γ-Glu-[$^{13}C_5$]-Thr) was generated in an amount of only 15.5 nmol/mL (**Figure 5**). It might be concluded that not only the specificity for heterotranspeptidation but also the activity for homotranspeptidation depend on the available acceptor amino acid. L-Methionine, L-lysine and L-histidine were good acceptors and simultaneously led to an increased generation of the homotranspeptidation product. L-Threonine was a poor γ-glutamyl acceptor but did facilitate the generation of γ-Glu-Gln-[$^{13}C_{10}$], whereas L-phenylalanine was a good γ-glutamyl acceptor, but seemed to suppress the generation of γ-Glu-Gln-[$^{13}C_{10}$], indicating an inhibitory effect as recently reported for glutathione.[36]

In order to compare the substrate specificity of milk's GGT in a competitive multicomponent mixture, raw milk was incubated with a mixture of the amino acids L-glutamine-[U-^{13}C], L-glutamic acid, L-histidine, L-methionine, L-phenylalanine, L-lysine, L-leucine, L-aspartic acid, and L-threonine. Quantitative LC-MS/MS analysis of the respective homo- and heterotranspeptidation products confirmed L-phenylalanine and L-methionine as the best γ-glutamyl acceptors yielding the respective ^{13}C-labeled peptides in concentrations of 797 and 635 nmol/mL, respectively (**Figure 6**). Moderate concentrations of ~50 nmol/L were found for γ-Glu-[$^{13}C_5$]-Lys and γ-Glu-[$^{13}C_5$]-His, while the other candidate peptides were detectable only in trace amounts (< 5 nmol/mL), thus confirming the trends observed in the binary incubation experiments (**Figure 5**).

Taking all data into consideration, the raw milk that is slightly heat-treated (55°C, 60 min) during Parmesan cheese manufacturing, rather than microorganisms seems to be the main GGT source in Parmesan cheese. On a quantitative basis, the findings of these precursor incubation experiments in milk (**Figure 5, 6**) did only partially reflect the results of the incubation experiments performed in Parmesan cheese (**Table 5**). In both experiments, γ-Glu-[$^{13}C_5$]-Lys, γ-Glu-[$^{13}C_5$]-His, γ-Glu-[$^{13}C_5$]-Gln, γ-Glu-[$^{13}C_5$]-Phe, and γ-

Glu-[$^{13}C_5$]-Met were identified as major peptides, but in Parmesan cheese matrix γ-Glu-[$^{13}C_5$]-Lys, γ-Glu-[$^{13}C_5$]-His, and γ-Glu-[$^{13}C_5$]-Gln were generated in a significantly higher concentration than γ-Glu-[$^{13}C_5$]-Phe and γ-Glu-[$^{13}C_5$]-Met, respectively. These data indicate that, besides varying concentrations of and GGT's specificity for acceptor amino acids, additional factors in the cheese matrix like short-chain peptides, released upon proteolysis of milk proteins, might modulate GGT's substrate specificity and heterotranspeptidation activity, a phenomenon that has been recently shown for the tripeptide glutathione,[36] and, in consequence, facilitate the generation of cheese-specific profiles of kokumi-enhancing γ-glutamyl dipeptides.

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SUPPORTING INFORMATION

Growth conditions, taxonomic identity and Neighbor-Joining dendrogram of the seven bacteria colonies (K1-K7) isolated from Parmesan cheese (PC-13) are available in the supporting information.

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FIGURE LEGEND

Figure 1. Glutamyltransferase (GGT)-catalyzed transformation of the γ-glutamyl donor amino acid γ-Gln-[$^{13}C_5$] (**1**) via the key intermediate **2** to give (**A**) the homotranspeptidation product γ-Glu-[$^{13}C_5$]-Gln (**3**) upon reaction with a second molecule of γ-Gln-[$^{13}C_5$], (**B**) the heterotranspeptidation product γ-Glu-[$^{13}C_5$]-X (**4**) upon reaction with another acceptor amino acid, and (**C**) hydrolysis to give L-glutamate (**5**). The MS/MS fragments selected for the MS-screening of Glu-[$^{13}C_5$]-X is given in the chemical structure of **4**. Isotope labeled carbon atoms are marked with asterisks.

Figure 2. HPLC-MS/MS chromatograms (MRM, ESI$^+$) of α-/γ-Glu-Gly (**A**), α-/γ-Glu-Val (**C**), α-/γ-Glu-Glu (**E**), γ-Glu-Met (**G**), γ-Glu-His (**I**), and γ-Glu-Phe (**L**) in aqueous reference solution and of their corresponding $^{13}C_5$-labeled twins γ-Glu-[$^{13}C_5$]-Gly (**B**), γ-Glu-[$^{13}C_5$]-Val (**D**), γ-Glu-[$^{13}C_5$]-Glu (**F**), γ-Glu-[$^{13}C_5$]-Met (**H**), γ-Glu-[$^{13}C_5$]-His (**K**), and γ-Glu-[$^{13}C_5$]-Phe (**M**) in the extract of PC-24 incubated with L-glutamine-[U-^{13}C] for 21 days at r.t..

Figure 3. HPLC-MS/MS chromatograms (MRM, ESI$^+$) of γ-Glu-Gln (**A**) and γ-Glu-Glu (**D**) in reference solution, their corresponding ^{13}C-labeled twins γ-Glu-Gln-[$^{13}C_{10}$] (**B**) and γ-Glu-[$^{13}C_5$]-Glu (**E**) in raw milk incubated for 30 min at 37 °C with L-Gln-[U-^{13}C] and L-Glu (5 mmol/L, each), and of γ-Glu-Gln-[$^{13}C_{10}$] (**C**) and γ-Glu-[$^{13}C_5$]-Glu (**F**) in control sample (raw milk, not incubated, but heated for 30 min at 37 °C).

Figure 4. Yields [nmol/mL milk ±SD; n = 3] of γ-Glu-[$^{13}C_5$]-Glu and γ-Glu-Gln-[$^{13}C_{10}$] in raw milk and in milk samples heat-treated for 10 min at 55, 60, 65, 70 and 75 °C, and then incubated (30 min, 37 °C) with L-glutamine-[U-^{13}C] and L-glutamic acid (5 mmol/L, each). The GGT activity [U/mL milk±SD; n = 3] of the same samples were measured by means of a photometric assay.

Figure 5. Yields (nmol/mL milk ±SD; n = 3) of ^{13}C-labeled γ-glutamyl peptides, generated upon the incubation (30 min, 37 °C) of raw milk samples spiked with binary mixtures of L-glutamine-[U-^{13}C] (5 mmol/L) and L-methionine (**A**), L-phenylalanine (**B**), L-lysine (**C**), L-histidine (**D**), L-leucine (**E**), L-aspartic acid (**F**), L-threonine (**G**), and L-glutamic acid (**H**), respectively (5 mmol/L, each).

Figure 6. Yields (nmol/mL milk ±SD; n = 3) of ^{13}C-labeled γ-glutamyl peptides generated upon the incubation (30 min, 37 °C) of raw milk samples spiked with a mixture of L-glutamine-[U-^{13}C] (40 mmol/L) and L-methionine, L-phenylalanine, L-lysine, L-histidine, L-leucine, L-aspartic acid, L-threonine, and L-glutamic acid (5 mmol/L, each).

Table 1. Measured Mass Transitions of γ-Glu-X Dipeptides and Calculated Mass Transitions for Detection of γ-Glu-[$^{13}C_5$]-X Dipeptides

Compound	measured mass transition of γ-Glu-X, [*m/z*] [a]	calculated mass transition of γ-Glu-[$^{13}C_5$]-X [*m/z*]
γ-Glu-Gly	205→76[b]; 205→84	210→76[b]; 210→88
γ-Glu-Ala	219→90[b]; 219→84	224→90[b]; 224→88
γ-Glu-Val	247→118[b]; 247→84	252→118[b]; 252→88
γ-Glu-Thr	249→119[b]; 249→84	254→119[b]; 249→88
γ-Glu-Asp	263→134[b]; 263→84	268→134[b]; 268→88
γ-Glu-Lys	276→130[b]; 276→84	281→130[b]; 276→88
γ-Glu-Glu	277→130[b]; 277→84	282→130[b]; 282→88
γ-Glu-Trp	334→188[b]; 334→145	339→188[b]; 339→88
γ-Glu-Leu/Ile	261→86[b]; 261→84	266→86[b]; 266→88
γ-Glu-Gln	276→130[b]; 276→84	281→130[b]; 281→88
γ-Glu-Met	279→150[b]; 279→84	284→150[b]; 284→88
γ-Glu-His	285→156[b]; 285→110	290→156[b]; 290→88
γ-Glu-Phe	295→166[b]; 295→84	300→166[b]; 300→88
γ-Glu-Tyr	311→165[b]; 311→84	316→165[b]; 316→88
γ-Glu-Gln	276→130[b]; 276→84	286→135[b,c]; 286→88[c]

[a] mass transitions for optimized MRM parameters of γ-glutamyl peptides. [b] mass transition used for quantification. [c] mass transitions of γ-Glu-[$^{13}C_{10}$]-Gln.

Table 2. Concentration [μmol/kg dm] of α- and γ-Glutamyl Dipeptides in Parmesan Cheese Ripened for 13 (PC-13), 24 (PC-24), and 30 Months (PC-30).

	concentration [μmol/kg dm ±SD][a]			ratio	
peptide	PC-13	PC-24	PC-30	24/13	30/24
α-Glu-Lys	90.2±14.0	243.1±30.9	106.9±25.6	2.7	0.4
α-Glu-Glu	64.7±4.7	178.7±14.9	108.3±10.4	2.8	0.6
α-Glu-Thr	18.5±2.9	48.6±3.8	37.9±5.3	2.6	0.8
α-Glu-Val	21.0±1.3	45.1±3.8	36.2±3.5	2.1	0.8
α-Glu-Gly	17.4±4.4	36.9±3.5	27.6±5.1	2.1	0.7
α-Glu-Asp	18.1±2.8	14.7±1.2	9.5±0.6	0.8	0.6
α-Glu-Ala	6.4±1.1	11.5±1.4	10.1±1.3	1.8	0.9
α-Glu-Trp	2.6±0.5	3.4±0.7	2.6±0.8	1.3	0.8
γ-Glu-His	2807.8±120.6	8486.9±593.2	6075.0±456.2	3.0	0.7
γ-Glu-Glu	1677.0±125.2	4564.9±259.5	3261.4±198.6	2.7	0.7
γ-Glu-Thr	1026.8±76.9	3533.0±178.6	2350.7±171.2	3.4	0.6
γ-Glu-Val	488.5±44.4	1785.2±127.4	1196.0±84.8	3.7	0.7
γ-Glu-Leu	1028.8±88.5	1804.2±75.5	1336.3±119.5	1.8	0.7
γ-Glu-Lys	384.3±22.9	1599.0±72.5	973.8±98.2	4.1	0.6
γ-Glu-Phe	526.3±39.2	1595.3±66.2	1067.5±61.2	3.0	0.7
γ-Glu-Gly	586.2±48.8	1449.2±106.8	1069.8±41.9	2.5	0.7
γ-Glu-Ile	384.2±28.8	1325.1±90.1	915.8±94.3	3.4	0.7
γ-Glu-Met	459.4±25.9	866.2±44.1	600.7±41.2	1.9	0.7
γ-Glu-Asp	99.1±11.4	376.2±32.4	264.2±11.5	3.8	0.7
γ-Glu-Ala	111.4±10.5	294.5±15.9	246.1±13.3	2.2	0.8
γ-Glu-Tyr	195.9±8.7	274.7±14.5	228.9±18.4	1.4	0.8
γ-Glu-Gln	237.7±18.3	210.9±19.4	102.5±6.5	0.9	0.5
γ-Glu-Trp	26.0±1.9	83.5±3.7	52.9±3.6	3.2	0.6
Σ α-Glu-X	238.9	582.0	339.1	2.4	0.6
Σ γ-Glu-X	10039.5	28248.9	19723.5	2.8	0.7
Σ α-/γ-Glu-X	10278.4	28830.9	20062.6	2.8	0.7
Σ γ/Σ α	42.0	48.5	58.2		

[a] Concentration is calculated on dry matter (dm) basis. ±SD = standard deviation (n = 3).

Table 3. Concentration [mmol/kg dm] of Free Amino Acids in Parmesan Cheese Ripened for 13 (PC-13), 24 (PC-24), and 30 Months (PC-30).

	conc [mmol/kg dm±SD][a]			ratio	
compound	PC-13	PC-24	PC-30	24/13	30/24
glutamic acid	193.5±5.9	282.2±6.4	273.3±8.2	1.5	0.9
lysine	150.8±1.1	213.9±5.7	195.3±2.7	1.4	0.8
proline	156.4±5.5	216.1±3.8	203.5±4.2	1.4	0.9
serine	110.7±3.6	172.8±2.9	189.8±1.4	1.6	1.1
valine	109.4±1.1	150.5±2.7	138.1±1.5	1.4	0.9
leucine	96.4±2.1	114.9±6.4	110.2±0.3	1.2	0.9
isoleucine	81.3±1.9	102.5±6.4	100.6±1.5	1.3	1.0
phenylalanine	69.1±1.0	90.7±0.9	82.7±1.3	1.3	0.9
glycine	59.7±4.9	83.1±0.7	68.5±1.6	1.4	0.8
aspartic acid	51.5±0.4	82.1±0.4	74.8±1.1	1.6	0.9
asparagine	53.0±1.3	72.4±2.5	62.9±0.3	1.4	0.9
threonine	49.4±1.5	73.1±0.7	68.1±0.9	1.5	0.9
arginine	58.7±3.7	60.6±3.1	70.7±0.4	1.0	1.2
histidine	31.1±0.8	47.8±0.9	40.1±0.2	1.5	0.8
alanine	43.9±0.6	52.0±0.7	45.9±0.4	1.2	0.9
methionine	26.7±0.2	35.1±0.3	33.5±1.2	1.3	1.0
tyrosine	21.1±0.9	20.3±0.7	21.7±1.0	1.0	1.1
tryptophan	4.4±0.1	6.7±0.1	6.1±0.1	1.5	0.9
glutamine	11.3±0.1	7.4±0.1	3.5±0.2	0.7	0.5
Σ	1378.3	1884.2	1789.3	1.4	0.9

[a] Concentration is calculated on dry matter (dm) basis. ±SD = standard deviation (n = 3).

Table 4. GGT Activity of Parmesan Cheese Ripened for 13 (PC-13), 24 (PC-24), and 30 Months (PC-30), Raw and Heat-Treated Milk Samples, and Bacteria Strains Identified in PC-13.

Sample	GGT activity[a] [U/g dm ±SD]
Parmesan cheese, 13 month (PC-13)	14.6±1.7
Parmesan cheese, 24 month (PC-24)	15.3±1.4
Parmesan cheese, 30 month (PC-30)	14.7±1.8
	GGT activity[a] [U/mL]
milk, raw	5.3±0.5
milk (55°C, 60 min)	4.5±0.2
milk (55°C, 10 min)	4.8±0.04
milk (60°C, 10 min)	4.8±0.05
milk (65°C, 10 min)	3.4±0.1
milk (70°C, 10 min)	1.4±0.03
milk (75°C, 10 min)	0.2±0.07
milk (100°C, 1 min)	n.d.
	GGT activity[a] [U/g protein]
Lactobacillus casei	n.d.
Lactobacillus harbinensis	n.d.

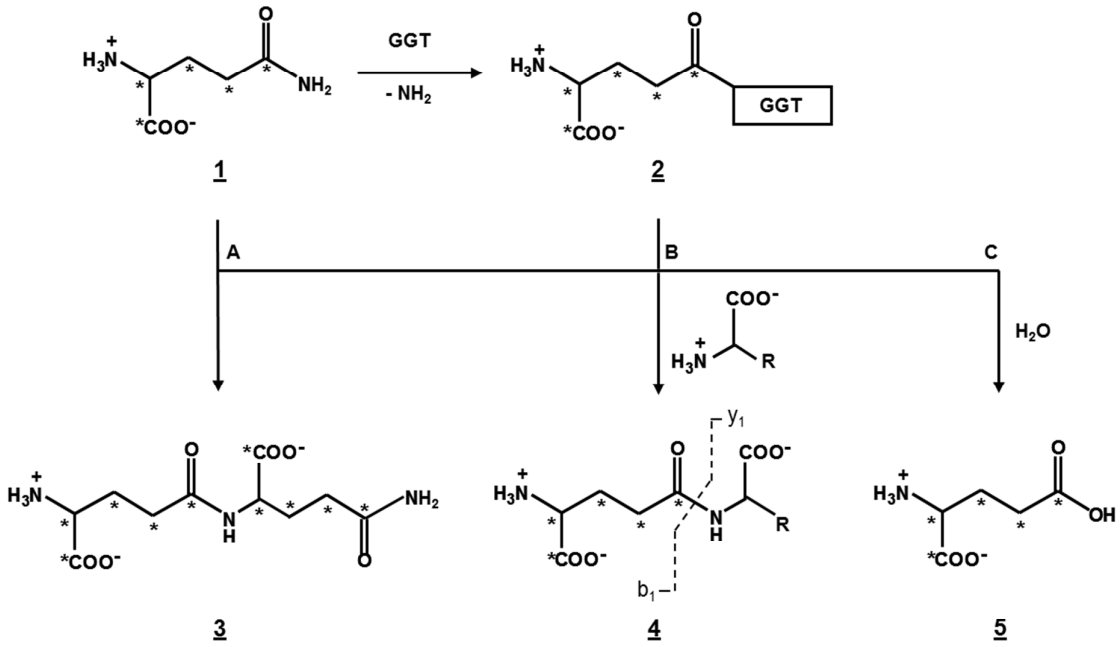
[a] GGT activity was determined by use of the photometric assay reported in the literature,[22] and is given as the mean value ±SD (n = 3). n.d. not detectable; dm dry mass.

Table 5. Concentrations (µmol/kg dry matter, µmol/mmol L-Gln-[U-^{13}C]) of γ-Glu-[$^{13}C_5$]-X Peptides in Parmesan Cheese (PC-24) Incubated with Gln-[U-^{13}C] for 21 days at R.T.

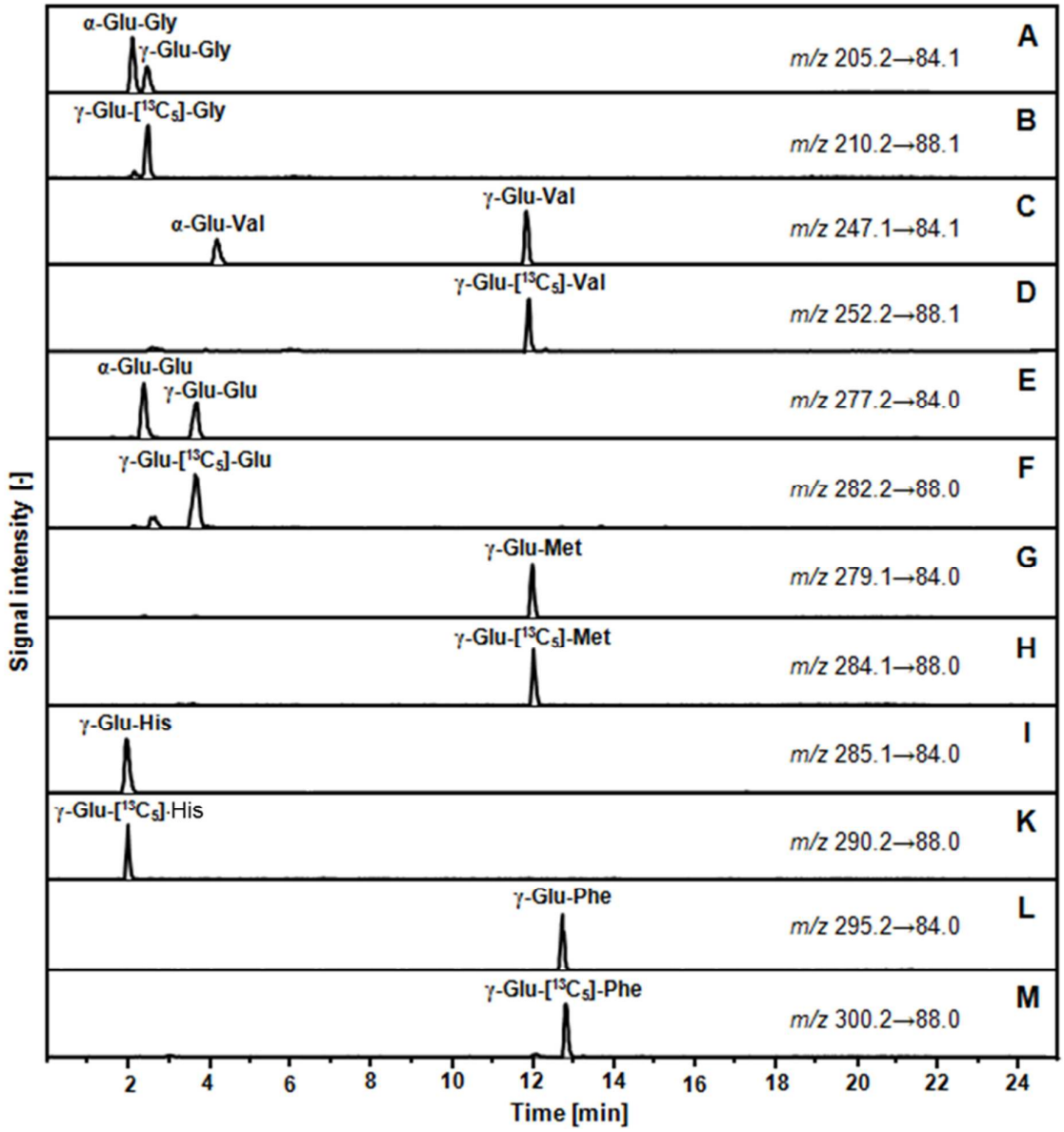
compound	conc. [µmol/kg dm ±SD][a]	µmol/mmol L-Gln-[U-^{13}C±SD][b]
γ-Glu-[$^{13}C_5$]-Lys	37.35±3.45	2.67±0.24
γ-Glu-[$^{13}C_5$]-His	10.30±0.97	0.73±0.07
γ-Glu-[$^{13}C_5$]-Gln	5.20±0.22	0.24±0.02
γ-Glu-[$^{13}C_5$]-Phe	3.36±0.29	0.24±0.02
γ-Glu-[$^{13}C_5$]-Glu	2.04±0.02	0.14±0.001
γ-Glu-[$^{13}C_5$]-Met	1.67±0.12	0.12±0.01
γ-Glu-[$^{13}C_5$]-Thr	1.53±0.10	0.11±0.01
γ-Glu-[$^{13}C_5$]-Gly	1.16±0.04	0.08±0.003
γ-Glu-[$^{13}C_5$]-Val	0.92±0.08	0.07±0.01
γ-Glu-[$^{13}C_5$]-Leu	0.79±0.07	0.06±0.005
γ-Glu-[$^{13}C_5$]-Asp	0.63±0.05	0.04±0.003
γ-Glu-[$^{13}C_5$]-Trp	0.42±0.03	0.03±0.002
γ-Glu-[$^{13}C_5$]-Tyr	0.38±0.05	0.03±0.004
γ-Glu-Gln-[$^{13}C_{10}$]	0.07±0.02	0.005±0.001
Σ	65.82	4.67

[a] concentration is calculated on dry matter basis (±SD, n=3). [b] concentration refers to mmol L-Gln-[U-^{13}C] (±SD, n = 3).

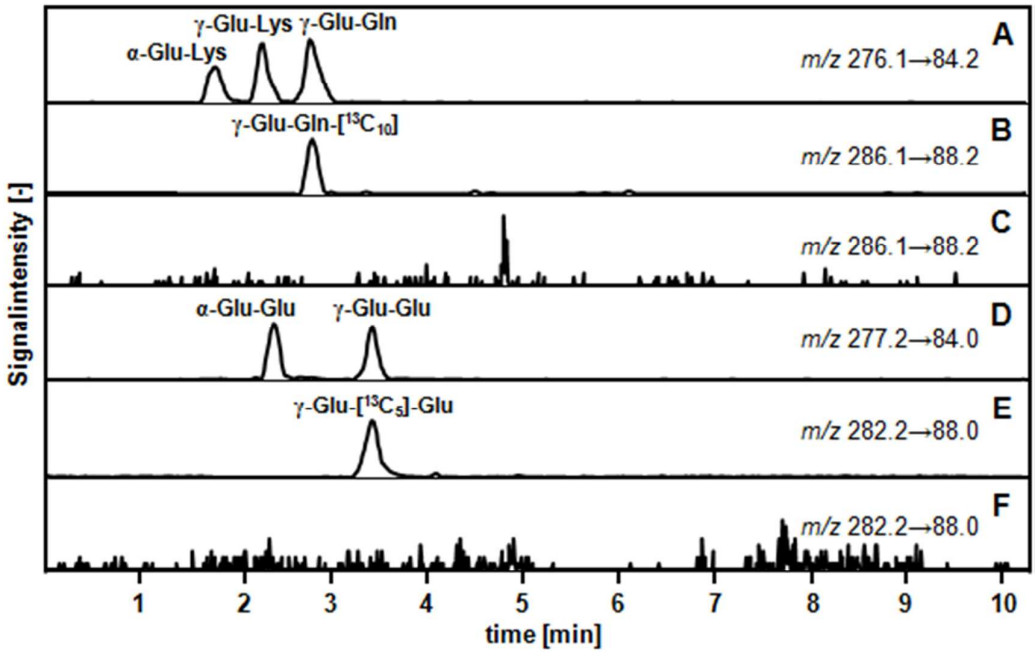
Hillmann et al., Figure 1



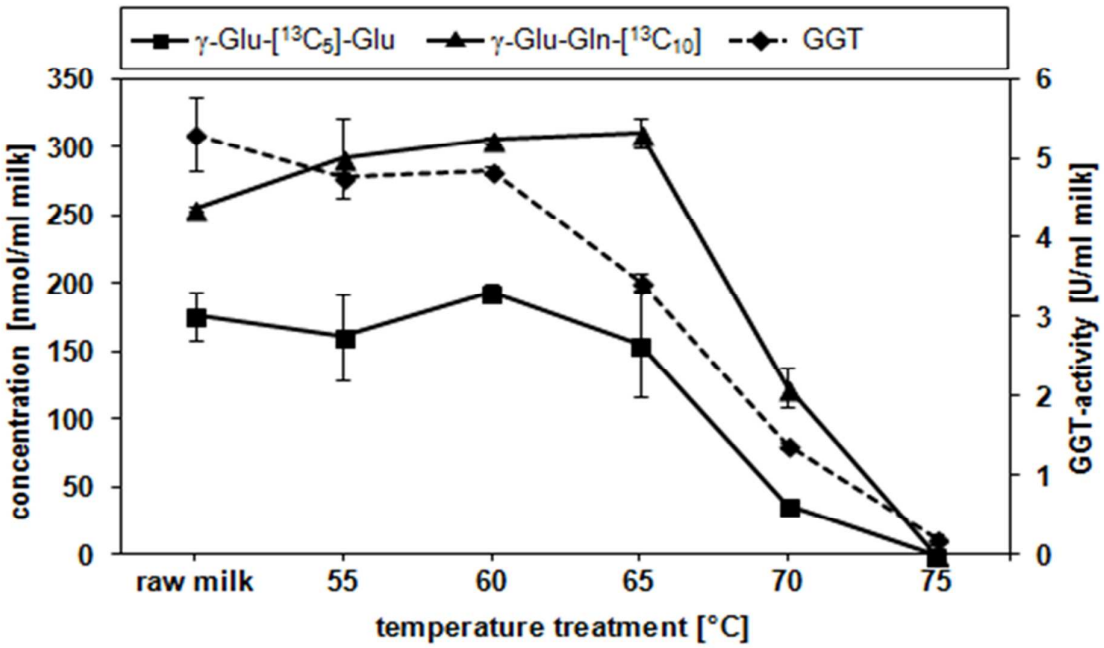
Hillmann et al., Figure 2



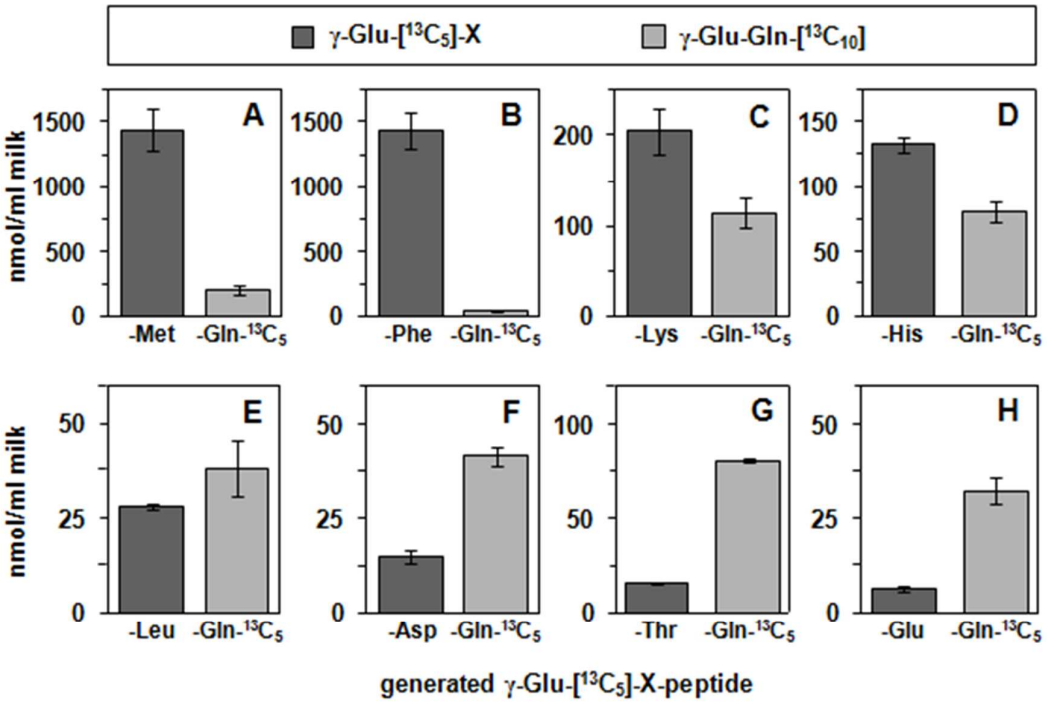
Hillmann et al., Figure 3



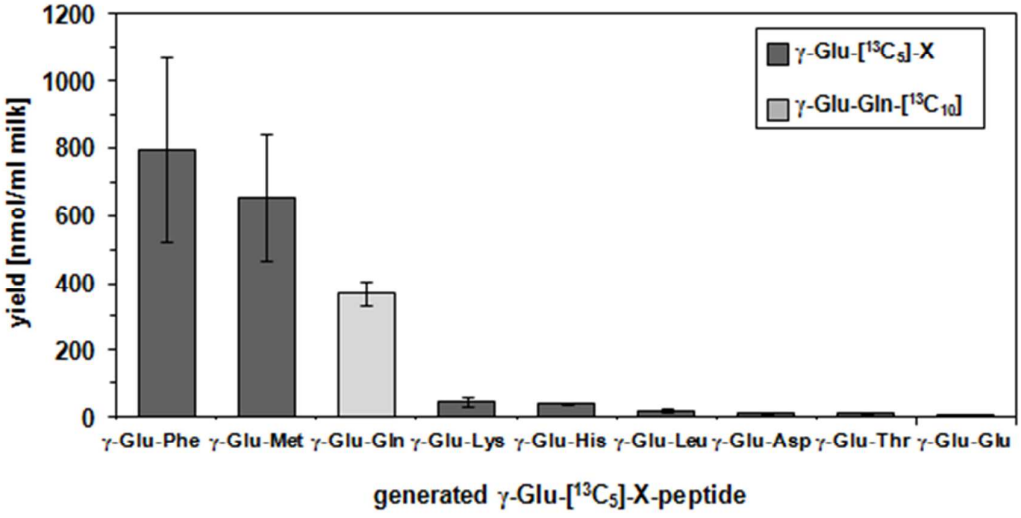
Hillmann et al., Figure 4

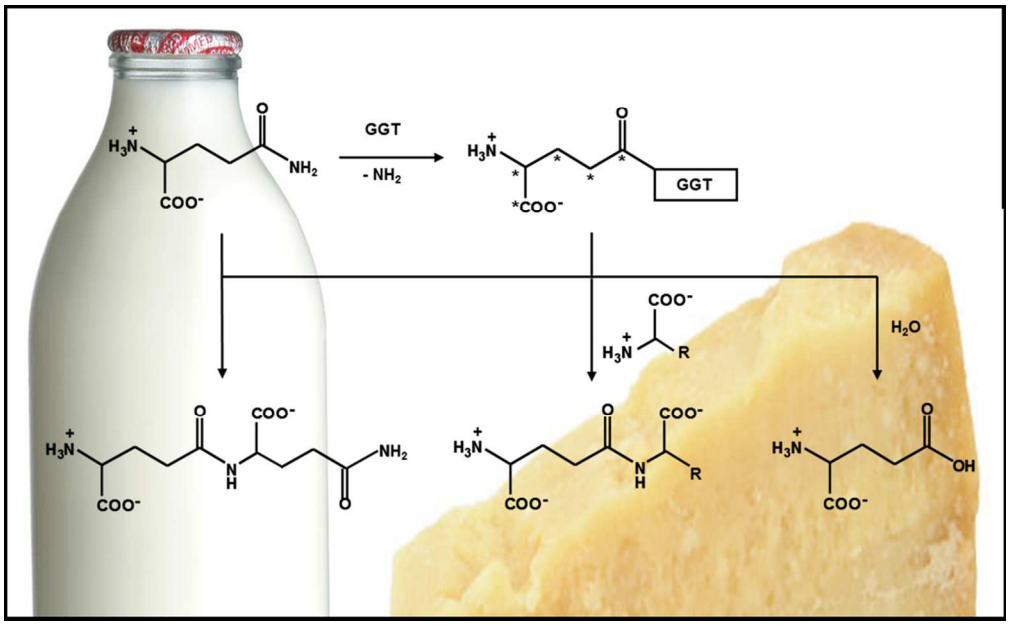


Hillmann et al., Figure 5



Hillmann et al., Figure 6





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