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Higher microbial diversity in raw than in pasteurized milk Raclette-type cheese enhances peptide and metabolite diversity after *in vitro* digestion

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ABSTRACT

Numerous bacteria are responsible for hydrolysis of proteins during cheese ripening. The raw milk flora is a major source of bacterial variety, starter cultures are needed for successful acidification of the cheese and proteolytic strains like *Lactobacillus helveticus*, are added for flavor improvement or acceleration of ripening processes.

To study the impact of higher bacterial diversity in cheese on protein hydrolysis during simulated human digestion, Raclette-type cheeses were produced from raw or heat treated milk, with or without proteolytic *L. helveticus* and ripened for 120 days.

Kinetic processes were studied with a dynamic (DIDGI®) *in vitro* protocol and endpoints with the static INFOGEST *in vitro* digestion protocol, allowing a comparison of the two *in vitro* protocols at the level of gastric and intestinal endpoints.

Both digestion protocols resulted in comparable peptide patterns after intestinal digestion and higher microbial diversity in cheeses led to a more diverse peptidome after simulated digestion.

1. Introduction

In several countries, dairy products are recommended as a key food for a healthy diet. Besides providing high amounts of bioavailable calcium, they are also an excellent source of proteins that contain high amounts of essential amino acids (Bos, Mahe, Gaudichon, Benamouzig, Gausseres, Luengo, et al., 1999). Milk proteins are highly digestible with a true ileal digestibility around 95%. However, it has been shown that hydrolyzed milk proteins are metabolized faster than native ones, causing a faster increase in amino acids in the bloodstream of human volunteers after ingestion when compared with intact proteins (Deglaire, Bos, Tome, & Moughan, 2009). Therefore, the state of hydrolysis of a protein is a key factor that will highly influence its metabolic use.

Among dairy products, cheese has one of the highest protein contents per gram and, therefore, is an interesting source of amino acids. After manufacture, some varieties of cheeses are ripened, leading to a breakdown of proteins over time. The proteolysis process is essential for the final quality of cheese: proteins will first be cleaved into peptides that are further degraded into amino acids that then will act as precursors for aroma compounds. Cheese proteolysis is mainly caused by milk indigenous enzymes (plasmin and cathepsin D), enzymes from the coagulant (chymosin or pepsin), and proteases that are released by the different types of bacteria present in cheese. These bacteria originate from the raw milk microbiome, from starter cultures needed for the successful acidification of the cheese curd, and from cultures for flavor improvement or acceleration of ripening processes, depending on the type of cheese.

After ingestion, cheese is digested in the gastrointestinal tract, and milk proteins are further degraded to provide free amino acids to the body. Cheese microstructure and texture have been shown to influence the rate of milk protein hydrolysis (Guinot, Rioux, Labrie, Britten, & Turgeon, 2019). The digestion of cheese was investigated because of its ability to release peptides into the gastrointestinal tract, being known for carrying different biological activities (Summer, Formaggioni, Franceschi, Di Frangia, Righi, & Malacarne, 2017), especially

Abbreviations: FAA, free amino acids; IVD, *in vitro* digestion; ACE, Angiotensin converting enzyme; DPP IV, dipeptidyl peptidase IV

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antihypertensive, or antioxidant (Barac, Vucic, Zilic, Pesic, Sokovic, Petrovic, et al., 2019; Stuknytė, Cattaneo, Masotti, & De Noni, 2015). All these studies have been performed using various static *in vitro* digestion (IVD) models.

Recently, a simple static IVD consensus model was elaborated by the INFOGEST network (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, et al., 2014). Interlaboratory experiments have shown that the protocol improved the comparability of experimental data (Egger, Ménard, Delgado-Andrade, Alvito, Assunção, Balance, et al., 2016). The comparison of protein hydrolysis between this harmonized IVD protocol with the *in vivo* pig digestion of skim milk powder (SMP) showed that the gastric and intestinal endpoints were similar in the two systems (Egger, Ménard, Baumann, Duerr, Schlegel, Stoll, et al., 2017). The harmonized static digestion protocol is now widely applied and has recently been updated (Brodkorb et al., 2019). However, static digestion models aim to simplify a highly complex and dynamic physiological process in which peristalsis, enzyme secretion, gastric emptying, and absorption processes, to name the most obvious, have a major influence on food disintegration. To obtain a better approximation of these physiological processes, dynamic IVD protocols have been developed, such as the DIDGI® system (Ménard, Picque, & Dupont, 2015). This system allows for the continuous addition of digestion juices and enzymes, the progressive adjustment of pH levels, and the gastric and intestinal emptying at predetermined rates. The comparability between the harmonized static and the dynamic IVD protocol in SMP was previously demonstrated at the gastric and intestinal endpoints (Egger, et al., 2017). Therefore, in this study, digestion kinetics were assessed with dynamic IVD and endpoints with both protocols. With this background in mind, the current study aimed to determine if bacterial strains in cheese could have an impact on protein hydrolysis during human digestion using *in vitro* models, and if so, if a higher microbial diversity could lead to the generation of a higher number or a different set of metabolites and bioactive peptides after digestion. Raclette-type cheese was selected as ideal model system, because it is available on the market as pasteurized or raw milk cheese, it is known at an international level and the heat treatment during scalding is not affecting the bacterial diversity of the raw milk. A gradual increase in microbial diversity in the cheeses was generated by producing Raclette-type cheeses with pasteurized or raw milk without and with proteolytic *L. helveticus* strains, respectively. The proteolytic properties of *L. helveticus* were previously shown (Griffiths & Tellez, 2013) and were in addition tested in Raclette-type cheeses (unpublished data).

2. Materials and methods

2.1. Cheese manufacturing and sampling

Raw milk was obtained at a local cheese producer and hygienic parameters were analyzed (Enterobacteriaceae 170 CFU/g, Propionic bacteria < 10 CFU/g, salt tolerant bacteria 2800 CFU/g, aerobic mesophilic bacteria 5400 CFU/g, butyric acid spores 53 /L). Four different Raclette-type cheeses in duplicates, 30 cm in diameter, were manufactured simultaneously in the pilot plant of the Agroscope Research Station (Liebefeld, Switzerland) in stainless steel vats and ripened for 120 days. The four different conditions were produced from milk with batch pasteurization in the cheese vat, with a holding temperature of 70 °C for 10 *sec* at the end of the heating process or without thermal treatment prior to cheese manufacturing. After the addition of the starter culture (Ceska Star C04, CSK Food Enrichment C.V., The Netherlands), an additional *Lactobacilus helveticus* strain mixture (*L. helveticus* FAM1172, *L. helveticus* FAM22157, *L. helveticus* FAM22243) with enhanced proteolytic properties (unpublished data, (Wyder, 2001)) was added to obtain the four different conditions: i) pasteurized (past -), ii) pasteurized plus proteolytic culture (past +), iii) raw milk (raw −), and iv) raw milk plus proteolytic culture (raw +). The cheese manufacturing process followed a recipe for Raclette-type cheese. Briefly, 0.35% of $CaCl_2$ was added to raw or pasteurized milk at 26 °C starting temperature. For pre-maturation and culture addition (Ceska star C 04, 0.6%, with total bacterial count ≥ 10E10 cfu/mL, Leuconostoc sp. ≥ 10E9 cfu/mL, Lactococcus lactis subsp. Lactis var. diacetylactis < 10E5 cfu/mL, respectively, added to all cheeses) and *L. helveticus* mix (*L. helveticus* FAM1172 (2.5E + 04), *L. helveticus* FAM22157 (3.65E + 04), *L. helveticus* FAM22243 (3.7E + 04)/ 90 L of cheese milk, added to past + and raw + cheeses) the milk was heated at 31 °C and kept at this temperature for 30 min. Rennet (18 mL , 80% Chymosin: 20% Pepsin, Clerici, Emmi, Switzerland, diluted in 1 L of H_2O) was incubated for 25 min after addition. The curd was cut in grains of 4–8 mm in size during 25 min, 20 L of H_2O were added per 90 L of milk and heated at 36 °C during 10 min and scalding was done at 36 °C for 40 min. The cheese were molded and pressed (4 bar) at 30 °C for 45 min, at 24–26 °C for 4 h, and finally 31 °C for 5 h. Salting was done at 11–13 °C for 26 h in the salt bath and ripening in the cellar at 10–11 °C, 90–96 °C relative humidity for a total of 120 days. Cheese samples were collected after 120 days of cheese ripening.

2.2. Chemicals and reagents

All chemicals and enzymes used for the simulated fluids were purchased from Sigma Aldrich as described in the original publication of the INFOGEST digestion protocol (Minekus et al., 2014). The enzymes for static IVD: pepsin (P7012), pancreatin (P7545), and bile extract (B8631) from porcine origin; for dynamic IVD: porcine pepsin (P6887), porcine pancreatin (P7545), and bovine bile extract (B3883).

2.3. Static in vitro digestion with the harmonized INFOGEST protocol

Prior to the digestion experiments (static or dynamic), all enzyme activities and bile salts concentrations were determined according to the harmonized IVD protocol (Minekus, et al., 2014). After 120 days of ripening, the eight cheeses were digested once with the harmonized *in vitro* protocol (Minekus, et al., 2014), resulting in duplicate digestions for each of the different cheese conditions. Here, 0.8 g of cheese were homogenized (2× 15000 rpm for 60 sech, Omni Prep, LabForce, Switzerland) with 5 mL of simulated salivary fluid (SSF). In order not to overload the digestion system, the ratio was 0.8:5 mL (w:v) generating a paste-like consistency according the the improved INFOGEST protocol published by Brodkorb et al. (Brodkorb, et al., 2019) For the oral phase, 5 mL of SSF were added without amylase and incubated at 37 °C for 2 min. For the gastric phase, 10 mL of simulated gastric fluid (SGF) containing pepsin were added to cover 2000 U of pepsin/mL of digesta), and individual tubes were stopped after different gastric time points of 0.1, 0.5, 1, 3, 5, 10, 20, 30, 60, and 120 min, with a maximal gastric endpoint of 120 min. Then, 20 mL of simulated intestinal fluid (SIF) were added for the intestinal phase, which contained pancreatin to cover 100 U of trypsin per mL of digesta and 10 mmol/L of bile salts in the total digesta; individual time points were taken after 0.1, 0.5, 1, 3, 5, 10, 20, 30, 60, and 120 min, with a maximal intestinal endpoint at 120 min. The incubations were performed on a rotating wheel at 37 °C. After the gastric phase, pepsin activity was stopped by the addition of NaOH until reaching a pH of 7, which was followed by immediate snap freezing in liquid nitrogen. Reactions in the intestinal phase were stopped by the addition of Pefabloc® (1 mmol/L) at the end of the time points of sample collection prior to the snap freezing.

2.4. Dynamic in vitro digestion of cheese

A dynamic *in vitro* gastrointestinal digestion system (DIDGI®, INRA, France) was used to simulate the digestion of the cheeses and helped in monitoring the kinetics of hydrolysis and disintegration of the food during digestion.

The computer-controlled system was set up to simulate adult gastrointestinal digestion. In Table 1 of the supplemental data, the

Table 1
Production conditions, composition, pH, and total free amino acids of the four types of Swiss Raclette-type cheeses after 120 days of ripening. LH: *L. helveticus* (proteolytic culture) (n = 2).

Cheese		pH	True protein		Fat		Dry matter		NaCl		Free R-NH $_2$		Free amino acids		Citrate		Succinate		Biogenic amines		Butanediols		GABA		Lipolysis:C6 to C20	Oxidation: C4 to C10
			g/kg	SD	g/kg	SD	g/kg	SD	g/kg	SD	g/kg	SD	g/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	mg/kg
milk	**LH**																									
raw	no	**5.6**	**221.1**	2.3	**307.0**	6.5	**606.0**	8.0	**15.9**	0.8	**21.4**	0.2	**12.6**	0.5	**0.0**	0.0	**537.3**	35.9	**120.3**	13.9	**108.2**	5.9	**51.0**	8.3	**674.6**	**75**
	yes	**5.6**	**201.5**	4.4	**279.0**	1.0	**565.0**	1.2	**20.8**	0.2	**23.1**	0.3	**14.6**	0.4	**0.0**	0.0	**496.0**	8.4	**293.4**	80.7	**139.7**	7.5	**89.2**	56.3	**633.8**	**77**
past	no	**5.6**	**209.9**	3.8	**290.3**	4.9	**580.5**	7.5	**18.9**	0.6	**20.6**	0.5	**11.6**	0.6	**403.5**	79.2	**0.0**	0.0	**66.5**	4.8	**56.4**	0.9	**4.3**	0.7	**518.0**	**68**
	yes	**5.6**	**202.3**	6.4	**282.8**	1.5	**568.0**	5.1	**21.0**	1.6	**21.9**	0.3	**13.4**	0.5	**264.2**	25.0	**70.9**	14.5	**59.8**	11.9	**56.5**	4.8	**13.7**	1.4	**513.9**	**70**

digestive conditions used are summarized. To simulate the gastric fasted volume, 24 mL of simulated gastric fluid adjusted at pH = 2 was introduced before food ingestion (Lentner, 1973). The gastric and intestinal emptying followed an exponential equation, as described previously (Elashoff, Reedy, & Meyer, 1982). For the gastric phase, a gastric emptying half-time ($T_{1/2}$) of 85 min and a β coefficient of 1.8 were applied. For the intestinal phase, a $T_{1/2}$ of 250 min and a β of 2.5 were used. These conditions were previously proposed (Lvova, Denis, Barra, Mielle, Salles, Vergoignan, et al., 2012) for simulating cheese digestion.

The gastric acidification followed an exponential equation (pH = 1.68 + 3.82(-t/42)) as observed after modeling the data obtained on adults after the ingestion of a meal (Malagelada, Longstreth, Summerskill, & Go, 1976). Regulation of the gastric pH was achieved using HCl 1 mol/L. The pepsin solution was prepared to cover 2000 U/mL of gastric content (Minekus, et al., 2014) and the amount of pepsin needed was calculated based on the activity measured in the powder. The intestinal pH conditions were set up, as previously reported (Minekus, Marteau, Havenaar, & Huis in T Veld, 1995). Intestinal pH was maintained at 6.8 during the first two hours and then at 6.6 until the end by addition of $NaHCO_3$ 1 mol/L.

The preparation of food for digestion experiments was as follows: 30 g of cheese were thawed and grated and mixed with 30 mL of simulated salivary electrolyte fluid (NaCl 2 mM, KCl 2 mM, $NaHCO_3$ 25 mM). This simulated oral phase was introduced in the DIDGI system for the subsequent digestion steps. Digestion experiments were performed over 4 h in duplicate for each cheese modality.

Each cheese was digested twice, resulting in quadruplicate digestions for each cheese condition (past -, past +, raw -, raw +). Samples were collected after 0.1, 15, 30, 60, 90, and 120 min of the gastric phase and after 30, 60, 90, 120, 150, 180, and 240 min of the intestinal digestion. Samples were frozen and freeze-dried after the addition of protease inhibitors, Pepstatin A (0.72 mmol/L) for gastric digesta and Pefabloc® (5 mmol/L) for intestinal digesta.

2.5. Confocal laser scanning microscopy (CLSM)

The microstructure was observed using a Nikon C1Si Laser Scanning Confocal Imaging System on an inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) operated with an argon laser (excitation at 488 nm) and two He-Ne lasers (excitation at 543 and 633 nm), as previously described (Bourlieu, Menard, De La Chevasnerie, Sams, Rousseau, Madec, et al., 2015) except that a 40X oil-immersion objective was used for all images. Three fluorescent dyes - fast green, Rd-DOPE®, and lipidtox® - were used to simultaneously label protein, amphiphilic compounds, and apolar lipids in the sample (Bourlieu, et al., 2015).

2.6. Sample preparation and gel electrophoresis

The samples were normalized according to the amount of protein, that is, the dilution of cheese during the digestion steps was used to load the samples with equal amounts of protein and directly observe on gel the proteolysis effect. Samples were mixed with a sample buffer (Tris-HCl 350 mmol/L, pH = 6.8, SDS 10%, DTT 100 mmol/L, glycerol 50%) prior to separation by SDS-PAGE. A molecular weight marker (Benchmark™, Invitrogen) was included. Thereafter, the gels were stained with colloidal Coomassie blue as previously described (Egger et al., 2017).

2.7. HPLC analysis of individual free amino acids (FAA)

Samples were normalized to the amount of cheese, as described for gel electrophoresis, prior to the analysis of free amino acids. Equal volumes containing equal amounts of cheese were analyzed by HPLC according to a previous method (Bidlingmeyer, Cohen, & Tarvin, 1984).

2.8. Total free amino acids and small peptides (OPA)

Total FAAs and di- and tripeptides were analyzed with the ophthaldialdehyde (OPA) method (Egger, Ménard Portmann, 2018). Briefly, the samples were diluted 10 times prior to precipitation with perchloric acid (0.5 mol/L), derivatized with OPA in the presence of 2-mercapto-ethansulfonic acid, and the produced 1-alkylthio-2-alkylisoindol compound was measured at 340 nm. The results were calculated based on a glutamic acid standard curve.

2.9. LC-MS peptide identification and length analysis

Analysis by MS was performed as previously described (Egger, et al., 2016; Egger et al., 2018). Briefly, samples were filtered through Microcon columns (Ultracel YM-30, Millipore); subsequently, peptides were separated on a Rheos 2200 HPLC (Flux Instruments, Switzerland) equipped with an XTerra MS C18 column (3.5 μm, 1.0 mm × 150 mm, Waters). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Switzerland) using an ESI interface. The *m/z* signals were measured between 100 and 1300 using multiple overlapping mass windows. The overlapping mass windows of these MSMS spectra were merged, and a Mascot search was performed for peptide identification (Matrix Science), an in-house database with all the typical milk proteins from different species was used. The peptides identified from the five main milk proteins (β-casein, α_{s1}-casein, α_{s2}-casein, κ-casein, and β-lactoglobulin) were monitored using the minimal criteria for peptide identification, that is, an ion score of 20, to obtain the first identification score. Questionable results were manually verified by checking the fragmentation pattern (peptide coverage and fragmentation pattern). Peptide abundance was visualized by summing up individual amino acids within the identified peptides from the abovementioned dairy proteins and representing them in an abundance color code. Amino acid counting was performed using an in-house generated Perl script extending the Mascot search engine, and the color codes were adjusted using Microsoft Excel. The colors range from blue over green and yellow to red, indicating low and high recurrence of specific amino acids, respectively. Unidentified protein sequences are shown as white stretches. The colors were normalized to the maximal number of identifications within the corresponding protein and digestion phase; for example, red in α_{s1}-casein can correspond to a different number than red in κ-casein.

2.10. GC–MS analysis

The samples were prepared to perform untargeted GC–MS metabolomics. The samples were prepared according to the Human Serum Metabolome Consortium procedure, with small modifications of the protocol (HUSERMET) (Dunn, Broadhurst, Begley, Zelena, Francis-McIntyre, Anderson, et al., 2011). U13C6-labeled D-fructose (0.125 g/L solution in water) was used as an internal standard (Cambridge Isotope Laboratories, Inc.).

Gastric and intestinal samples were centrifuged at 15,800 g for 15 min, and the supernatant was taken for further analysis.

Samples from the simulated oral phase (SSF) were diluted before analysis (150 μL SSF + 600 μL water) and centrifuged at 370 g for 15 min; the supernatant was taken for further analysis, and 50 μL of internal standard was added to 100 μL of the sample. The mixture was precipitated with 300 μL of cold methanol (Merck, Darmstadt) and centrifuged at 15,800 g for 15 min. The supernatant (380 μL) was lyophilized with a vacuum concentrator at 30 °C. A two-step derivatization was applied to the dry extract. A quantity of 50 μL of a 20 g/L methoxyamine hydrochloride solution in pyridine (Acros Organics and Merck Chemicals) was added to the extract before heating at 80 °C for 15 min. This was followed by the addition of 50 μL *N*-methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma Aldrich) and a second heating at 80 °C for 15 min. Finally, 20 μL of a retention index solution was added before centrifugation (15800 g, 15 min), and 100 μL of supernatant was used for GC–MS measurement. The retention index solution consisted of a mix of eight *n*-alkanes at 0.6 g/L in pyridine (C10, C12, C15, C19, C22, C27, C31, and C34; Merck Chemicals). GC–MS measurement was performed on an Agilent 7890B/5977A GC–MS system, 70 kV, equipped with a DB-5MS column 60 m × 0·250 mm × 0·25 μm (Agilent Technologies). The temperature program was 70 °C for 2 min, 5 °C/ min to 160 °C (acquisition time 0 min), and 10 °C/ min to 300 °C (hold time 36 min). Peak integration was performed using the Agilent MassHunter Quantitative Analysis Software (V.B.7.00; Agilent Technologies).

2.11. RNA-based 16S rRNA transcript (cDNA) sequencing and analysis

Bacterial 16S rRNA amplicon libraries were prepared by RT-PCR amplification of the V1V2 hypervariable region of the SSU rRNA using universal 8F (5′AGAGTTTGATCMTGGCTCAG3′) and 355R (5′GCWGCCTCCCGTAGGAGT3′) sequences. Single-end sequencing was performed on a Life Science Ion Torrent PGM on a chip. RNA extraction, cDNA synthesis, library preparation, and sequencing were performed as described in the Supplemental Material and Methods. On average, 320 bp long raw sequences were primer trimmed and quality filtered in DADA2 (Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016). Amplicon sequence variances (ASVs) were obtained in DADA2 with the parameter POOL = "pseudo" using in-house priors. Taxonomic annotation was performed using DAIRYdb v1.2.2 (Meola, Rifa, Shani, Delbès, Berthoud, & Chassard, 2019) with IDTAXA (Murali, Bhargava, & Wright, 2018). Biostatistical analyses were done in PHYLOSEQ (McMurdie & Holmes, 2013).

2.12. Additional methods

GC–MS analysis of propane and butane-diols in cheeses: Propane and butane-diols were analyzed by GC–MS as previously reported (Badertscher, Freiburghaus, Wechsler, & Irmler, 2017).

Biogenic amine determination: Biogenic amines were determined by HPLC as previously carried out (Ascone, Maurer, Haldemann, Irmler, Berthoud, Portmann, et al., 2017).

3. Results and discussion

3.1. Cheese composition and bacterial diversity

Four different Raclette-type cheeses with different bacterial backgrounds (n = 2) were produced at the Agroscope pilot plant and ripened for 120 days. The four conditions with expected increasing bacterial diversity were made from pasteurized milk, without (past-) and with (past +) or from raw milk, without (raw -) and with (raw +) the addition of a mix of proteolytic *L. helveticus* cultures of known proteolytic activity (unpublished data) (Griffiths & Tellez, 2013; Wyder, 2001), respectively. The ripened cheeses had comparable compositions of fat, NaCl, protein, dry matter, and pH values. Cheeses containing the proteolytic culture (raw +, past +) had a slightly higher amount of total free R-NH_2 groups (OPA analysis) and free amino acids (Table 1) when compared with the corresponding cheese without the *L. helveticus* strains (raw -, past -). Some compounds were found at different concentrations in raw and pasteurized cheeses, such as Citrate, D-Tagatose, volatile diols, namely 2,3-butanediol and 1,3-propanediol, Succinate, gamma-amino butyric acid (GABA), and biogenic amines (Table 1, Supplemental Fig. 4). These differences persisted throughout IVD and were identified by GC–MS analysis after the intestinal phase of IVD (Supplemental Table 2), indicating that the digestion process did not affect these molecules. Most of these compounds are relevant for cheese flavor, excepted GABA and biogenic amines, which have physiological effects after digestion. GABA is an inhibitory neurotransmitter with positive effects on the brain, leading to decreased anxiety, depression, sleeplessness, or memory loss and acting on the immune system by

reducing inflammatory signaling (Carafa, Stocco, Nardin, Larcher, Bittante, Tuohy, et al., 2019). On the other hand, raw milk cheeses had higher lipolysis and lipid oxidation (Table 1), as well as more biogenic amines (Table 1, Supplemental Fig. 4B). Three different biogenic amines were found mainly in the raw milk cheeses, namely histamine, tyramine, and cadaverine (Fig. 4B). All three compounds can induce food poisoning when consumed in higher amounts, depending on the susceptibility of the consumer (Stratton, Hutkins, & Taylor, 1991). The actively present bacteria after 24 h and 120 d were analyzed by sequencing the 16S rRNA transcripts. At the very early stages after cheese manufacturing (24 h), all the cheeses had a low diversity (data not shown), and after 120 days of cheese ripening, the raw milk cheeses had a higher number of bacterial species, shown as relative abundancies (Supplemental Fig. 1). In all cheeses, 33 different bacterial species were detected, with *Lactococcus lactis* dominating the bacterial community at a relative abundance ranging from 52% to 97% (Supplemental Fig. 1). Raw milk cheeses contained in addition dominant amounts of *Lactobacillus plantarum* and *Weissella paramesenteroides*. The proteolytic bacterium *L. helveticus* was detected in the cheeses, where it was added as an adjunct culture. The abundance of *L. helveticus* throughout cheese ripening was determined in addition by quantitative real-time PCR (qPCR). With this method, no copies were detected in cheeses without added *L. helveticus* culture, whereas past + ($6.5E^{+6}$c/g) and raw + ($12.5E^{+6}$c/g) cheeses contained similar copy numbers. The lower relative abundance of *L. helveticus* in raw milk cheeses (raw +) compared to pasteurized cheeses (past +) by 16S rRNA transcript analysis (Supplemental Fig. 1), can be explained by the growth of raw milk bacteria leading to a higher bacterial diversity and a lower relative abundance *of L. helveticus*.

3.2. Evolution of the cheese microstructure during dynamic in vitro digestion

Fig. 1 shows the evolution of the cheese microstructure before and after 85 min of dynamic gastric and gastrointestinal digestion (85 min represents the gastric emptying half-time corresponding to the middle of the gastric digestion). A small difference between the raw and pasteurized cheeses could be seen in the proteins present: whereas proteins appeared as a loose network in the raw milk cheeses, they formed a denser network in the pasteurized ones. Pasteurization has been previously shown to lower plasmin activity (Somers, Guinee, & Kelly, 2002). A higher plasmin activity in raw milk cheeses could possibly result in a modification of the cheese casein network during ripening. After 85 min of gastric digestion, most of the protein network of the pasteurized cheeses had disappeared; only small aggregates remained visible, which was also the case for the raw milk cheese with the proteolytic strain. In contrast, the raw milk cheeses without proteolytic bacteria exhibited more protein aggregates. In all the digested samples, lipids appeared only as few small droplets, but there was an accumulation of amphiphilic compounds that could be seen in the digested pasteurized cheese containing the proteolytic bacteria. During gastric digestion, a lipid layer was observed for all the cheeses on the top of the gastric bowl; this is not visible on the images, where only small droplets are visible. After 85 min of intestinal digestion, most of the proteins and lipids had disappeared, indicating that they were hydrolyzed. Only a few spots of amphiphilic compounds were visible, probably resulting from the digestion process, such as aggregation of peptides, but also from bile salts included in the intestinal step, as well as mixed micelles of amphiphilic compounds and apolar lipids. In Gouda cheese, the disintegration of the protein network mainly occurred in the intestinal phase, this is slightly different to what was observed in Raclette-type cheese, where protein and lipid disintegration occurred in the gastric

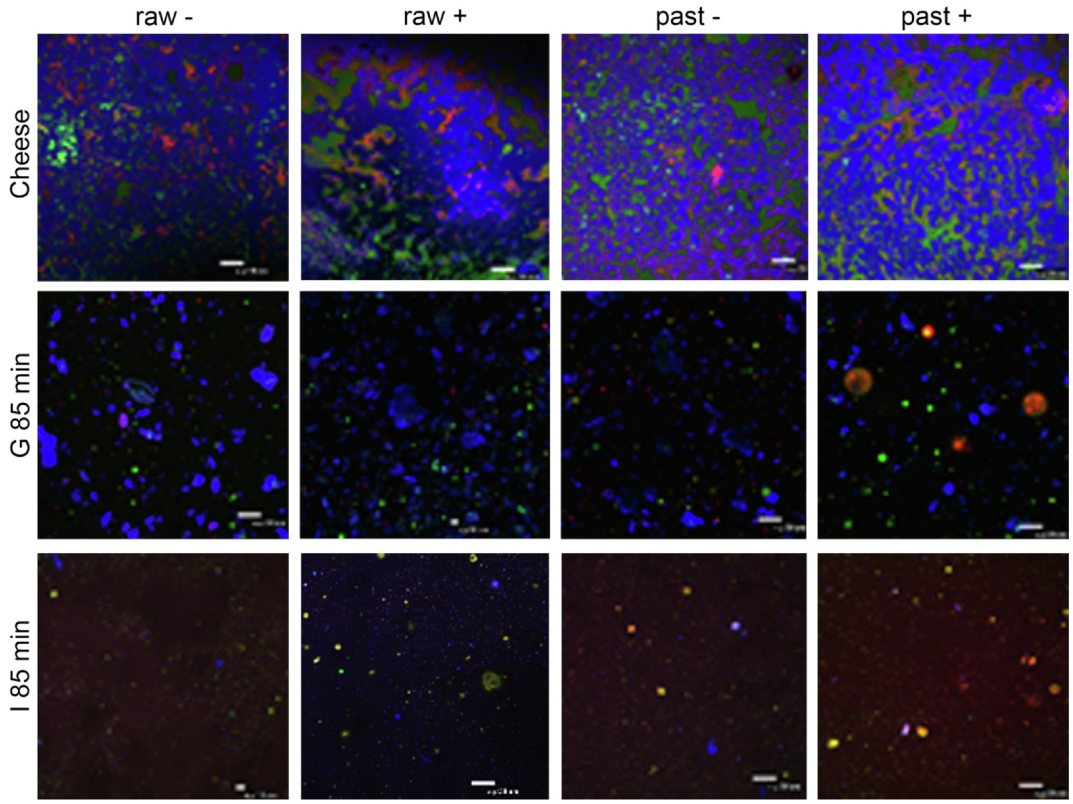

Fig. 1. Microstructure of the cheese matrix and disintegration during dynamic *in vitro* digestion. Confocal microscopy of the cheese samples (Cheese) and after 85 min of digestion (samples withdrawn from the gastric bowl (G85 min) and the intestinal bowl (I85 min)) of *in vitro dynamic* digestion. Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®), and polar lipids in red (Rhodamine-PE®) (Bourlieu, et al., 2015). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

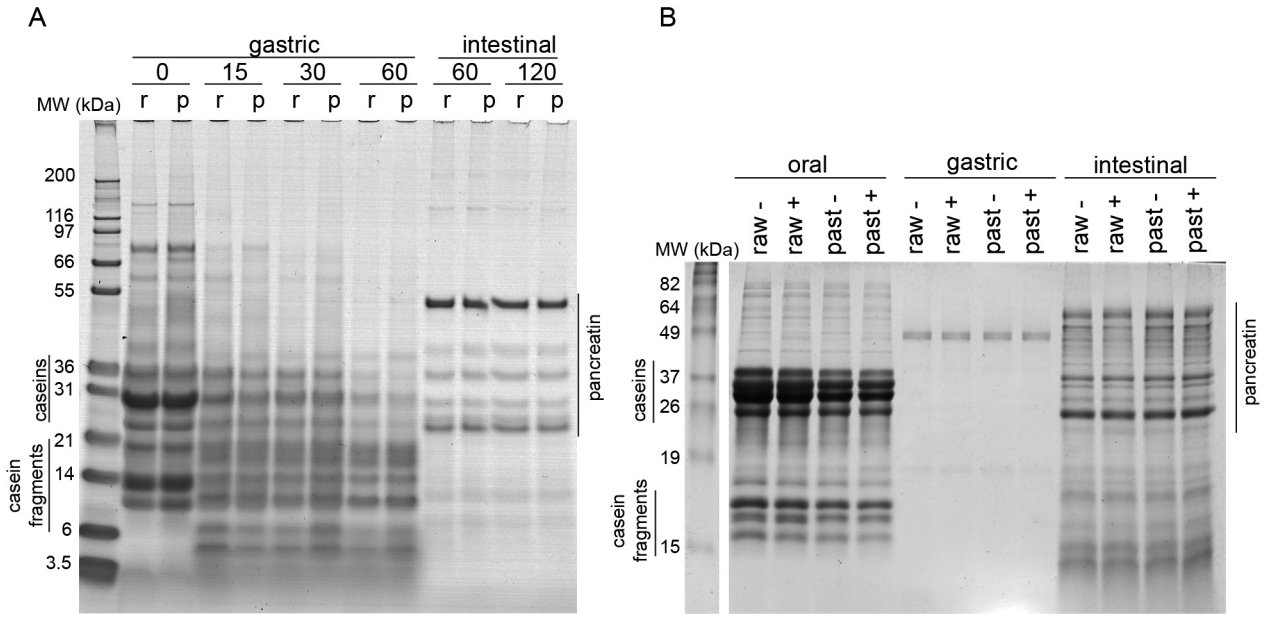

Fig. 2. Protein hydrolysis during *in vitro* digestion. Representative Coomassie blue-stained SDS-PAGE at times 0, 15, 30, and 60 min of gastric and 60 and 120 min of intestinal dynamic IVD. r corresponds to raw milk cheeses and p to pasteurized milk cheeses (A). Endpoints of oral, gastric, and intestinal static digestion of the four different cheese conditions made from raw or pasteurized milk (raw, past), with or without additional proteolytic culture (−/+ LH)(B).

phase (Žolnere, Arnold, Hull, & Everett, 2019). However, the direct comparison is difficult due to the different digestion models (static and dynamic) and incubation times used for the experiments. The structure evolution during gastrointestinal digestion did not differ among the different cheeses.

3.3. Proteolysis followed by SDS-PAGE

All samples were analyzed by SDS-PAGE, and one representative gel is shown (Fig. 2). Fig. 2A shows the evolution of the proteins and their breakdown products during dynamic IVD, here indicating a gradual breakdown of intact caseins over time during the gastric phase. After 60 min of digestion, intact casein bands were dramatically reduced, but large fragments of caseins and peptides of around 4 to 6 kDa remained visible. At 120 min of digestion, no intact caseins were visible on the gels (data not shown). This is in contrast to intestinal digestion, where caseins had fully disappeared after 60 min of digestion and only the bands corresponding to the pancreatin were still visible. Static IVD experiments were performed with raw and pasteurized cheese samples to visualize the possible differences between the cheeses in hydrolysis of whole proteins (Fig. 2B) after the oral, gastric, and intestinal endpoints of IVD. Small differences in band intensities were visible after the oral phase between raw and pasteurized cheeses, but there were no differences between the samples with or without the proteolytic culture. Only pepsin was identified as a double band at 49 kDa in the gastric phase and bands from pancreatin were found in the intestinal phase. Kinetics of casein hydrolysis at the gastric and intestinal endpoints of both digestion protocols are in agreement with previous data (Egger, et al., 2017, 2016).

3.4. Proteolysis followed by LC-MS

The time-resolved generation of peptides during dynamic IVD was analyzed by mass spectrometry in all different cheeses (n = 4) and at different time points during dynamic IVD (Fig. 3, Supplemental Fig. 2). Peptide patterns of the four caseins (β-casein, αs_1-casein, αs_2-casein, κ-casein) were analyzed semi-quantitatively as previously described (Egger, et al., 2016) and represented in heatmaps. As previously shown for digestion of SMP (Egger, et al., 2017), a clear evolution in peptide generation during the gastric and intestinal digestion phases was observed for all caseins. After the gastric phase, pasteurized samples without the added proteolytic culture had a lower number of peptides at the C-terminal ends of the caseins (Fig. 3, Supplemental Fig. 2). These differences were no longer visible after the completion of the intestinal digestion phase, where no clear differences in the peptide patterns between the four cheeses could be found. Only few casein peptides were left after 240 min of intestinal digestion, indicating that most of the casein peptides were smaller than 5 amino acids in length which is the detection size for the method. Although many peptides are already present in ripened cheeses due to bacterial proteolysis, the kinetics of casein hydrolysis during Raclette-type cheese and earlier performed SMP digestion (Egger, et al., 2017) were very similar.

3.5. Generation of free amino acids and small peptides

The kinetics of free amino acid (HPLC) and free primary amine release (OPA method) in the cheese digesta during dynamic digestion are shown in Fig. 4. The amount of free amino acids and free R-NH_2 at G0 corresponded to the level of protein breakdown that occurred during the ripening process, without addition of digestive enzymes. The level of R-NH_2 release was higher than the level of FAA release because with this method, small peptides remaining in solution after precipitation with perchloric acid were detected as well. Compared with the intestinal phase, very few amino acids and small peptides were released in the gastric phase independent of the cheeses, which was as well observed in a study with Gouda cheese (Žolnere, Arnold, Hull, & Everett, 2019), in Emmental cheese (Parrot, Degraeve, Curia, & Martial-Gros, 2003), and in SMP (Egger, et al., 2017; Parrot, Degraeve, Curia, & Martial-Gros, 2003). The release of free R-NH_2 reached its highest levels after 15 min of gastric digestion and decreased after 60 min of this phase because of gastric emptying. Intestinal digestion showed a rapid and important increase of FAA and free R-NH_2 up to 180 min and was followed by a decrease after 240 min of intestinal digestion because of an increase in the intestinal emptying rate together with less entry from the gastric compartment. Comparing the four cheeses, no significant difference in the extent of proteolysis was observed, with a higher level being liberated in the intestinal phase compared with the gastric phase because of the high susceptibility of caseins toward pancreatic

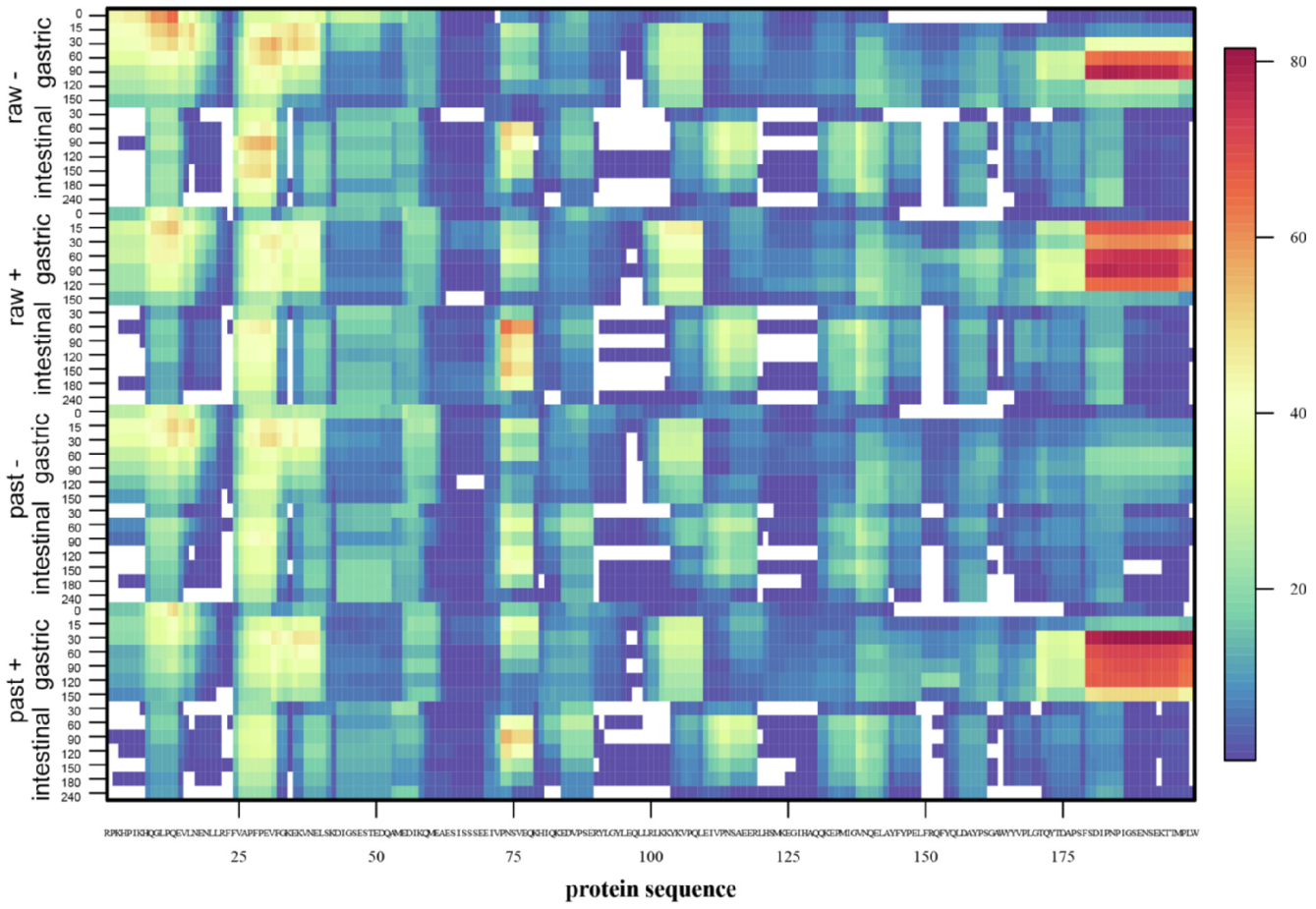

Fig. 3. Peptide patterns of α-s$_1$ casein showing the evolution in peptide generation during dynamic IVD. Comparison of average (n = 2) peptide patterns between cheeses produced either from raw (raw −, raw +) or pasteurized (past −, past +) milk with the addition of a proteolitic *L. helveticus* (LH) culture (−, +). Samples were taken after 0, 15, 30, 60, 90, 120, and 150 min of gastric digestion and after 30, 60, 90, 120, 150, 180, and 240 min of intestinal digestion. The number of identified peptides is shown as a color code, with a high number of identifications being represented in red over yellow and a low number of identifications in green and blue. Unidentified stretches are left white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteases, such as trypsin, similar to observations in Gouda cheese (Žolnere, Arnold, Hull, & Everett, 2019). A higher amount of free amino acids observed in cheeses containing *L. helveticus* was not observed after digestion, indicating that the proteolytic activity of *L. helveticus* was restricted to cheese fermentation and had no effect on the degree of hydrolysis during the digestive process.

3.6. Generation of specific peptides regarding the bacterial diversity

The overall peptide patterns of the four cheeses (data not shown)

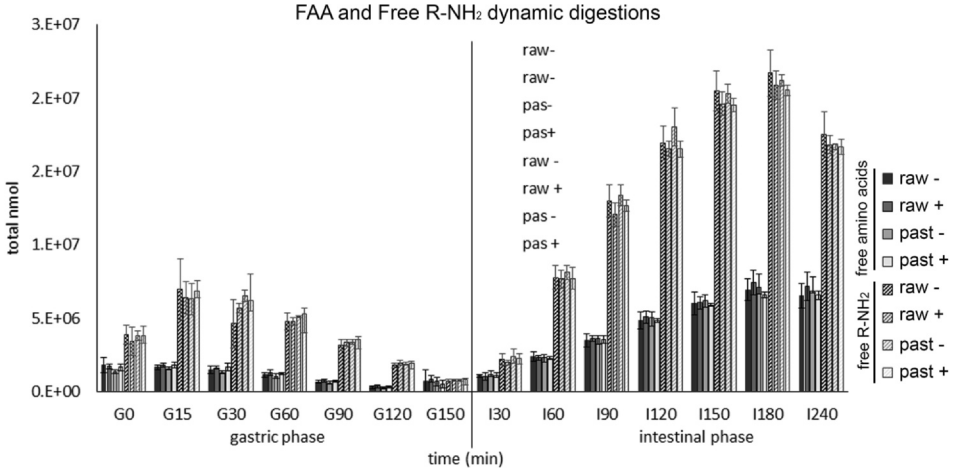

Fig. 4. Liberation of free amino acids and R-NH$_2$: during dynamic IVD. Release of free amino acids and R-NH$_2$ during dynamic IVD in the four different cheese conditions. Error bars indicate the standard deviation of two individual digests and two cheeses with the same conditions (n = 4).

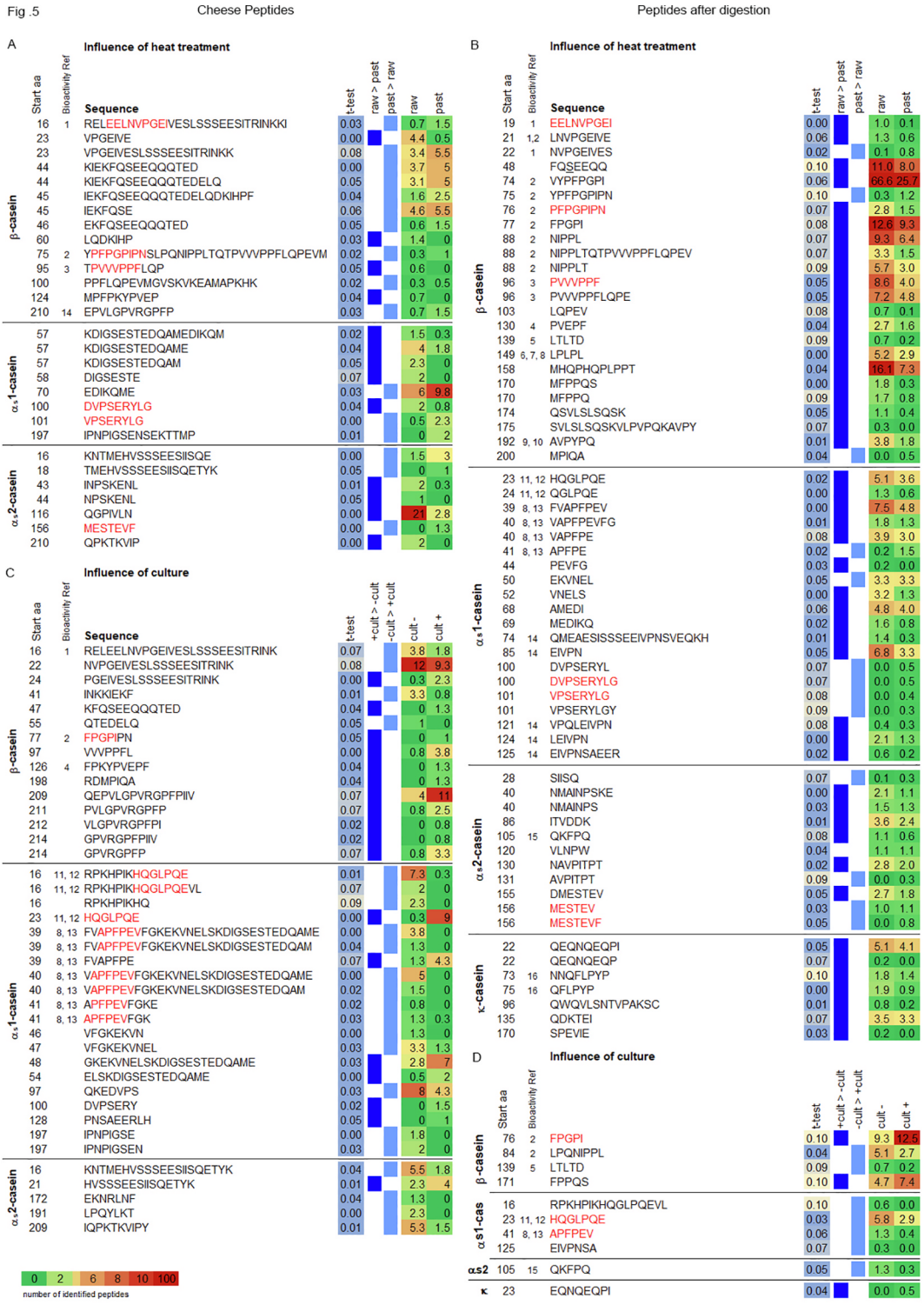

Fig. 5. Peptides in cheese and peptides present at the end of static and dynamic IVD depending on the thermal milk treatment prior to cheese making or the addition of a proteolytic culture. Summed up amino acids identified within casein peptides for the four cheese conditions in the cheeses (a, c) and after complete static and dynamic IVD (intestinal phase 120 min, b, d). Significant differently generated peptides between raw and pasteurized conditions are shown (*t*-test, ≤ 0.1, n = 12). Dark blue squares indicate a higher number of peptides in raw milk cheeses (raw > past) or in samples without the added proteolytic culture (-cult > +cult), and light blue squares indicate a higher number of peptides under pasteurized conditions (past > raw) or with the added culture (+cult > -cult). The start amino acid within the respective protein is indicated for each peptide (start aa). Digestion-resistant peptides that were already present in cheeses are highlighted in red; references are indicated for known bioactive peptides (bioactive Ref) and are listed in Supplemental Table 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and at the end of intestinal IVD were not different (Fig. 3, Supplemental Fig. 2), but by performing an additional semi-quantitative analysis at the level of individual peptides of the four caseins (αs_1-, αs_2-, β-, and κ-casein), differences in specific peptides could be observed (*t*-test, $p \leq 0.1$, Fig. 5) in dependence of milk heat treatment or presence of the *L. helveticus* strains. As no differences were found at the intestinal endpoints of dynamic (n = 8) and static (n = 4) IVD, the results from both IVD protocols were evaluated together. In the cheeses, a total of 29 peptides were found to be different between the raw and pasteurized conditions (Fig. 5A), and 40 peptides were different depending on the presence or absence of the proteolytic strain (Fig. 5C). In contrast to what has been observed in raw and pasteurized ovine cheeses (Pisanu, Pagnozzi, Pes, Pirisi, Roggio, Uzzau, et al., 2015), in the Raclette-type cheeses, a higher bacterial diversity (raw > past and + > -) did not lead to higher peptide numbers in general (Fig. 5A and C, dark blue), but in raw milk cheeses they were shorter (Supplemental Fig. 3, $p < 0.001$). This could be explained by a higher plasmin activity due to milk pasteurization (Santiago-López, Aguilar-Toalá, Hernández-Mendoza, Vallejo-Cordoba, Liceaga, & González-Córdova, 2018) that was compensated by the activity bacterial proteases present in raw milk cheeses. A similar balancing effect was observed during ripening of Parmigiano-Reggiano over 24 month, where the constant generation and degradation of peptides was studied in detail by a peptidomics approach and was explained by the differential activity of bacterial endo- and exopeptidases (Sforza, Cavatorta, Lambertini, Galaverna, Dossena, & Marchelli, 2012). In contrast to the cheeses, in the intestinal digesta, a higher number of peptides, namely 62 of the identified digestion resistant peptides were different between raw and pasteurized cheeses, and 50 of these peptides were present in a higher number in raw milk cheeses. Only 10 peptides were different, depending on the presence of the proteolytic strain (Fig. 5D). These results suggest that the influence of *L. helveticus* observed in the cheeses was counterbalanced during IVD by proteases present in the gastric and intestinal fluids and the higher bacterial diversity present in raw milk cheese had a dominant influence on peptide generation. In a study focusing on the impact of peptide generation by *L. helveticus* during simulated IVD, the influence of raw milk bacteria was excluded by heat treatment (Matar, Amiot, Savoie, & Goulet, 1996), making a direct comparison difficult. Interestingly, most of the identified digestion-resistant peptides have been previously identified in human jejunal effluents by Boutrou et al. (Boutrou, Gaudichon, Dupont, Jardin, Airinei, Marsset-Baglieri, et al., 2013), indicating that the IVD model is close to human physiology. Moreover, for 34 of the identified digestion-resistant peptides a bioactive function has been reported (Fig. 5 B, Bioactivity Ref), such as acting as ACE inhibitors, DPP IV inhibitors, calcium binding properties, or having antimicrobial effects (Supplemental Table 3).

4. Conclusions

The impact of bacterial diversity was investigated in four different Raclette-type cheeses before, during, and after completion of IVD. Digestion kinetics were assessed by applying a dynamic (DIDGI®) protocol and for digestion endpoints, the harmonized both the static IVD (M. Minekus, et al., 2014) and the dynamic model were considered; according to the previously reported comparability of the two systems (Egger & Ménard, 2017).

Bacterial diversity ranging from low, where the milk microbiome was reduced by pasteurization (past -), to a degree of higher diversity with the addition of proteolytic *L. helveticus* strains (past +), to high diversity using raw milk without (raw -), and to even higher with *L. helveticus* cultures (raw +), could be confirmed after 120 days of ripening (Supplemental Fig. 1). At this time point, the cheeses had no differences for the major compounds (dry weight, fat, and protein). Only a slight increase in proteolysis ($R\text{-}NH_2$, Table 1), specific metabolites and microstructure (Fig. 1) could be related to the higher bacterial complexity (raw -, raw +). Moreover, differences in specific peptides were equally influenced by heat treatment (Fig. 5A) or the presence of *L. helveticus* strains (Fig. 5C).

Digestion kinetics revealed a small difference in peptide hydrolysis in the gastric phase, where fewer peptides were generated at the C-terminal ends of all caseins (Fig. 3 and Supplemental Fig. 2), in digesta of pasteurized cheeses without *L. helveticus*, which was no longer present after full intestinal digestion. However, at the intestinal endpoint, a higher number of small peptides (5–6 aa in length, $p < 0.001$) in digests of raw milk cheeses, and a non-significant higher number of longer peptides (11-x aa in length) in digests from pasteurized cheeses (Supplemental Fig. 3) were observed, indicating that proteolysis was slightly more advanced after digestion of raw milk cheeses. Moreover, specific digestion-resistant peptides with and without bioactive function, were increased in digesta of raw milk cheeses (Fig. 5B), allowing the conclusion, that a higher bacterial diversity, had an impact on IVD and was leading to a higher number of bioactive peptides that could be beneficial for consumers.

Credit author statements

E. L., M. O., Di. D. and P. R.: Conceptualization and Data curation. **E. L., M. O., P. R., A. L., De. D., B. R., F. C., M. M., B. H., S. H., C. B.:** Formal analysis. **E. L.:** Project administration, Writing - original draft. **E. L., M. O., Di. D.:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Ascone, P., Maurer, J., Haldemann, J., Irmler, S., Berthoud, H., Portmann, R., ... Wechsler, D. (2017). Prevalence and diversity of histamine-forming Lactobacillus parabuchneri strains in raw milk and cheese – A case study. *International Dairy Journal, 70*, 26–33.

Badertscher, R., Freiburghaus, C., Wechsler, D., & Irmler, S. (2017). Validated method for the determination of propane-1,2-diol, butane-2,3-diol, and propane-1,3-diol in cheese and bacterial cultures using phenylboronic esterification and GC–MS. *Food Chemistry, 230*, 372–377.

Barac, M., Vucic, T., Zilic, S., Pesic, M., Sokovic, M., Petrovic, J., ... Milincic, D. (2019). The Effect of In Vitro Digestion on Antioxidant, ACE-Inhibitory and Antimicrobial Potentials of Traditional Serbian White-Brined Cheeses. *Foods, 8*(3), 94.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography B, 336*(1), 93–104.

Bos, C., Mahe, S., Gaudichon, C., Benamouzig, R., Gausseres, N., Luengo, C., ... Tome, D. (1999). Assessment of net postprandial protein utilization of 15N-labelled milk nitrogen in human subjects. *British Journal of Nutrition, 81*(3), 221–226.

Bourlieu, C., Menard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M. N., ... Dupont, D. (2015). The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during in vitro gastric digestion. *Food Chemistry, 182*, 224–235.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., ... Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition, 97*(6), 1314–1323.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... Recio, I.

(2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*. https://doi.org/10.1038/s41596-018-0119-1.

Callahan, B. J., Sankaran, K., Fukuyama, J. A., McMurdie, P. J., & Holmes, S. P. (2016). Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses. In F1000Res 2016/11/17 ed., vol. 5 (pp. 1492).

Carafa, I., Stocco, G., Nardin, T., Larcher, R., Bittante, G., Tuohy, K., & Franciosi, E. (2019). Production of Naturally γ-Aminobutyric Acid-Enriched Cheese Using the Dairy Strains Streptococcus thermophilus 84C and Lactobacillus brevis DSM 32386. Frontiers in Microbiology 10, 93-93.

Deglaire, A., Bos, C., Tome, D., & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition, 102*(12), 1752–1759.

Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., ... Goodacre, R. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature Protocols, 6*(7), 1060–1083.

Egger, L., & Ménard, O. (2017). Update on bioactive peptides after milk and cheese digestion. *Current Opinion in Food Science, 14*, 116–121.

Egger, L., Ménard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., ... Portmann, R. (2017). Digestion of milk proteins: Comparing static and dynamic in vitro digestion systems with in vivo data. *Food Research International*.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., ... Portmann, R. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International, 88*, 217–225.

Egger, L., Ménard, O., Portmann, R. (2018). Interdisciplinary Approaches to Food Digestion, Chapter: Quantitative Characterization of Digestion Processes (Springer ed.): Springer.

Elashoff, J. D., Reedy, T. J., & Meyer, J. H. (1982). Analysis of gastric emptying data. *Gastroenterology, 83*(6), 1306–1312.

Griffiths, M. W., & Tellez, A. M. (2013). Lactobacillus helveticus: The proteolytic system. *Frontiers in Microbiology, 4*, 30.

Guinot, L., Rioux, L.-E., Labrie, S., Britten, M., & Turgeon, S. L. (2019). Identification of texture parameters influencing commercial cheese matrix disintegration and lipid digestion using an in vitro static digestion model. *Food Research International, 121*, 269–277.

Lentner, C. (1973). *Geigy Scientific Tables, 1*.

Lvova, L., Denis, S., Barra, A., Mielle, P., Salles, C., Vergoignan, C., ... Feron, G. (2012). Salt release monitoring with specific sensors in “in vitro” oral and digestive environments from soft cheeses. *Talanta, 97*, 171–180.

Malagelada, J. R., Longstreth, G. F., Summerskill, W. H., & Go, V. L. (1976). Measurement of gastric functions during digestion of ordinary solid meals in man. *Gastroenterology, 70*(2), 203–210.

Matar, C., Amiot, J., Savoie, L., & Goulet, J. (1996). The Effect of Milk Fermentation by Lactobacillus helveticus on the Release of Peptides During In Vitro Digestion. *Journal of Dairy Science, 79*(6), 971–979.

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE, 8*(4), Article e61217.

Ménard, O., Picque, D., & Dupont, D. (2015). The DIDGI® System. In K. Verhoeckx, P. Cotter, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, ... H. Wichers (Eds.). *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (pp. 73–81). Cham: Springer International Publishing.

Meola, M., Rifa, E., Shani, N., Delbès, C., Berthoud, H., & Chassard, C. (2019). DAIRYdb: A manually curated reference database for improved taxonomy annotation of 16S rRNA gene sequences from dairy products. *BMC Genomics, 20*(1), 560.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function, 5*(6), 1113–1124.

Minekus, M., Marteau, P., Havenaar, R., & Huis in T Veld, J. H. J. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine (Vol. 23). Nottingham, ROYAUME-UNI: Fund for the Replacement of Animals in Medical Experiments.

Murali, A., Bhargava, A., & Wright, E. S. (2018). IDTAXA: A novel approach for accurate taxonomic classification of microbiome sequences. *Microbiome, 6*(1), 140.

Parrot, S., Degraeve, P., Curia, C., & Martial-Gros, A. (2003). In vitro study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. *Nahrung, 47*(2), 87–94.

Pisanu, S., Pagnozzi, D., Pes, M., Pirisi, A., Roggio, T., Uzzau, S., & Addis, M. F. (2015). Differences in the peptide profile of raw and pasteurised ovine milk cheese and implications for its bioactive potential. *International Dairy Journal, 42*, 26–33.

Santiago-López, L., Aguilar-Toalá, J. E., Hernández-Mendoza, A., Vallejo-Cordoba, B., Liceaga, A. M., & González-Córdova, A. F. (2018). Invited review: Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science, 101*(5), 3742–3757.

Sforza, S., Cavatorta, V., Lambertini, F., Galaverna, G., Dossena, A., & Marchelli, R. (2012). Cheese peptidomics: A detailed study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24 months of aging. *Journal of Dairy Science, 95*(7), 3514–3526.

Somers, J. M., Guinee, T. P., & Kelly, A. L. (2002). The effect of plasmin activity and cold storage of cheese milk on the composition, ripening and functionality of mozzarella-type cheese. *International Journal of Dairy Technology, 55*(1), 5–11.

Stratton, J. E., Hutkins, R. W., & Taylor, S. L. (1991). Biogenic Amines in Cheese and other Fermented Foods: A Review. *Journal of Food Protection, 54*(6), 460–470.

Stuknytė, M., Cattaneo, S., Masotti, F., & De Noni, I. (2015). Occurrence and fate of ACE-inhibitor peptides in cheeses and in their digestates following in vitro static gastrointestinal digestion. *Food Chemistry, 168*, 27–33.

Summer, A., Formaggioni, P., Franceschi, P., Di Frangia, F., Righi, F., & Malacarne, M. (2017). Cheese as Functional Food: The Example of Parmigiano Reggiano and Grana Padano. *Food Technol Biotechnol, 55*(3), 277–289.

Wyder, M. T. (2001). Einsatz von Lb. helveticus in der Raclette-Fabrikation. FAM Information(412).

Žolnere, K., Arnold, M., Hull, B., & Everett, D. W. (2019). Cheese proteolysis and matrix disintegration during in vitro digestion. *Food Structure, 21*, Article 100114.