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## **Influence of raw milk microflora and starter cultures in cheese on protein hydrolysis and peptide generation during digestion**

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## Context

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Do bacterial strains in cheese have an impact on protein hydrolysis during human digestion, and if so, does a higher microbial diversity lead to the generation of a higher number of different peptides after digestion? To answer this question, four different cheeses made from milk with or without microflora, plus or minus the addition of a proteolytic strain were produced and ripened for 120 days, followed by *in vitro* digestion experiments, applying a static (Infogest) and a dynamic (DIDGI) oro-gastrointestinal *in vitro* model. The digesta were characterized at a multiscale level, such as, confocal laser microscopy, gel electrophoresis, mass spectrometry, and HPLC, to investigate the influence of the pre-proteolysis occurring during cheese ripening, on the global proteolysis during the digestion.

## Objectives

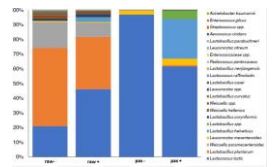
### Cheese fabrication and composition

## Dynamic and Static *in vitro* digestions

Swiss **Raclette** cheeses were produced either from raw or pasteurized milk, with or without the addition of a proteolytic bacterial strain (*Lactobacillus helveticus*) and ripened during 120 days. 2 cheeses of each condition were produced: - raw milk without starter (**raw -**) n=2 - raw milk plus starter (**raw +**) n=2 - pasteurized milk without starter (**past -**) n=2 - pasteurized milk plus starter (**past +**) n=2

characterization after 120 days of ripening

	fat	NaCl	true protein	NPN	pH	OPA
	g/kg	g/kg	g/kg	g/kg		mmol/kg
raw -	290.3	38.9	244.4	5.7	5.6	151.3
raw +	295.8	37.0	250.3	5.6	5.6	151.5
past -	284.8	21.0	247.4	5.7	5.6	143.2
past +	288.3	38.9	238.3	5.8	5.6	145.2



A standardised static *in vitro* digestion method suitable for food – an international consensus!  
M. Minekus,<sup>1\*</sup> M. Alminger,<sup>2\*</sup> P. Alvito,<sup>3</sup> S. Ballance,<sup>4\*</sup> T. Bohm,<sup>5</sup> C. Bourlieu,<sup>6</sup> F. Carrière,<sup>7</sup> R. Bourrou,<sup>8</sup> M. Corredig,<sup>9</sup> D. Dupont,<sup>10</sup> C. Dufour,<sup>11</sup> L. Egger,<sup>12</sup> M. Goding,<sup>13</sup> S. Karakaya,<sup>14</sup> B. Kikinas,<sup>15</sup> S. Le Feunteun,<sup>16</sup> U. Lemmens,<sup>17</sup> A. Macierzanka,<sup>18</sup> A. Mackie,<sup>19</sup> S. Marze,<sup>20</sup> J. McClements,<sup>21</sup> O. Ménard,<sup>1\*</sup> I. Recio,<sup>22</sup> C. N. Santos,<sup>23</sup> R. P. Singh,<sup>24</sup> G. E. Vegarud,<sup>25</sup> M. S. J. Wickham,<sup>26</sup> W. Weichenthal,<sup>27</sup> and A. Brodsky<sup>28</sup>

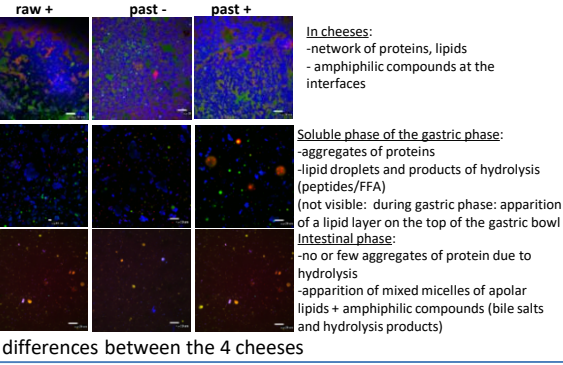
**Parameters**  
**Gastric:**  
 -Pepsin (2000U/ml) :0.5ml/min  
 -HCL1M to achieve:pH=1.68±3.52<sup>(1/4)</sup>  
 -Emptying  $t_{1/2}$ =85min  $\beta$ =1.8  
 -duration: 150min  
**Intestinal digestion:**  
 -Bile (2%) 0.5ml/min  
 -Pancreatin (7%)0.25ml/min  
 -NaHCO<sub>3</sub> 1M to achieve pH=6.8  
 -Emptying  $t_{1/2}$ =250min  $\beta$ =2.5  
 -duration: 240min

**Parameters**  
**Gastric:**  
 -Pepsin (2000U/ml)  
 -pH=3  
 -duration: 120min  
**Intestinal digestion:**  
 -Bile salts 10mM  
 -Pancreatin: to cover 100 U of trypsin/ml  
 -pH=7  
 -duration: 120min

## Results

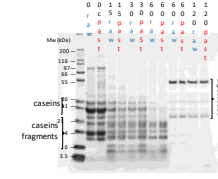
### Confocal microscopy (CSLM)

Observations of cheeses and after 85min ( $t_{1/2}$ ) of dynamic gastric and intestinal digestions



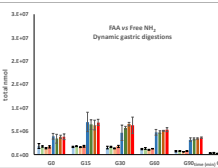
## PROTEOLYSIS

### Dynamic digestion

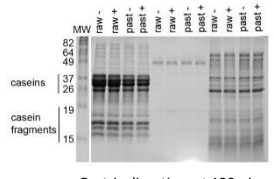


**Gastric digestion:**  
 - rapid hydrolysis of intact caseins in all cheeses  
 - at 60min of digestion no intact caseins remained but large fragments still detected

**Intestinal digestion:**  
 - only proteins from pancreatin visible

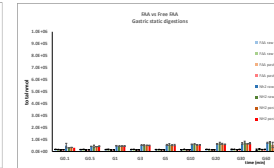


### Static digestion

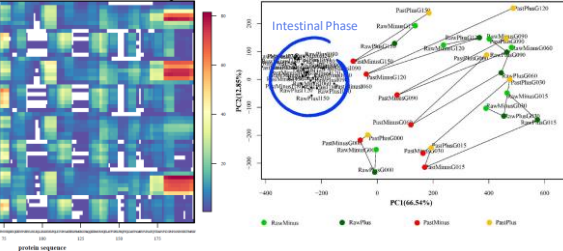


**Gastric digestion at 120min:**  
 - no intact caseins detected

**Intestinal digestion at 120min:**  
 - only proteins and peptides from pancreatin



### Mass spectrometry: Peptide patterns and PCA of dynamic digestions

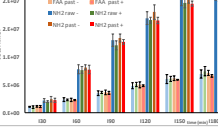


**Gastric digestion:**  
 - lower number of peptides at the c-terminus of the past – cheese sample (observed in all caseins)

**Intestinal digestion:**  
 - differences are equalized through the action of intestinal proteases

**Gastric digestion:**  
 - past – cheese samples are different from the others during the whole gastric phase  
 - no differences observed in the intestinal phase for the four different cheeses

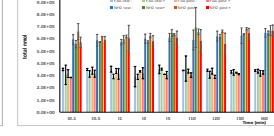
### Dynamic digestion



**Gastric digestion:**  
 - very low liberation of amino-acids (FAA) during the gastric phase  
 - liberation of Free NH<sub>3</sub> after 15min but decrease from 60min due to increase in gastric emptying

**Intestinal digestion:**  
 - high proteolysis with a maximal value after 180 min  
 - at t240min decrease due to intestinal emptying

### Static digestion



**Gastric digestion:**  
 - no or low liberation of FAA & Free NH<sub>3</sub> in the 4 cheeses

**Intestinal digestion:**  
 - same pattern for the 4 cheeses with a rapid proteolysis at the beginning of the intestinal phase leading to a drastic increase of FAA & Free NH<sub>3</sub>, followed by a plateau that could be explained by a high susceptibility of caseins towards trypsin and/or an inhibition of the proteases

In both digestion systems: no difference between the four cheeses

## Conclusion

1. A lower bacterial diversity (past - cheese) leads to a decreased peptide generation during the gastric phase which is equalized at the beginning of the intestinal phase
2. Dynamic vs static digestion:  
 Gastric phase: no difference between the 2 digestion models due to a low proteolysis at the molecular level  
 Intestinal phase: different pattern is observed with a progressive increase of FAA and Free NH<sub>3</sub> in the dynamic system compared to a drastic and instantaneous liberation of FAA and Free NH<sub>3</sub> in the static model. The results are in accordance with previous studies (Egger et al; 2018 Digestion of milk proteins: comparing static and dynamic *in vitro* digestion systems with *in vivo* data)