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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

**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

**Bacterial RNA at 120 days**

## Dynamic and Static *in vitro* digestions

**A standardised static *in vitro* digestion method suitable for food – an international consensus!**

**Parameters**

**Dynamic (DIDGI) dig n=2 cheeses: 4X2**

**Static (INFOGEST) dig n=1 cheeses: 4X2**

**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

**GR probes**  
**Apolar lipids**  
**Proteins**  
**Amphiphiles**

**raw - raw + past - past +**

**In cheeses:**  
-network of proteins, lipids  
- amphiphilic compounds at the interfaces

**Soluble phase of the gastric phase:**  
-aggregates of proteins  
-lipid droplets and products of hydrolysis (peptides/FFA)  
(not visible: during gastric phase: apparition of a lipid layer on the top of the gastric bowl)

**Intestinal phase:**  
-no or few aggregates of protein due to hydrolysis  
-apparition of mixed micelles of apolar lipids + amphiphilic compounds (bile salts and hydrolysis products)

no structural differences between the 4 cheeses

### PROTEOLYSIS

**Dynamic digestion**

**Static 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

**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

**Intestinal Phase**

**Gastric digestion:**  
- lower number of peptides at the c-terminus of the past – cheese sample (observed in all caseins)  
- differences are equalized through the action of intestinal proteases

**Intestinal 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

### PROTEOLYSIS

**Dynamic digestion**

**Static 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

**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)