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Olivia Ménard, Reto Portmann, Didier Dupont, Helena Stoffers, Hélène Berthoud, Marco Meola, Guy Vergères, Lotti Egger

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Influence of raw milk microflora and starter cultures in cheese on proteolysis during *in vitro* digestion

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Swiss Confederation

Federal Department of Economic Affairs, Education and Research EAER
Agroscope

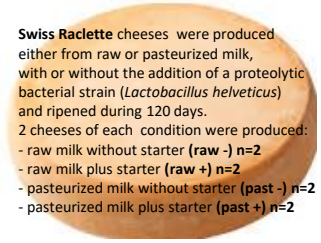
Context

O Ménard[1*], R Portmann[2], D Dupont[1], L Abbuehl[2], H Stoffers[2], H Berthoud[2], M Meola[2], G Vergères[2], L Egger[2]

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

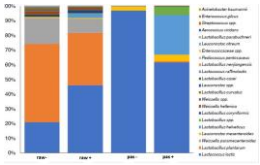


Swiss Raclette cheeses were produced either from raw or pasteurized milk, with or without the addition of a proteolytic bacterial strain (*Lactobacillus helveticus*) and ripened during 120 days.
2 cheeses of each condition were produced:
- raw milk without starter **(raw -) n=2**
- raw milk plus starter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

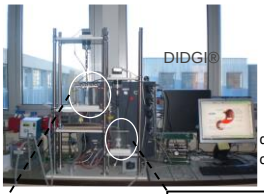
characterization after 120 days of ripening

	fat	NaCl	true protein	NPN	pH	OPA
	g/kg	g/kg	g/kg	g/kg		mmol/kg
raw -	290.3	18.9	244.4	5.7	5.6	151.3
raw +	295.8	17.9	250.3	5.6	5.6	151.5
past -	284.8	21.0	247.4	5.7	5.6	143.2
past +	288.3	18.9	238.3	5.8	5.6	145.2

Bacterial RNA at 120 days



Dynamic and Static *in vitro* digestions



dig n=2
cheeses: 4X2

Gastric :
-Pepsin (2000U/ml) :0.5ml/min
-HCL1M to acheive:pH=1.68+3.52$^{(-t/42)}$
-Emptying $t_{1/2}$=85min β= 1.8
- duration: 150min

Intestinal digestion:
-Bile (2%) 0.5ml/min
-Pancreatin (7%)0.25ml/min
-$NaHCO_3$ 1M to acheive pH=6.8
-Emptying $t_{1/2}$=250min β= 2.5
-duration: 240min

Parameters

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

M. Minekus,[1,2] M. Alminger,[3,4] P. Alvito,[5,6] S. Ballance,[7,8] T. Bohn,[9,10] C. Bourlieu,[11,12] F. Carrière,[13] R. Boutrou,[14] M. Corredig,[15] D. Dupont,[16] C. Dufour,[17] L. Egger,[18] M. Golding,[19] S. Karakaya,[20] B. Kirkhus,[21] S. Le Feunteun,[22] U. Lesmes,[23] A. Macierzanka,[24] A. Mackie,[25] S. Marze,[26] D. J. McClements,[27] O. Ménard,[28] I. Recio,[29] C. N. Santos,[30] R. P. Singh,[31] G. E. Vegarud,[32] M. S. J. Wickham,[33] W. Weitschies[34] and A. Brodkorb[35]

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dig n=1
cheeses: 4X2

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

Parameters

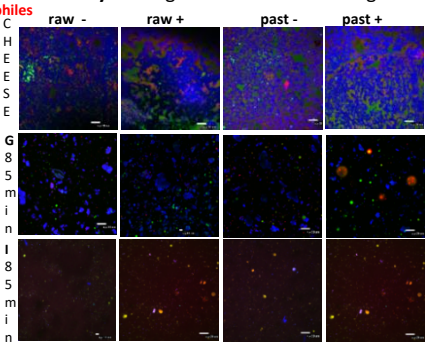
Results

Confocal microscopy (CSLM)

GR probes
Apolar lipids
Proteins
Amphiphiles

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

STRUCTURE



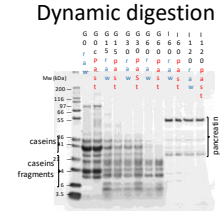
In cheeses:
-network of proteins, lipids
- amphiphilic compounds at the interfaces

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

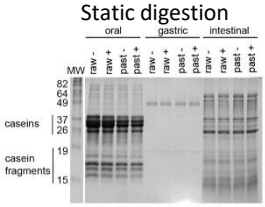
PROTEOLYSIS

Dynamic digestion



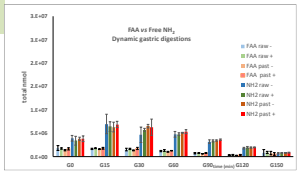
Gastric digestion:
- rapid hydrolysis of intact caseins in all cheeses
- at 60min of digestion no intact caseins remained but large fragments still detected
Intestinal digestion:
- only proteins from pancreatin visible

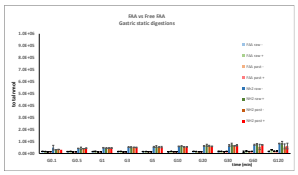
Static digestion

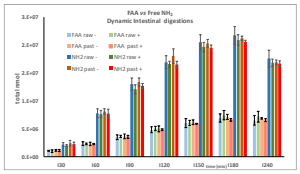


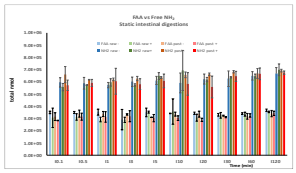
Gastric digestion at 120min:
- no intact caseins detected

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









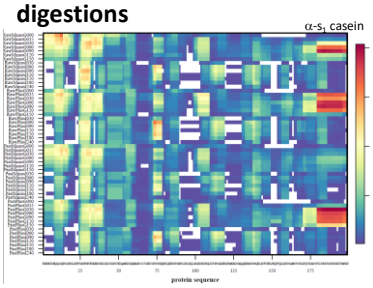
Gastric digestion:
- very low liberation of amino-acids (FAA) during the gastric phase
- liberation of Free NH_2 after 15min but decrease from 60min due to increase in gastric emptying
Intestinal digestion:
- high proteolysis with a maximal value after 180 min
- at t240min decrease due to intestinal emptying

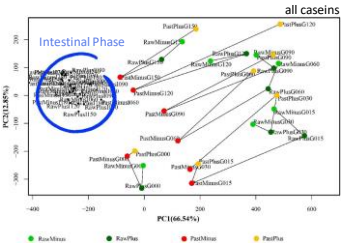
Gastric digestion:
- no or low liberation of FAA & Free NH_2 in the 4 cheeses
Intestinal digestion:
- same pattern for the 4 cheeses with a rapid proteolysis at the beginning of the intestinal phase leading to a drastic increase of FAA & Free NH_2, followed by a plateau that could be explained by a high susceptibility of caseins towards trypsin and /or an inhibition of the proteases

In both digestion systems :no difference between the four cheeses

PROTEOLYSIS

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

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_2 in the dynamic system compared to a drastic and instanteneous liberation of FAA and Free NH_2 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)

STLO

[1]STLO, UMR 1253, INRA, Agrocampus Ouest, 35000 Rennes, France
[2]Agroscope, Schwarzenburgstr. 161, 3003 Bern, Switzerland

*olivia.menard@inra.fr

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Schweizerische Eidgenossenschaft
Confédération suisse
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