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Par

Mme Leticia Hernández Galán

POTENTIAL OF CHEESE MICROORGANISMS ECOSYSTEMS FOR THE PRODUCTION OF BIOACTIVE PEPTIDES AND EFFECT OF THE DAIRY MATRICES ON THE SURVIVAL OF DAIRY MICROORGANISMS THROUGH DIGESTION.

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Mme Silvia L. Amaya Llano, Professeur, Universidad Autónoma de Querétaro, Mexique	**Rapporteur**
M. Eric Beuvier, Ingénier de Recherche (HDR), INRA, France	**Rapporteur**
Mme Catherine Béal, Professeur, AgroParisTech, France	**Président du jury**
Mme Sandra T. Martín del Campo B, Professeur, Tecnológico de Monterrey, Mexique	**Directrice de Thèse**
M. Henry Eric Spinnler, Professeur, AgroParisTech, France	**Directeur de Thèse**
M. Daniel Picque, Ingénier de Recherche, INRA, France	**Co-encadrant**
M. Micloth López del Castillo L., Professeur, Universidad Veracruzana, Mexique	**Co-encadrant**

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TESIS QUE PRESENTA

LETICIA HERNÁNDEZ GALÁN

DOCTORADO EN CIENCIAS DE INGENIERÍA

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LETICIA HERNÁNDEZ GALÁN

Directora: Dra. Sandra Teresita Martín del Campo Barba

Director: Dr. Henry Eric Spinnler

Comité de tesis:

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	Dra. Catherine Béal	Vocal
	Dra. Sandra Teresita Martín del Campo Barba.	Vocal
	Dr. Henry Eric Spinnler.	Vocal

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List of abbreviations

	English	**Français**
ACE	angiotensin converting enzyme	enzyme de conversion de l'angiotensine
ASN	acid (pH 4.6) soluble nitrogen	azote soluble dans acide (pH 4,6)
BA	*Brevibacterium aurantiacum*	*Brevibacterium aurantiacum*
CFU	colony forming unit	unité formant colonie
CLSM	confocal laser scanning microscopy	microscopie confocal à balayage laser
DIDGI	gastrointestinal dynamic digester	digesteur dynamique gastro-intestinal
DPPH	2,2- diphenyl-1-picrylhydrazyl	2,2-diphényl-1-picrylhydrazyle
EtOH-NSN	non ethanol soluble nitrogen	azote insoluble dans l´éthanol
EtOH-SN	ethanol soluble nitrogen	azote soluble dans l´éthanol
GRM	rennet gel from whole milk	gel de pressure à partir de lait entier
GSM	rennet gel from skimmed milk	gel de présure à partir de lait écrémé
HA	hippuric acid	acide hippurique
HA	*Hafnia alvei*	*Hafnia alvei*
HHL	hippuryl-histidyl-leucine	hippuryl-histidyl-leucine

HI	hydrophilic peptide	hydrophile peptide
HO	hydrophobic peptide	hydrophobe peptide
NP	raw	cru
NPN	non-protein nitrogen	azote non protéique
P	pasteurized	pasteurisée
RM	whole milk	lait entier
S	synthetic medium	milieu synthétique
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis	électrophorèse sur gel de poluacrylamide-dodécylsulfate de sodium
SM	skimmed milk	lait écrémé
ST	*Streptococcus thermophilus*	*Streptococcus thermophilus*
TN	total nitrogen	azote total

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Résumé détaillé en français

INTRODUCTION

Le fromage est un aliment diversifié, apprécié et consommé partout dans le monde et dont l'impact sur la santé humaine a été récemment mis en évidence (Adouard *et al.*, 2016; Lopez-Exposito & Recio, 2008; Silva *et al*., 2012). L'association lait-microorganismes (fromage microbiote) qui développe les caractéristiques sensorielles des fromages, produit aussi des peptides bioactifs, qui peuvent potentiellement affecter le cours de certaines maladies (Choi, Sabikhi, Hassan, & Anand, 2012; De Simone *et al.*, 2009). La présence de ces microorganismes dans les produits laitiers a attiré l´attention du fait de leurs effets sur la santé lorsqu'ils sont consommés, en particulier parce que certains d'entre eux résistent à la digestion et interagissent avec le microbiote intestinal (Adouard *et al.*, 2016; Lay *et al*., 2004). Cette résistance à la digestion a été associée à un comportement des produits laitiers qui agiraient comme matrice protectrice pour ces microorganismes (Do Espirito Santo, Perego, Converti, & Oliveira, 2011; Lay *et al.*, 2004; Saxelin *et al*., 2010).

C´est dans ce contexte qu'est apparu l'intérêt de travailler de façon conjointe, pour l´élaboration de cette thèse, par une cotutelle entre le Tecnológico de Monterrey et l´AgroParisTech-INRA. Elle a pour objectif d'étudier la présence de peptides bioactifs avec des activités comme l'activité antioxydante ou activité inhibitrice de l´ enzyme de conversion de l'angiotensine (ECA) dans les fromages mexicains; et d'observer l'effet de matrices laitières dans la survie des microorganismes laitiers au cours de la digestion.

REVUE BIBLIOGRAPHIQUE

Peptides bioactifs dans les fromages

Les peptides bioactifs sont des fragments de protéines spécifiques qui ont un impact positif sur les fonctions du corps et pourraient influer sur l'état de santé (Kitts & Weiler, 2003). la structure des peptides bioactifs est généralement constituée de 3 à 20 acides aminés par molécule (Hajirostamloo, 2010). Dans les fromages, les peptides bioactifs sont produits par protéolyse pendant la fermentation, l´affinage ou même lors de la digestion (Gupta, Mann,

Kumar, & Sangwan, 2009; Gupta, Mann, Kumar, & Sangwan, 2013; Hernández-Ledesma, del Mar Contreras, & Recio, 2011; Theolier, Hammami, Fliss, & Jean, 2014)

Certaines fonctionnalités trouvées dans les fromages ont été associées à la présence de peptides bioactifs comprenant une activité antibactérienne (Pritchard, Phillips, & Kailasapathy, 2010; Theolier *et al.*, 2014); des propriétés cytomodulatoires (De Simone *et al.*, 2009); une activité antioxydante (Pritchard *et al.*, 2010) et l'activité anti-hypertensive (Ong, Henriksson, & Shah, 2007; Paul & Van Hekken, 2011; Torres-Llanez, González-Córdova, Hernandez-Mendoza, Garcia, & Vallejo-Cordoba, 2011). Dans ces travaux, nous allons seulement décrire le caractère antioxydant et les activités d'inhibition de l´ enzyme de conversion de l'angiotensine (ECA).

L'activité antioxydante

Les peptides bioactifs ayant une activité antioxydante ont été signalés sur le Cheddar (Pritchard *et al*., 2010), Caciocavallo (Perna, Intaglietta, Simonetti, & Gambacorta, 2015), Parmigiano Regigiano (Bottesini *et al.*, 2013) et les "cottage cheeses" (Abadía-García *et al.*, 2013). Par exemple, Perna *et al.* (2015) ont observé que l'activité antioxydante présente dans le Caciocavallo augmente au cours de l´affinage. Bottesini *et al*., (2013) ont observé que l'activité antioxydante du Parmigiano Reggiano est restée stable au cours d´affinage. Aussi, Gupta *et al.,* (2009) ont observé que l'activité antioxydante du fromage cheddar augmente au cours de l´affinage mais une protéolyse trop intense réduit cette activité. D'autre part, Timón, Parra, Otte, Broncano, et Petrón (2014) ont observé que l'origine de la présure utilisée pour la coagulation des fromages de type Burgos influe sur la concentration de peptides antioxydants.

Activité antihypertensive

Saito, Nakamura, Kitazawa, Kawai, et Itoh (2000) ont découvert que des peptides dérivés des caséines αs1- et ß de fromage Gouda affinés 8 mois sont capables de diminuer la pression artérielle systolique chez des rats spontanément hypertendus. Ryhänen, Pihlanto-Leppälä, et Pahkala (2001) ont observé une activité inhibitrice de l'ECA, dans le fromage Festivo, produite par des souches probiotiques (*Lactobacillus acidophilus* et bifidobactéries). Les deux études ont conclu que l'activité inhibitrice de l´ECA a augmenté pendant l´affinage jusqu'à un certain niveau, et à diminuer plus tard, ce qui suggère que les peptides bioactifs

sont naturellement formés dans les fromages et ne restent actifs que pour une période limitée, avant d'être hydrolysés.

Une activité inhibitrice de l'ECA a également été trouvée dans les extraits de fromages frais mexicains fabriqués avec des bactéries lactiques spécifiques (BAL) (Torres-Llanez *et al.*, 2011). Dans une autre étude, des extraits solubles de fromages frais modèles (Mexicain), fabriqués à base de lait pasteurisé, sans cultures starters, présentaient une activité inhibitrice de l´ECA significativement plus élevée à huit semaines de stockage que juste après fabrication. Cependant, les fromages ne sont plus consommables à ce moment là. Cette activité a été attribuée à la détérioration bactérienne (Paul & Van Hekken, 2011).

Le cas des fromages mexicains

Au Mexique, la plupart des fromages proviennent de fabricants de fromages artisanaux, dont l'origine géographique et de terroir, fournissent un lien fort à leurs caractéristiques et décrit, en quelque sorte, l'histoire et la culture des communautés qui les produisent (González-Córdova *et al.*, 2016). Cependant, en dépit de son importance, on sait très peu de choses sur les fromages mexicains et leurs propriétés biologiques (Abadía-García *et al.*, 2013; Torres-Llanez *et al.*, 2011).

Néanmoins, l'étude d'un aliment et son impact sur la santé peut être extrêmement complexe, principalement parce qu'il dépend d'abord de sa structure et de sa composition (initiale et / ou lors de la digestion) et ensuite parce qu'il peut agir directement sur l'hôte ou par la modulation potentielle de l'activité et des fonctions de la microflore intestinale.

Effet d'une matrice alimentaire dans la survie des microorganismes

Les microorganismes consommés avec de la nourriture, comme le fromage, doivent survivre au processus digestif (stress du à la haute acidité de de l'estomac et des sels biliaires dans l'intestin) avant de se fixer dans l'intestin et d'interagir avec le microbiote intestinal (Sumeri, Adamberg, Uusna, Sarand, & Paalme, 2012). Certains auteurs affirment que la présence d'une matrice alimentaire pourrait améliorer la survie de ces micro-organismes lors de la digestion (Do Espirito Santo *et al.*, 2011; Lay *et al.*, 2004; Saxelin *et al.*, 2010). Cependant, les rapports de la littérature sont contradictoires. Par exemple Faye, Tamburello, Vegarud, and Skeie (2012) ont observé que la survie de *L. lactis ssp cremoris* lors

d'expériences *in vitro* a été plus élevée dans le lait fermenté que dans un milieu synthétique. Par contre, Sumeri *et al.*, (2012) ont observé que les mêmes micro-organismes ne survivent pas au cours d'expériences *in vitro* lorsque du fromage semi-dur a été utilisé. La viabilité de *S. thermophilus* au cours de la digestion *in vivo* est plus grande lorsqu'il est cultivé dans le fromage camembert que dans le yogourt (Lay *et al.* , 2004). Adouard *et al.*, (2016) ont, quant à eux, mesuré la survie de *B. auranticum* et *H. alvei* au cours de digestion *in vitro*. Pour ces deux espèces, au contraire, cette survie est plus elevée lorsqu'elles sont cultivées dans un milieu synthétique que lorsqu´elles sont cultivées dans le fromage.

Des mécanismes de protection des matrices

En conséquence, l'effet réel de la matrice alimentaire dans la survie des micro-organismes n'a pas été complètement élucidé. Il a été proposé trois mécanismes pour essayer d´expliquer un effet protecteur: a) l'effet de préadaptation du micro-organismes à la matrice alimentaire; b) les effets de macrostructure pour la capacité tampon et / ou la vitesse de vidange gastrique; et c) les effets de la microstructure et ses interactions avec les micro-organismes.

L´effet de la préadaptation des microorganismes est liée à ses mécanismes de réponse au stress qui s'expriment lors de la fabrication des aliments. Cette réaction produit une adaptation croisée à un stress digestif (Begley, Gahan, & Hill, 2005). Pitino *et al.,* (2012) ont montré que la survie de *L. rhamnosus* dans le fromage au cours de la digestion *in vitro* était associé à la production de polysaccharides extracellulaires. De même Uriot *et al.*, (2016) ont observé que la viabilité de souches différentes de *S. thermophilus* pendant la digestion *in vitro* est dépendante de l'activation de l'uréase et de petites protéines de choc thermique.

D'autre part, les effets de macrostructure sont principalement liés à la capacité tampon de la matrice dans l'estomac. Gardiner, Ross, Collins, Fitzgerald, et Stanton (1998) ont suggéré que la viabilité plus élevée d´ *E. faecium* contenu dans le Cheddar ou le yaourt, après digestion *in vitro*, était due à la capacité tampon des produits laitiers par rapport au milieu synthétique utilisé comme témoin. D´autre part, la macrostructure influence le procédé de digestion, par exemple, la coagulation du lait augmente la viscosité de la matrice, perturbe la vidange gastrique et le transit intestinal (Turgeon & Rioux, 2011).

La microstructure joue également un rôle clé dans la survie des microorganismes. Dans une matrice fromagére Pitino *et al.,* (2012) ont observé la préférence de *L. rhamnosus* au réseau de caséine. Sumeri *et al*., (2012) ont observé que la survie des bactéries lactiques lors de digestion *in vitro* a été plus élevée dans les fromages que dans un milieu synthétique. Ils ont suggéré que la teneur élevée en protéines et les globules gras constituent une barrière physique, ce qui permet une surface d'interaction supplémentaire avec les microorganismes. Selon Hannon, Lopez, Madec, et Lortal (2006) la survie de *L. lactis* lors d'expériences *in vitro* a été plus élevée lorsque le fromage était pressuré à pH 5,2 que à 6,2, parce que le pH plus bas produit une matrice plus homogène.

Donc, le rôle de la matrice alimentaire est encore mal connu. Différents mécanismes sont proposés mais il est encore difficile de déterminer leurs contributions à la survie des microorganismes.

MATERIELS ET METHODES

Les fromages

Le fromage Cotija fait partie de la production régulière d'une ferme laitière artisanale située dans la région de Cotija. Ce fromage a été affiné 6 mois à 25 °C sans contrôle de l'humidité. Par contre, le fromage de chèvre frais a été produit à partir de lait cru et lait pasteurisé (63 °C 30 min). Ce fromage a été analysé frais sans affinage.

Fractions azotées

Les extraits de fromage ont été analysés pour mesurer l'azote total (TN), l'azote soluble dans l'acide (ASN), l'azote non protéique (NPN), l'azote soluble (EtOH-SN) et l'azote insoluble (EtOH-NSN) dans 70% d'éthanol. Ces fractions ont été déterminées selon la méthode de Guerra-Martínez, Montejano, and Martín-del-Campo (2012). La teneur en azote a été quantifié en utilisant des procédés Kjeldahl et micro-Kjeldahl.

Les fractions azotées, une analyse par RP-HPLC

Les fractions azotées (ASN, NPN, EtOH-SN et EtOH-NSN) ont été sélectionnés pour déterminer leurs profils peptidiques selon la méthode Abadía-García *et al.,* (2013). Les peptides des chromatogrammes résultants ont été divisés en fonction de leur temps de

rétention en peptides hydrophiles (HI) et peptides hydrophobes (HO) suivant les critères de Gonzalez de Llano, Polo, et Ramos (1995).

Détermination de l'activité antioxydante

Les fractions ASN et NPN ont été testés avec la méthode du DPPH (2,2- diphényl-1-picrylhydrazyl) selon la méthode de Abadía-García *et al*., (2013).

Détermination *in vitro* de l'activité inhibant de l´ECA

Les fractions EtOH-SN et EtOH RSN ont été testées pour leur activité inhibitrice de l'ECA suivant la méthode de Wang *et al*., (2013), l'acide hippurique (HA) a été utilisé comme standard et le hippuryl-histidyl-leucine (HHL) comme équivalent de 100% de l'activité de l'ECA.

Souches bactériennes

Streptococcus thermophilus 257 TIL (ST) a été cultivée dans du bouillon M17 à 37 °C, *Brevibacterium aurantiacum* ATCC 9174 (BA) et *Hafnia alvei* GB01 (HA) ont été cultivées dans du BHI à 25 °C.

Matrices laitières

Toutes les matrices laitières ont été fabriquées à partir de lait écrémé (SM). Pour les matrices faites avec du lait entier, la matière grasse laitière anhydre a été ajoutée à la SM. Pour des gels emprésurés, a présure a été ajoutée au lait, et les caractéristiques rhéologiques des gels ont été déterminés avec un rhéomètre rotatif.

Le modèle dynamique *in vitro* du tractus gastrointestinal

Le digesteur Dynamique Gastro-Intestinal (DIDGI®, INRA Versailles-Grignon, France) se compose de trois compartiments séparés. Ils simulent l'estomac, le duodénum et l'intestin grêle, comme décrit précédemment par Adouard *et al.,* (2016). Les paramètres de la digestion ont été contrôlés par le logiciel SToRMs pour le règlage de la vidange de l'estomac et la surveillance (Guillemin, Perret, Picque, Menard, & Cattenoz, 2010).

Digestion *in vivo*, et analyses métagénomiques et metatranscriptomiques

Vingt-quatre souris mâles (CH3 / HeN) ont été séparées en 3 groupes: le groupe témoin avec une alimentation standard, le groupe alimenté avec la matrice laitière et le groupe alimenté avec la matrice + les microorganismes. Leurs fécès ont été collectées pour l'extraction de l'ADN (Godon, Zumstein, Dabert, Habouzit, & Moletta, 1997), ensuite le gène codant l'ARNr 16S a été amplifié (Lazuka *et al*., 2015).

Microscopie à balayage laser confocal (CLSM)

Les composants laitiers ont été marqués avec différents colorants, Bodipy 493/503 (couleur verte) pour la matière grasse, DyLightTM 550 NHS Ester 562/576 (couleur jaune) pour les protéines et SYTO® 61 colorant d'acide nucléique 628/645 (couleur rouge) pour les microorganismes. La viabilité du micro-organisme dans un milieu synthétique lors d'un stress acide a été mesurée par LIVE/ DEAD® tests du biofilm qui marque les bactéries vivantes en vert et les bactéries mortes en rouge. Tous les échantillons ont été observés avec un microscope confocal inversé.

Calcul de la capacité tampon des matrices laitières

Les courbes de addition de HCL, obtenus au cours de la digestion gastrique (pour descendré le pH à 2), ont été utilisées pour calculer la capacité tampon par l'équation de Salaün, Mietton, and Gaucheron (2005).

Mesure de viscosité au cours de la digestion

Les caractéristiques rhéologiques de matrices laitières ont été mesurées pendant la digestion gastrique avec le même rhéomètre utilisé pour la caractérisation dès les gels. Ces expériences ont été conduites simultanément avec ceux de DIDGI®.

Dégradation des matrices laitières au cours de la digestion

La dégradation de la matière grasse et des protéines lors de la digestion a été mesurée par électrophorèse sur gel de polyacrylamide au Sodium Dodécyl Sulfate (SDS-PAGE), et par la mesure de la libération des acides gras mesurés avec par Chrotographie en Phase Gazeuse - Spectrométrie de Masse.

Analyses statistiques

Toutes les analyses statistiques ont été effectuées avec le logiciel Statistica V12 (Statsoft Inc., Tulsa, OK, USA).

RESULTATS ET DISCUSSION

La première partie du travail a été concentrée sur le fromage Cotija. L'activité anti-oxydante, mesurée avec le test au DPPH, sur les fractions ASN et NPN de fromages Cotija détecte des peptides bioactifs avec une activité antioxydantes, dès le début de l'affinage. Les valeurs observées au cours de la première semaine ont été de 67,3% pour l'azote NPN et 19,22% pour l'azote ASN. L´activité antioxydante dans les deux fractions ont augmenté pendant l´affinage. Au sein de l'azote NPN, l'activité a augmenté modérément de 67,9 ± 1,2% au cours du premier mois à 75,7 ± 9,70% apres six mois d´affinage. Pendant ce temps, l'activité antioxydante ASN était significativement plus faible (p <0,05), par rapport à NPN. Pour l'azote ASN l´activité du premier mois a été de 17,6 ± 2,0%, et est resté relativament stable jusqu'au quatrième mois (19,9 ± 10,1 %). Après cette période l´activité antioxydante de l'ASN a augmenté de manière significative à 66,4 ± 19,1%, égalant ainsi les valeurs observées pour l'azote NPN dans le fromage Cotija d´un mois.

L'activité antioxydante la plus élevée, observée pour le fromage Cotija était 75,66 ± 9,70% en NPN et 66,4 ± 19,1% ASN pour le fromage de six mois. Ces valeurs étaient inférieures à celle observée par Silva *et al.,* (2012) dans des extraits de fromage Coalho (77,9 - 91,1%), mais semblable à celui rapporté par Gupta *et al.,* (2009) dans des extraits de Cheddar de 4 mois (~ 70%).

L'activité antioxydante était significativement plus élevée (p <0,05) dans NPN que dans ASN. Cela pourrait être liè à la taille des peptides contenus dans chaque fraction. Bien que NPN contenait de petits peptides et des acides aminés solubles à 12% de TCA (McSweeney & Fox, 1997; Panizzolo, Arujo, Taroco, Rodriguez, & Schopf, 2011) suggèrent que les petits peptides, exercent une activité antioxydante plus élevée que les peptides de plus grand taille comme cela dans ASN.

Entre temps, l'augmentation d´activité antioxydante à la fois dans les fractions ASN et NPN au cours de l´affinage du fromage Cotija, est en accord avec les résultats de Perna *et al.*, (2015) dans les fromages Caciocavallo. Par contre, Bottesini *et al.*, (2013) ont observé que l'activité antioxydante des extraits de Parmigiano Reggiano, est resté stable à 41 mois d'affinage. Mais Gupta *et al.*, (2009) ont observé que l'activité antioxydante du fromage Cheddar a augmenté au cours de l´affinage, mais seulement jusqu'à un certain niveau, car une protéolyse très poussée réduit l'activité antioxydante.

La tendance à l'augmentation de l'activité antioxydante dans les fractions azotées ASN et NPN est compatible avec l'augmentation du contenu de l'azote dans les deux fractions (ASN / NT et NPN / NT) (chapitre 2.1.3) Par conséquent, l'activité antioxydante semble être liée à la formation de peptides solubles comme l´ont suggéré Gupta *et al.*, (2009) et Barác *et al.*, (2016) dans les fromages Cheddar et Blanc Saumuré respectivement.

De plus, il en résulte que, après quatre mois d'affinage, il y avait une augmentation significative de la protéolyse (chapitre 2.1) qui pourrait être liée à des changements dans le microbiote fromager résultant de peptides plus courts et des acides aminés avec des activités biologiques supérieures (McSweeney, 2004).

Les fractions ASN et NPN ont ensuite été étudiées par RP-HPLC pour lier le contenu en peptide avec l'activité biologique. Les chromatogrammes obtenus ont été divisés en peptides hydrophiles (HI) et hydrophobes (HO) en fonction de leurs temps de rétention (chapitre 2.1). L'activité antioxydante de l'ASN a été fortement corrélée à sa teneur en peptides HI (0,81). Ce type de peptides dans ASN a augmenté linéairement en même temps que l'activité antioxydante dans cette fraction.

Entre temps, l´actitité antioxydante dans la fraction NPN était fortement corrélée avec les deux type de peptides HI et HO (0,92 et 0,79 respectivement, pour 14 degrés de liberté), mais sa corrélation était légérement plus élevé avec les peptides HI.

Dans des études antérieures, l'activité antioxydante a été principalement attribuée à des peptides hydrophobes (Pihlanto, 2006), mais dans nos résultats, nous avons trouvé que cette activité était, la plupart du temps, en corrélation avec la teneur en peptide HI, nous ne

pouvions pas expliquer complètement ce comportement. Cela pourrait être lié aux propriétés des acides aminés dans les peptides, qui va au-delà de leur nature hydrophobe ou hydrophile des peptides, mais plutôt à des propriétés de chélation, ou la capacité de produire autres antioxydants (comme la capacité du la cystéine pour produire glutathion) (Erdmann, Cheung, & Schröder, 2008).

D´un autre côté, on a vérifié la présence de peptides inhibiteurs d´ECA dans le fromage Cotija dans ses fractions EtOH-SN et EtOH-NSN. L´activité inhibitrice dans EtOH-SN au cours du premier mois était de 82,2 ± 3,8% et a augmenté linéairement pendant l´affinage, pour arriver à 100% d'inhibition de l´ECA après le quatrième mois.

Entre temps, dans la fraction EtOH-NSN, l´actvite inhibitrice pendant le premier mois était de 49,9 ± 18,4% et il a augmenté au cours du l´affinage et arrive a, 97,4 ± 3,3% (valeurs plus élevés), au cours du cinquieme mois, et restant constant jusqu'à la fin del affinage.

Selon nos résultats, le fromage Cotija posses de une haute teneur en peptides bioactifs capables d'inhiber l'ECA. Le fromage du trois mois avait presque 100% d´activité d'inhibition (~ 100% dans EtOH-SN et > 90% dans EtOH-NSN). Cela revêt une importance particulière si l'on considère que le fromage de Cotija commercialisable doit etre affiné au moins trois mois. Les valeurs obtenues pour le fromage Cotija étaient supérieures à celles observées dans un rapport précédent(Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, and Recio, 2008) dans des extraits des variétés espagnoles (56,6 à 76,1%); et celles observées par Ong *et al.*, (2007) dans des extraits de Cheddar de différents âges (<80%). Ryhänen *et al.*, (2001), de leur coté, ont rapporté ~50% d´activité inhibitrice de l´ECA dans des extraits de fromage Festivo de trois mois.

En général, les deux fractions EtOH-SN et EtOH-NSN possèdent une haute activité inhibitrice d´ECA qui augmente au cours de l´affinage. Toutefois l'activité dans la fraction EtOH-SN était significativement plus élevée (p <0,05) que dans EtOH-NSN. Cela pourrait être dû à la taille des peptides contenus dans chaque fraction, puisque la fraction EtOH-SN contenait les plus petits peptides et que, selon Espejo-Carpio, De Gobba, Guadix, Guadix, and Otte (2013) and Hong *et al.*, (2008), ils sont les plus capables d'interagir avec les sites actifs

de l'ECA. La fraction EtOH-NSN contient de plus gros peptides qui ont plus des difficultés conformationnelles pour interagir avec des sites où ils sont actifs sur l´ECA.

Ces résultats sont en accord avec les travaux de Lignitto *et al.,* (2010), qui ont observé que les peptides plus petits dans les extraits de fromage Asiago avaient une plus grande contribution à l'activité inhibitrice de l´ECA que les gros peptides. Cependant, nos résultats suggèrent que de plus gros peptides contenus dans la fraction EtOH-NSN du fromage Cotija pourraient également exercer une activité inhibitrice importante sur l'ECA: En outre, les deux fractions EtOH-SN et EtOH-NSN de fromages Cotija maturé entre trois et six mois ont exercé près de 100% de l'activité inhibitrice de l´ECA.

L´augmentation de l'activité inhibitrice sur l´ECA pendant l´affinage du fromage Cotija, peut être partiellement comparée à celle de travaux antérieurs (Gupta *et al.,* 2013; Lignitto *et al.,* 2010; Pritchard *et al.,* 2010) où les auteurs observent que cette activite dans des extraits de fromage Asiago d'allevo et Cheddar a augmenté au cours de l´affinage, mais seulement jusqu'à une certaine durée d'affinage et à diminuer au delà, ce qui suggère que les peptides inhibiteurs de l'ECA (et probablement tous les types de peptides bioactifs) dépendent d'un équilibre entre leur cinétique de formation et leur catabolisme. Toutefois, l´activité inhibitrice de l´ECA dans les fractions EtOH-SN et EtOH-NSN de fromage Cotija augmente au cours des six mois d´affinage sans aucun signe de diminution. Le désaccord avec nos résultats pourrait être due à des différences dans le temps d´affinage. Alors que Cotija a été mature pendant 6 mois, les fromages d´Asiago d'allevo et Cheddar ont été affinés pendant 18 mois et 9 mois respectivement.

Dans la fraction EtOH-NSN l´activité inhibitrice de l´ECA a été fortement corrélée avec les peptides HO (0,60). Par contre, l'activité dans EtOH-SN n'a pas été significativement corrélée ni avec les peptides HI ni avec les HO, (R^2 0,23 et 0,28 respectivement,pour 2 degrés de liberté). L´activité inhibitrice de l´ECA a toujours été liée à l'hydrophobie des peptides (Espejo-Carpio *et al.,* 2013; Meisel, 2004), ce qui explique la corrélation significative entre les peptides et l'activité HO dans EtOH-NSN. Par contre, l'absence de corrélation de ces peptides dans la fraction EtOH-SN montre qu'il existe sans doute d'autres caractéristiques importantes et que le caractère hydrophobe des peptides présents n'est pas suffisant. Il pourrait s'agir de la charge électrostatique des acides aminés ou la conformation du peptide (Meisel,

2004). Toutefois, d'autres études doivent être effectuées pour comprendre la relation entre ce type de peptides et l´activité biologique.

Jusqu'à présent, la majorité des études portant sur les peptides bioactifs du fromage se sont concentrés sur les fromages affinés. Par conséquent, la deuxième partie de ce travail a été concentrée sur le fromage de chèvre frais, et son contenu en peptides bioactifs (activité antioxydante et inhibitrice de l'ECA) afin d´observer en même temps l'effet de la pasteurisation sur ces bioactivités.

Les fromages de chèvre frais fait avec du lait cru (NP) et du lait pasteurisé (P) présentent une activité antioxydante dans les fractions NPN et ASN. Les valeurs observées pour les fromages NP étaient de 22,3 ± 2,1% et 61,6 ± 1,9% pour l'ASN et NPN respectivement. Pour les fromages P les valeurs de cette activité étaient de 21,9 ± 5,4% et 64,22 ± 0,04% obtenus respectivement pour les fractions ASN et NPN.

Nos résultats suggèrent qu'il n'y avait pas de différences significatives ($p > 0,05$) dans l'activité antioxydante entre les fromages NP et P. Mais, l'activité antioxydante était significativement plus élevée ($p < 0,05$) dans NPN que dans ASN pour tous les fromages.

L'activité antioxydante (en fraction NPN) mesurée dans le fromage de chèvre frais, (62,9%) est plus bas que celui rapporté par Silva *et al.,* (2012) dans l'extrait soluble de fromage Coalho (77,9 à 91,1%), mais plus élevé que celui rapporté par Meira *et al.,* (2012) dans du fromage de type Feta (32,7 ± 1,8%). Si on considère le fromage Cotija, l´activité antioxydante dans le fromage de chèvre frais était semblable à l'observation faite pendant une semaine sur le fromage frais (67,3% pour les NPN et 19,2% pour ASN).

Le manque de différences dans l'activité antioxydante entre fromages de chèvre frais NP et P est en désaccord avec le travail précédent de Silva, Pihlanto, and Malcata (2006) où les auteurs ont observé que les fromages ovins et caprins, fait avec du lait cru, ont une activité antioxydante plus élevée que celles faites avec du lait stérilisé. Toutefois, les auteurs ont travaillé avec le lait stérilisé (non pasteurisé) et leurs fromages étaient âgés de 45 jours, alors que notre fromage était frais. McSweeney *et al.,* (1993) ont observés que les différences de protéolyse entre les fromages crus et pasteurisés apparaissent et augmentent à mesure que

l´affinage a progressé, par conséquent, en dépit que nos fromages de chèvre frais n´ont montré aucune différences entre les uns et les autres, ces différences pourraient apparaître dans les fromages plus âgés.

Le profil de peptide de l'ASN et les fractions NPN ont été corrélées à l'activité antioxydante. L'activité de l'ASN a été positive en corrélation avec les peptides HI (0,85). Pour NPN, l´activité antioxydante a été fortement et négativement corrélé aux peptides HI et HO (-0,94 et -0,98 respectivement). Ces résultats sont partiellement en accord avec l´observation précédente pour le fromage Cotija où l'activité de l'ASN a été positive et corrélée avec des peptides HI et NPN. Toutefois, d'autres études doivent être menées pour comprendre la relation précise entre le type de peptides et l´activité biologique.

Nos résultats suggèrent que la libération de ces peptides peuvent également se produire pendant la fabrication du fromage où, selon McSweeney (2004), la protéolyse de la caséine est principalement due à la présure retenue dans le caillé, des enzymes endogènes ou des microorganismes de démarrage (ajoutés ou autochthones). Pour nos fromages de chèvre frais, le rôle des microorganismes n'a pas été significative, observée par l'absence de différences entre fromages NP et P. Par contre, des études antérieures ont sondé l'existence de peptides antioxydants présents naturellement dans le lait de chèvre (Bezerra *et al.*, 2013; El-Salam & El-Shibiny, 2012). Nous pouvons donc suggérés que des peptides bioactifs avec une activité antioxydante sont présents dans nos fromages de chèvre frais. Sans doute naturellement présents dans le lait de chèvre ou libérés par les coagulants mis en oeuvre.

Les deux types de fromage, NP et P présentaient une forte activité inhibitrice de l'ECA, attribué à son contenu en peptides bioactifs. Les valeurs pour les fromages NP étaient de 89,4 ± 14,4% et 94,7 ± 3,0% et pour les fractions EtOH-NSN et EtOH-SN respectivement. Pour les fromages P les valeurs obtenues étaient de 99,8 ± 0,2% et 85,2 ± 11,2% pour EtOH-SN et EtOH-NSN respectivement. Malgré les légères différences observées, les analyses ANOVA n'ont montré aucune différence statistique ($p > 0,05$) entre les fromages ou fractions.

L´activité inhibitrice d'ECA dans le fromage de chèvre frais, (variant entre 73,0% et 100%), était supérieur à ceux rapporté par Meira *et al.,* (2012) dans des extraits solubles de fromage Féta (46,5 %) et similaire aux rapports de Torres-Llanez *et al.,* (2011) et Silva *et al.,*

(2012) dans les extraits solubles de fromage Mexican Fresco (95,3% et 99,8%) et les fromages Coalho (75,9 % et 91,1%).

Il est intéressant de remarquer que, l´activité inhibitrice dans le fromage de chèvre frais était plus élevée que celle rapportée par Ong *et al.,* (2007) dans des extraits solubles de fromage Cheddar à différents étapes d´affinage (<80%). Aussi, l´activité inhibitrice de l´ECA dans le fromage de chèvre frais était semblable à l´activité observée en fromage Cotija de six mois mais supérieure à celle'observée chez les plus jeunes Cotija.

La libération de peptides inhibiteurs de l'ECA dans le fromage a toujours été considérée comme dépendant du degré de protéolyse au cours d´affinage (Gupta *et al.*, 2009; Ong *et al.*, 2007; Pritchard *et al.,* 2010). La plupart du temps, les études faites sur les fromages frais fabriqués avec la flore naturelle, attribuent la libération de peptides inhibiteurs d´ECA à l'action enzymatique des cultures starter (Torres-Llanez *et al.*, 2011). Néanmoins, notre fromage de chèvre frais a été non maturé et fabriqué sans culture starter.

Notre premiers résultats suggèrent qu'il n'y avait pas de différence dans l'activité inhibitrice de l'ECA entre fromages NP et P. Ce en désaccord avec ce qui avait été précédemment observé par Silva *et al.,* (2006) dans des fromage fait avec du lait de chèvres, où les auteurs ont observés que les fromages au lait cru avaient une inhibition plus élevée de l'ECA (44,9 et 79,4%) que celles faites avec le lait stérilisé (16,6 et 33,7%). En revanche, Paul and Van Hekken (2011) ont observé que l'activité inhibitrice de l´ECA dans les fromages mexicain « Queso Fresco », faite sans cultures starter, était similaire dans les fromages au lait cru et pasteurisé (87 à 93%). Les auteurs attribuent cette absence de différences à l´altération dans les deux types de fromages par des bactéries.

L´activité inhibitrice de l´ECA dans les fromages de chèvre frais, a été positivement corrélée avec les peptides HO (0,57) dans la fraction EtOH-SN ; et corrélée négativement avec des peptides HI (-0,96) de EtOH-NSN.

Il est bien connu que la pasteurisation du lait avant la fabrication du fromage peut affecte la protéolyse (Albenzio *et al.*, 2001; Kırmacı, Hayaloglu, Özer, Atasoy, & Türkoglud, 2014). Cependant, son impact sur le fromage de chèvre frais n'était pas évident à première

vue. Bien qu'une analyse statistique plus profonde, une analyse en composantes principales (PCA) a montré que la pasteurisation a augmentée la production de peptides HO (chapitre 2.2) qui sont fortement corrélés à l'activité biologique. Cette variation de peptides HO affecte l'activité inhibitrice de l´ECA dans la fraction EtOH-SN et a permis de faire une distinction entre fromages NP et P. Ces observations sont en accord avec l'observation précédente sur le fromage Cotija, où une forte corrélation a été observée entre les peptides HO et l'activité inhibitrice de l´ECA. Par conséquent, malgré les premiers résultats, l'effet de la pasteurisation sur l'activité biologique des fromages de chèvre frais ne peut être rejeté.

En bref, selon nos résultats, les fromages Mexicains, Cotija et fromage de chèvre frais contenaient des peptides avec des activités similaires aux inhibiteurs de l´ECA et antioxydantes, qui peuvent être libérés lors de la fabrication du fromage, par les coagulants ou par action d'autres enzymes protéolytiques endogènes (dans le fromage de chèvre frais), avec une augmentation au cours de l´affinage (dans le fromage Cotija) probablement à cause de l'action enzymatique de microbiote fromager (McSweeney, 2004). Ces micro-organismes ont été récemment mis en évidence parce qu' après la consommation, certains d'entre eux sont capables de survivre à la digestion et continuer à libérer des peptides bioactifs, ou d'interagir avec le microbiote intestinal produisant d'autres effets sur la santé (Adouard *et al.*, 2015; Ibrahim *et al.*, 2010; Lay *et al.*, 2004). A cet égard, il a été émis l'hypothèse que les matrices laitières pourraient améliorer leur résistance grâce à l'interaction entre les microorganismes et les composants de la matrice (Do Espirito Santo *et al.*, 2011; Lay *et al.*, 2004; Saxelin *et al.*, 2010).

Ainsi, cette partie du travail a été consacrée à l'étude de l'effet des matrices laitières dans la survie des micro-organismes laitiers pendant la digestion. Cependant, étant donné que le microbiote dans le fromage Cotija et dans le fromage de chèvre frais n'ont pas encore été caractérisés et que leur étude serait le sujet d'une autre thèse, nous avons décidé de sélectionner des microorganismes laitiers qui ont déjà été identifiés par leur rôle dans la production de yaourt et de fromage (Irlinger *et al.*, 2012; McSweeney *et al.*, 1993; Spinnler & Gripon, 2004): *Streptococcus thermophilus* TIL 257 (ST), *Brevibacterium aurantiacum* ATCC9174 (BA) et *Hafnia alvei* GB01 (HA). Ces micro-organismes ont récemment suscité un intérêt pour leur effet potentiel sur la santé humaine (Adouard *et al.*, 2015; Uriot *et al.*,

2016). Par conséquent, toutes les informations concernant sa survie au cours du stress digestif doit être intéressant.

La première étape consiste à caractériser la survie de chaque microorganisme, dans un milieu synthétique (S) au cours de la digestion; pour observer ultérieurement si leur inclusion dans une matrice laitière pourrait améliorer leur survie.

Les matrices laitières testées ont été fabriquées à partir du même lait écrémé en poudre, mais différenciées les unes des autres par leur composition, avec ou sans matière grasse; et leur structure, liquide et gel (comme dans le fromage de présure): lait écrémé (SM), le lait entier (WM), gel de présure de lait écrémé (GSM) et le gel de la présure de lait entier (GWM).

La digestion *in vitro* a été réalisée sur un digesteur dynamique (DIDGI) avec trois compartiments séparés qui simule l'estomac, le duodénum et l'intestin grêle (jejune et iléon), en tenant compte du réel dans les interactions *in vivo* entre les compartiments et ses aspects cinétiques et séquentiels. Les paramètres de la digestion (pH, de la vidange gastrique et le transit intestinal) sont les mêmes pour toutes les matrices testées. Les résultats *in vitro* ont été comparés à des expériences *in vivo* avec des souris. Et la dégradation des matrices laitières au cours de la digestion *in vitro* a été analysée par électrophorèse et GC-MS.

Les microorganismes ont été cultivés en culture pure et après ont été inclus ensemble dans les matrices laitières juste avant la digestion. Bien que la réponse de ces souches à un stress digestif le n’ait pas été entièrement caractérisée, son développement au cours de l'affinage du fromage nous donne une trace pour expliquer leur comportement pendant la digestion.

BA était modérément sensible au stress gastrique, il est resté viable les 50 premiers - 60 min (6,3 ± 0,8 log UFC/mL) diminue ensuite en dessous de notre seuil de détection. Dans le duodénum *BA* était tolérant au stress des sels biliaires et a perdu en moyenne 1,7 log UFC/mL. Dans l'intestin grêle la viabilité (5,8 ± 0,5 log UFC/mL) a diminué 1,07 log UFC/mL au cours de 140 min quelle que soit la matrice. Au-delà de 170 min, *BA* n'a été trouvé que dans S et SM (4,2 ± 0,7 log UFC/mL, respectivement).

Nos résultats sont en accord avec les travaux de Adouard *et al.,* (2015) et Adouard *et al.,* (2016) où les auteurs ont étudié la viabilité *B. auranticum* au cours de la digestion *in vitro* en trouvant que cette souche était sensible au stress gastrique, mais résistant aux conditions duodénales.

Le sensibilité de *BA* au stress gastrique n'était pas surprenante puisque ce microorganisme croît après désacidification du milieu à pH 6-7 (Irlinger *et al.*, 2012). À notre connaissance, les mécanismes de réponse à un stress acide par *BA* n'ont pas été décrits précédemment. La seule étude liée à cette réponse est celle effectuée par Halgasova, Bukovska, Ugorcakova, Timko, and Kormanec (2002) sur *Brevibacterium flavum* une espèce proche de *B. aurantiacum*, où les auteurs on déchiffré le rôle de sigB sur la croissance et la viabilité de *B. flavum* comme réponse au stress. Mounier, Rea, O´Connor, Fitzgerald, et Cogan (2007) ont étudié les caractéristiques de croissance de *B. aurantiacum* 16-01-58 isolés de fromage et ont constaté que l'activité uréasique, un mécanisme de réponse très effectif contre le stress acide, n'a pas été détectée dans ce microorganisme.

D'autre part, la tolérance de *BA* aux sels biliaires pourrait être liée à sa capacité à survivre à des concentrations de sel élevées lors de la fabrication du fromage (Irlinger *et al.*, 2012; Mounier *et al.*, 2007). Dans ce domaine, autres études ont observé que la tolérance au sel de ces microorganismes pourrait être lié à la présence de des transporteurs osmoprotecteurs dans son génome (Monnet, Landaud, Bonnarme, & Swennen, 2014) qui pourrait fournir *BA* avec une nouvelle adaptation croisée à d'autres formes de stress (Begley *et al.*, 2005). En plus, l'exposition de *BA* à un pH acide, précédemment à son passage dans l'intestin pourrait aussi augmenter sa résistance à la bile, en sélectionnant déjà des individus plus résistants aux stress.

D´un autre côté, *HA* est très résistant au stress gastrique avec une perte de seulement 2,78 log UFC/mL. Et cela a été la seule souche capable de survivre pendant toute la phase gastrique. Lors du passage à la phase duodénale *HA* était également très tolérant aux sels biliaires, ne perdant que 1,08 log UFC/mL. Dans l'intestin, la viabilité des *HA* dans des matrices sans matière grasse (S, SM et GSM) est resté stable pensée toute la phase (6,52 ±

0,41 log UFC/mL). Par contre, dans les matrices avec de la matière graisse (WM et GWM), la viabilité était significativement plus faible (p <0,05) dans la phase intestinale.

Nos résultats sont en accord avec ceux de Adouard *et al.,* (2015) et Adouard *et al.,* (2016) qui ont observé que *H. alvei* en milieu synthétique était capable de survivre au cours de la digestion *in vitro*. La survie de *HA* au stress gastrique pourrait être expliquée par les caractéristiques génétiques de ce microorganisme: c'est une bactérie Gram négative proche de *E. coli* (Janda & Abbott, 2006), une bactérie capable de survivre le stress digestif. En plus, les études cliniques précédentes ont suggéré la capacité de certaines souches de *H. alvei* à coloniser le tractus gastro-intestinal (Bobko, Tyras, & Jachymek, 2013; Vivas *et al.*, 2008). Dans ce domaine, d'autres auteurs ont observé que ce microorganisme est une bactérie ornithine et uréase positive (Janda & Abbott, 2006), les catabolismes de ces deux amines sont deux mécanismes de réponse très efficaces contre le stress acide.

Finalement, *ST* était très sensible aux stress gastrique, il est seulement stable au cours des 30 premières minutes dans l´estomac et n'a pas été trouvé au-delà du premier compartiment. Ce comportement est partiellement surprenant car *ST* a été largement considéré comme un microorganisme probiotique (Mater *et al.*, 2005; Uriot *et al.*, 2016). Cependant, quand on regarde son rôle dans l'industrie laitière, *ST* est une souche normalement utilisée comme culture initiale pour réduire le pH du lait, mais dans une mesure limitée (pH ~5), par conséquent, son intolérance à un pH très bas dans la phase stomacale est cohérant. Dans ce domaine Uriot *et al.,* (2016) ont observé que la survie de différentes souches de *ST* à la digestion est principalement dépendante du métabolisme de l'uréase et de la production des petites protéines de choc thermique (small Heat Shock Proteins, sHSP).

Donc, les trois souches testées, *BA, HA* et *ST* ont des comportements différents lors de la digestion cependant, pour chacun d'eux le pH acide a représenté la contrainte la plus importante au cours de la digestion; comme cela a été ensuite confirmé par les images obtenues par microscopie confocale, (CLSM). La mortalité des trois micro-organismes lorsque le pH est tombé de 4 à 2, a été plus marquée pour ST et BA que pour HA (40, 11 et 2 % de cellules endommagées, ImageJ, outils statistiques). Dans ces images de microscopie, nous avons également remarqué la préférence de ces microorganismes à se placer dans la

matrice protéique, en accord avec les observations de Pitino *et al.,* (2012) autour de la localisation de *L. rhamnosus* dans l'interface protéine-matière grasse d'un matrice fromagère.

Dans notre travail, nous avons analysé l'effet de la composition et de la structure de matrices laitières différentes - avec ou sans matières grasses, liquide ou gel- dans la survie de *BA, HA* et *ST*, pendant la digestion.

Pour *BA*, l'effet des matrices laitières n'a pas été significatif. Au cours de la phase intestinale SM donnait de meilleurs résultats que d'autres matrices laitières, mais semblable à celle observée pour le milieu synthétique (S), donc l'utilisation d'une matrice laitière n'a pas augmenté la survie de *BA* au cours de la digestion. Nos résultats contrastent avec les observations antérieures de Adouard *et al.,* (2016), où ces auteurs ont observé que la survie de *B. aurantiacum* à la digestion *in vitro* était plus élevée quand elle était au sein d'un gel de présure que dans un milieu synthétique.

Si on considère *HA*, les matrices laitières avec de la matière grasse (WM et GWM) ont eu un effet négatif sur la survie pendant la phase intestinale. Nos résultats sont partiellement comparables à ceux de Adouard *et al.,* (2016). Ces auteurs ont en effet observé que *H. alvei* cultivé dans un milieu synthétique a mieux survécu au stress gastrique que dans le fromage; cependant, dans les compartiments suivant, la survie était identique dans les deux matrices.

Nos résultats sont intéressants, car certain travaux précédents de Ranadheera, Evans, Adams, and Baines (2012), ont suggéré que la matière grasse dans les matrices fournie une meilleure protection à un stress acide et de la bile. La mortalité de *HA* en matrices avec matières grasses peut être dû à l'effet de lipases résiduelles qui libérent des acides gras avec des effets antimicrobiens (Sun, O´Connor, & Roberton, 2002). Cependant, dans notre travail, la diminution de la viabilité de *HA* n'a pas pu être corrélée avec la concentration en acides gras libérés à partir de la matière grasse du lait lors de la digestion.

Pour *ST* les matrices laitières n´ont pas montré d'effet sur la survie pendant la digestion. *S. thermophilus* a déjà été étudié pour observer sa capacité à survivre le stress digestif dans une matrice laitière (del Campo *et al.,* 2005; Lay *et al.,* 2004; Mater *et al.,* 2005). Cependant, les résultats sont contradictoires. La comparaison de nos résultats avec la

littérature a donc été difficile. Mater *et al.,* (2005) ont observé que *S. thermophilus* issu de yaourt frais, était capable de survivre à la digestion *in vivo*. Mais, dans un travail similaire, del Campo *et al.,* (2005) n'a trouvé aucune viabilité de *S. thermophilus* après la digestion *in vivo* du yaourt. Lay *et al.,* (2004) ont constaté que *S. thermophilus* était capable de survivre à la digestion *in vivo* quand il est présent dans du fromage Camembert mais pas quand il faisait partie du lait fermenté; suggérant qu'une matrice solide est plus protectrice que celle d'un liquide.

Dans ce même travail, Lay *et al.,* (2004) ont conclu que *H. alvei* dans un Camembert ne survit pas pendant la digestion *in vivo*. Cette dernière observation est en désaccord avec notre observation montrant qu' *H. alvei* est assez résistant à la digestion *in vitro* (quelle que soit la matrice). Une différence importante avec nos résultats pourrait être relatif à la méthode de Lay *et al.,* (2004) qui ont utilisé l'acide nalidixique pour éviter toute contamination, ce qui est suceptible de causer des interférences car ce médicament est également toxique pour *H. alvei* et d'autres bactéries à coloration de Gram négative (McBee & Schauer, 2006).

Dans une approche différente, nos résultats de microscopie confocale, ont permis d'observer la survie *de BA, HA* et ST. Leurs survies n'ont pas été affectées par la différence de microstructure entre la matrice liquide et le gel. Ceci est en désaccord avec les travaux antérieurs de Hannon *et al.,* (2006), qui ont observé que la survie de *L. lactis* est plus élevée dans une matrice de fromage plus homogène.

Finalement, notre analyse *in vivo* n'a pas démontré la survie de *BA; HA* et *ST* après la digestion d'une matrice laitière. Les traces de *Streptococcus* trouvés dans les matières fécales de souris ont été plutôt attribués à une souche similaire partie du tractus gastro-intestinal comme suggéré précédemment par Bogovic-Matijasic *et al.,* (2015).

Ainsi, selon nos résultats et en dépit de sa grande capacité tampon (plus élevé dans le gel que dans le lait) les matrices laitières testées WM, SM, GSM et GWM n'ont montré aucun effet protecteur clair sur la survie de *BA, HA* et *ST* au cours de la digestion. Nos résultats sont en désaccord avec les travaux antérieurs de (Conway, Gorbach, & Goldin, 1987; Charteris, Kelly, Morelli, & Collins, 1998) qui, dans différents travaux avec des probiotiques ont suggéré que les protéines de lait ont amélioré la tolérance au transit gastrique de

microorganismes, en raison de la capacité tampon du lait. Pourtant, nos résultats sont en accord avec le travail de Adouard *et al, 2016* qui ont observé que la survie de *Corynebacterium casei* et *Staphylococcus equorum* lors de la digestion *in vitro* était similaire lorsqu'ils étaient contenus dans le fromage ou dans un milieu synthétique. Donc, la composition semble avoir un effet qui dépend du micro-organisme (HA versus ST et BA en présence de matière grasse) mais le rôle de la structure de matrices laitieres dans la survie des microorganismes pendant la digestion reste incertain.

CONCLUSIONS ET PERSPECTIVES

Nos résultats ont prouvé que les fromages Cotija et fromage de chèvre frais ont une activité antioxydante importante. Cette activité était plus élevée en fractions riches en petits peptides, et a été corrélée avec les peptides du type hydrophile.

Le fromage Cotija jeune et le fromage de chèvre frais ont une activité antioxydante similaire qui a augmenté (en raison de la production de peptides solubles) tout au cours d´affinage et le fromage Cotija de six mois eu une activité antioxydante plus élevée que le fromage de chèvre frais. Par rapport au fromage de lait de chèvre, la pasteurisation n'a pas provoqué de changement significatif sur l'activité antioxydante.

D'autre part, les fractions peptidiques de Cotija et de fromage de chèvre frais, ont également montré, une forte inhibition de l'ECA, qui était légèrement plus élevée dans le fromage de chèvre frais que dans le jeune fromage Cotija (deux mois), mais les valeurs ont augmenté tout au long d´affinage et le fromage Cotija de trois mois a exercé les mêmes valeurs que le fromage de chèvre frais. Cette activité a été fortement corrélée avec les peptides de type hydrophobes. Pour cette activité, la pasteurisation du lait en fromages de chèvre frais, a augmenté la quantité de peptides hydrophobes, et, pour conséquence, a provoqué un changement de l'activité inhibitrice de l´ECA qui a permis de différencier les fromages frais fait avec du lait pasteurisés et ceux fait avec du lait cru.

Par contre il y a encore des questions qui restent posées par rapport a ces résultats et d´autres études doivent être faites : A) Pour mieux comprendre la relation précise entre le type

de peptides (hydrophobie) et l´activité biologique, on propose de séparer les peptides hydrophiles et hydrophobes et de tester leurs activités respectives.

B) Ensuite, après avoir selectioné les peptides ayant l'activité la plus élevée, les identifier.

C) Analyser l'impact de la pasteurisation sur les peptides bioactifs de fromages affinés.

D) Il serait également intéressant d'observer l'effet de temps d´affinage plus long sur des peptides bioactifs dans le fromage de Cotija, puisque dans d'autres fromages ces peptides semblent disparaitre et leur activité diminuer.

E) Regarder si les peptides bioactifs dans le fromage de chèvre frais sont libérés par l'action de la présure ou sont typiques du lait.

F) Etudier la stabilité de ces peptides dans le Cotija et des fromages de chèvre frais pendant la digestion, pour nous assurer que leur bioaccessibilité est maintenue.

G) Caractériser le microbiote du fromage Cotija et ensuite l'associer à la libération de peptides bioactifs.

H) Evaluer l'effet potentiel de ces microorganismes sur la santé humaine et leur capacité a survivre à la digestion.

La deuxième partie de cette thèse est issue de cette question. Elle est consacrée à l'étude de l'effet des matrices laitières sur les microorganismes au cours de la digestion. Pour cela, nous nous sommes concentrés sur l'effet de l'interaction entre les microorganismes et les composants laitiers et / ou la microstructure; ainsi que sur l'effet tampon exercé par ces matrices lors de la digestion gastrique.

Les microorganismes sélectionnés, avaient des taux de survie differents au cours de la digestion in vitro. *H. alvei* était très résistant au stress gastrique et duodénal; *B. auranticum* était modérément résistant au stress gastrique, mais très résistant au stress duodénale et *S. thermophilus* a été très sensible au stress gastrique et n'a pas été trouvé dans le compartiment duodénale. Par contre, notre analyse *in vivo* par analyse desgènes codant l' ARNr 16S par metabarcoding n'a pas réussi à confirmer ces observations, car les microorganismes testés n´ont pas été détectés dans les fèces de souris.

Donc, le stress le plus important pour les micro-organismes lors de la digestion est l´acidité dans le compartement de l'estomac, comme cela a été confirmé ultérieurement par

des images obtenus avec le microscope confocal à balayage laser (CLSM). Cette CLSM a également souligné la préférence des micro-organismes pour le réseau de protéines, quelquesoit le type de microstructure (lait ou gel).

Dans le compartiment gastrique, l´effet tampon du aux matrices laitières était dépendant de la microstructure de la matrice (plus élevée en gel que dans le lait), mais n'a pas été affecté par l'addition de matières grasses.

Toutefois, malgré cet effet tampon élevé des matrices laitières, l'inclusion des microorganismes dans cette matrice n'a pas amélioré la survie des microorganismes pendant la digestion. Au lieu de cela, l'addition de matières grasses a augmenté la mortalité de *H. alvei* lors de son passage dans la phase intestinale.

En conclusion, nos résultats suggèrent que l'effet protecteur des matrices laitières sur la survie des microorganismes lors de la digestion n´est pas dépendant de la capacité tampon ou des interactions entre les microorganismes et les composants et/ou la microstructure de la matrice. Donc, l'hypothèse la plus utilisée sur l'effet tampon n'a pas suffi à améliorer la survie des microorganismes lors de la digestion; et l'interaction avec certains composants laitiers comme la matière grasse, pourrait même diminuer la survie. Ce qui suggère que l'effet protecteur des matrices laitières est principalement dépendant de la souche ou liée à des changements dans les paramètres digestifs associés à la matrice, deux paramètres qui ne sont pas évalués dans ce travail.

À propos de l'effet de la matière grasse, nous n'avons pas pu faire de lien entre la concentration des acides gras libérés au cours du le digestion intestinale avec la survie des microorganismes, donc d'autres analyses doivent être effectuées dans ce domaine. Nous recommandons un test de sensibilité spécifique de *H. alvei* contre les acides gras libérés de la matière grasse du lait. En outre, l'étude des interactions possibles entre les acides gras et les peptides bioactifs avec des propriétés antimicrobiennes, libérées de la matrice laitière, peut également donner des résultats intéressants; à propos de la survie des microorganismes mais aussi sur l'efficacité du peptide.

References

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., . . . Amaya-Llano, S. L. (2013). Influence of probiotic strains added to Cottage Cheese on potentially-antioxidant peptides generation, anti-Listerial activity, and survival of probiotic microorganisms in simulated astrointestinal conditions. *International Dairy Journal, 33*(2), 191–197. doi:10.1016/j.idairyj.2013.04.005

Adouard, N., Foligné, B., Dewulf, J., Bouix, M., Picque, D., & Bonnarme, P. (2015). In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese. *International Journal of Food MIcrobiology, 197*, 98-107. doi:10.1016/j.ijfoodmicro.2014.12.011

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Albenzio, M., Corbo, M. R., Rehman , S. U., Fox, P. F., De Angelis, M., & A. Corsetti , A. S., M. Gobbetti. (2001). Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food MIcrobiology, 67*. doi:10.1016/S0168-1605(00)00533-X

Barác, M., Smiljanic, M., Zilic, S., Pesic, M., Stanojevic, S., Vasic, M., & Vucic, T. (2016). Protein profiles and total antioxidant capacity of water soluble and insoluble protein fractions of white cow cheese at different stage of ripening. *Mljekarstavo, 66*(3), 187-197. doi:10.15567/mljekarstvo.2016.0303

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29*, 625-651. doi:10.1016/j.femsre.2004.09.003

Bezerra, V. S., Campos, J. F., da Silva, R. A., Porto, T. S., de Lima-Filho, J. S., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. *Food Science and Technology, 33*(3), 513-520. doi:10.1590/S0101-20612013005000074

Bobko, E., Tyras, M., & Jachymek, W. (2013). Reprint of ‘‘New complete structure of *Hafnia alvei* clinical isolate strain PCM 2670 semi-rough lipopolysaccharide". *Carbohydrate*

Research, 378, 71-78. doi:10.1016/j.carres.2013.06.021

Bogovic-Matijasic, B., Obermajer, T., Lipoglavsek, L., Sernel, T., Locatelli, I., Kos, M., . . . Rogelj, R. (2015). Effects of synbiotic fermented milk containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. lactis BB-12 on the fecal microbiota of adults with irritable bowel syndrome: A randomized double-blind, placebo-controlled trial. *Journal of dairy science, 99*, 1-14. doi:10.3168/jds.2015-10743

Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., . . . Sforza, S. (2013). Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. *Food Science and Nutrition, 68*(8), 963-968. doi:10.3109/09637486.2013.821696

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of Lactic Acid Bacteria in the Human Stomach and Adhesion to Intestinal Cells. *Journal of dairy science, 70*(1), 1-12. doi:10.3168/jds.S0022-0302(87)79974-3

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology, 84*, 759-768. doi:10.1046/j.1365-2672.1998.00407.x

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12. doi:10.1111/j.1471-0307.2011.00725.x

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., & Vanacore, D. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258. doi:10.1002/psc.1093.

del Campo, R., Bravo, D., Cantón, R., Ruiz-Garbajosa, P., García-Albiach, R., MOntesi-Libois, A., . . . Baquero, F. (2005). Scarce Evidence of Yogurt Lactic Acid Bacteria in Human Feces after Daily Yogurt Consumption by Healthy Volunteers. *Applied and Enviromental Microbiology, 71*(1), 547-549. doi:10.1128/AEM.71.1.547–549.2005

Do Espirito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2011). Influence of food matrices on probiotic viability - A review focusing on the fruity bases. *Trends in Food Science and Technology, 22*, 377-385. doi:10.1016/j.tifs.2011.04.008

El-Salam, M. H. A., & El-Shibiny, S. (2012). Bioactive Peptides of Buffalo, Camel, Goat, Sheep, Mare, and Yak Milks and Milk Products. *Food Reviews International, 29*(1), 1-23. doi:10.1080/87559129.2012.692137

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654. doi:10.1016/j.jnutbio.2007.11.010

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183. doi:10.1016/j.idairyj.2013.04.002

Faye, T., Tamburello, A., Vegarud, G. E., & Skeie, S. (2012). Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human astric and duodenum juice. *Journal of dairy science, 95*, 558-566. doi:10.3168/jds.2011-4705

Gardiner, G., Ross, R. P., Collins, J. K., Fitzgerald, G., & Stanton, C. (1998). Development of a Probiotic Cheddar Cheese Containing Human-Derived *Lactobacillus paracasei* Strains. *Applied and Enviromental Microbiology, 64*(6), 2192-2199.

Godon, J. J., Zumstein, E., Dabert, P., Habouzit, F., & Moletta, R. (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Applied and Environmental Microbiology, 63*(7), 2802-2813.

Gómez-Ruiz, J. Á., López-Expósito, I., Pihlanto, A., Ramos, M., & Recio, I. (2008). Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC–MS/MS. *European Food Research and Technology, 227*(4), 1061-1067. doi:10.1007/s00217-008-0820-3

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gonzalez de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of Proteolysis in Liquid Chromatography Artisanal Cheeses: High Performance of Peptides. *Journal of dairy science, 78*, 1018-1024. doi:10.3168/jds.S0022-0302(95)76717-0

Guerra-Martínez, J. A., Montejano, J. G., & Martín-del-Campo, S. T. (2012). Evaluation of proteolytic and physicochemical changes during storage of fresh Panela cheese from Queretaro, Mexico and its impact in texture. *CyTA - Journal of Food, 10*(4), 296-305.

doi:10.1080/19476337.2011.653791

Guillemin, H., Perret, B., Picque, D., Menard, O., & Cattenoz, T. (2010). Logiciel StoRM-stomach and duodenum regulation and monitoring. *IDDNFR001230009000RP201000031235, 290*.

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Hajirostamloo, B. (2010). Bioactive component in milk and dairy product. *World Acad Sci Eng Technol, 72*, 162-166.

Halgasova, N., Bukovska, G., Ugorcakova, J., Timko, J., & Kormanec, J. (2002). The *Brevibacterium £avum* sigma factor SigB has a role in the environmental stress response. *FEMS Microbiology Letters, 216*, 77-84. doi:10.1111/j.1574-6968.2002.tb11418.x

Hannon, J. A., Lopez, C., Madec, M. N., & Lortal, S. (2006). Altering Renneting pH Changes Microstructure, Cell Distribution, and Lysis of *Lactococcus lactis* AM2 in Cheese Made from Ultrafiltered Milk. *Journal of dairy science, 89*(3), 812-823.

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*(1), 23-35. doi:10.1016/j.cis.2010.11.001

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*, 1062-1071. doi:10.1016/j.peptides.2008.02.005

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2010). Probiotics and immunosenescence: cheese as a carrier. *FEMS Immunology and Medical Microbiology, 59*, 53-59. doi:10.1111/j.1574-695X.2010.00658.x

Irlinger, F., Ah Yuen In Yung, S., Sarthou, A., Delbès-Paus, C., Montel, M., Coton, M., . . . Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food MIcrobiology, 153*, 332-338. doi:10.1016/j.ijfoodmicro.2011.11.022

Janda, J. M., & Abbott, S. L. (2006). The Genus Hafnia: from Soup to Nuts. *Clinical Microbiology Reviews, 19*(1), 12-18. doi:10.1128/CMR.19.1.12–28.2006

Kırmacı, H. A., Hayaloglu, A. A., Özer, H. B., Atasoy, A. F., & Türkoglud, H. (2014). Evolution of proteolysis in Urfa cheese made from ewe's milkby wild type starter culture systems. *Small Ruminant Research, 119*, 120-129. doi:10.1016/j.smallrumres.2014.01.006

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323. doi:10.2174/1381612033454883

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C. (2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D. P., O'Donohue, M., & Hernandez-Raquet, G. (2015). Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. *Bioresource Technology, 196*, 241-249. doi:10.1016/j.biortech.2015.07.084

Lignitto, L., Cavatorta, V., Balzan, S., G., G., Galaverna, G., Novelli, E., . . . Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal, 20*, 11-17. doi:10.1016/j.idairyj.2009.07.001

Lopez-Exposito, I., & Recio, I. (2008). Protective Effect of Milk Peptides: Antibacterial and Antitumor Properties. In Z. Bösze (Ed.), *Bioactive components of milk* (Vol. 606, pp. 271-293). New York: Springer.

Mater, D. D. G., Bretigny, L., Firmesse, O., Flores, M., Mogenet, A., Bresson, J., & Corthier, G. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiology Letters, 250*, 185-187. doi:10.1016/j.femsle.2005.07.006

McBee, M. E., & Schauer, D. B. (2006). The Genus Hafnia. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes: Volume 6: Proteobacteria: Gamma Subclass* (pp. 215-218). New York, NY: Springer New York.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127-144. doi:10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H., & Fox, P. F. (1997). Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait, 77*, 41-76.

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N., & Cogan, T. M. (1993). Contribution of the Indigenous Microflora to the Maturation of Cheddar Cheese. *International Dairy Journal, 3*, 613-634. doi:10.1016/0958-6946(93)90104-8

Meira, S. M. M., Daroit, D. J., Helfer, V. E., Corrêa, A. P. F., Segalin, J., Carro, S., & Brandelli, A. (2012). Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and Uruguay. *Food Research International, 48*(1), 322-329. doi:10.1016/j.foodres.2012.05.009

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Monnet, C., Landaud, S., Bonnarme, P., & Swennen, D. (2014). Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters, 263*(1), 1-9. doi:10.1093/femsle/fnu025

Mounier, J., Rea, M. C., O´Connor, P. M., Fitzgerald, G. F., & Cogan, T. M. (2007). Characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Staphylococcus* spp. Isolated from Surface-Ripened Cheese. *Applied and Enviromental Microbiology, 73*(23), 7723-7739. doi:10.1128/AEM.01260-07

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Panizzolo, L. A., Arujo, A. C., Taroco, L. V., Rodriguez, A., & Schopf, G. (2011). Evolución de la proteólisis durante la maduración de quesos Danbo elaborados con distintos cultivos iniciadores. *REVISTA DEL LABORATORIO TECNOLÓGICO DEL URUGUAY, 6*, 24-27.

Paul, M., & Van Hekken, D. L. (2011). Assessing antihypertensive activity in native and model Queso Fresco cheeses. *Journal of dairy science, 94*, 2280-2284. doi:10.3168/jds.2010-3852

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2015). Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening. *Journal of dairy science, 98*, 3690-3694. doi:10.3168/jds.2014-9097

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63. doi:10.1016/j.fm.2012.02.013

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548. doi:10.1016/j.foodres.2010.03.007

Ranadheera, C. S., Evans, C. A., Adams, C. A., & Baines, S. K. (2012). In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International, 49*, 619-625. doi:10.1016/j.foodres.2012.09.007

Ryhänen, E.-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal, 11*, 441-447. doi:10.1016/S0958-6946(01)00079-6

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese. *Journal of dairy science, 83*(7), 1434-1440. doi:10.3168/jds.S0022-0302(00)75013-2

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal, 15*, 95-109. doi:10.1016/j.idairyj.2004.06.007

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., . . . Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food MIcrobiology, 144*, 293-300. doi:10.1016/j.ijfoodmicro.2010.10.009

Silva, R. A., Lima, M. S. F., Viana, J. B. M., Bezerra, V. S., Pimentel, V. S., Porto, A. L. F., . . . Lima-Filho, J. L. (2012). Can artisanal ‘‘Coalho’’ cheese from Northeastern Brazil be used as a functional food? *Food Chemistry, 135*, 1533-1538. doi:10.1016/j.foodchem.2012.06.058

Silva, S., Pihlanto, A., & Malcata, F. (2006). Bioactive Peptides in Ovine and Caprine Cheeselike Systems Prepared with Proteases from< i> Cynara cardunculus</i>. *Journal of dairy science, 89*(9), 3336-3344. doi:10.3168/jds.S0022-0302(06)72370-0

Spinnler, H. E., & Gripon, J. C. (2004). Surface Mould-ripened Cheeses. In P. F. Fox, P. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: Chemistry, Physics and Microbiology (1 ed., Vol. 2, pp. 157-174). Retrieved from

http://www.sciencedirect.com/science/article/pii/S1874558X04800435. doi:10.1016/S1874-558X(04)80043-5

Sumeri, I., Adamberg, S., Uusna, R., Sarand, I., & Paalme, T. (2012). Survival of cheese bacteria in a gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41. doi:10.1016/j.idairyj.2011.12.016

Sun, C. Q., O´Connor, C. J., & Roberton, A. M. (2002). The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chemico-Biological Interactions, 140*, 185-198.

Theolier, J., Hammami, R., Fliss, I., & Jean, J. (2014). Antibacterial and antifungal activity of water-soluble extracts from Mozzarella, Gouda, wiss, and Cheddar commercial cheeses produced in Canada. *Dairy Science and Technology, 94*, 427-438. doi:10.1007/s13594-014-0170-9

Timón, M. L., Parra, V., Otte, J., Broncano, J. M., & Petrón, M. J. (2014). Identification of radical scavenging peptides (<3 kDa) from Burgos-type cheese. *LWT - Food Science and Technology, 57*(1), 359-365. doi:10.1016/j.lwt.2014.01.020

Torres-Llanez, M., González-Córdova, A., Hernandez-Mendoza, A., Garcia, H., & Vallejo-Cordoba, B. (2011). Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. *Journal of dairy science, 94*(8), 3794-3800. doi:10.3168/jds.2011-4237

Turgeon, S. L., & Rioux, L. (2011). Food matrix impact on macronutrients nutritional properties *Food Hydrocolloids, 25*, 1915-1924. doi:10.1016/j.foodhyd.2011.02.026

Uriot, O., Galia, W., Awussi, A. A., Perrin, C., Denis, S., Chalancon, S., . . . Roussel, Y. (2016). Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. *Food Microbiology, 53*, 18-29. doi:10.1016/j.fm.2015.05.007

Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., & Acosta, F. (2008). Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. *Veterinary microbiology, 129*, 150-155. doi:10.1016/j.vetmic.2007.11.007

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910

INTRODUCTION

Introduction

Cheese is a very diverse type of food, appreciated and consumed all over the world. The association between milk-microorganism (lactic bacteria, ripening microorganisms) that develop the characteristics of each kind of cheese also lead to the release of a wide variety of peptides (during proteolysis) that in some cases, could have effects on human health (Bezerra *et al.*, 2013; De Simone *et al.*, 2009; Gupta *et al.*, 2009). The presence of these peptides known as bioactive peptides has been observed in various European cheeses but very little is known about their presence in Mexican varieties.

In Mexico, most of the cheeses are made by artisan cheese makers and therefore, the geographical characteristic provides them with a strong link to their place of origin describing the history and culture of the communities that produce them (González-Córdova *et al.*, 2016). Despite its importance, very little is known about Mexican cheeses with a special lack of information about its biological properties.

However, the study of the impact of a type of food on health can be extremely complex, mainly because it depends first on its structure and composition (initial and/or during digestion) that impacts the bioaccesibility of the bioactive components (i.e. bioactive peptides); and second because it can act directly on the host or through modulation of the functional potential of the intestinal microbiota.

This intestinal microbiota is the diversity of microorganisms that live in the colon and that have control on some aspects of human health. This microbiota is a very delicate ecosystem that can be easily altered by interaction with food components or other microorganisms (Lay *et al.*, 2004).

Therefore, the previously mentioned association between milk-microorganisms in cheeses has raised questions about the effect of those microorganisms when consumed. Cheese, is a food matrix with a large variety of microorganisms (10^8 – 10^9 viable cells/g of cheese, lactic acid bacteria and ripening microorganisms) that enter into the gut tract after consumption.

Some of those microorganisms are capable to reach alive the colon and interact with the intestinal microbiota (Adouard *et al.*, 2016). However, before reaching the colon those microorganisms need to pass through stomach and intestinal stress, represented mainly by the high acidity and bile salts respectively (Sumeri *et al.*, 2012). Previous studies in this field claimed that the presence of a food matrix (structure and/or composition) could modify the survival of these microorganisms during digestion. However literature reports are contradictory.

It is in this context that surges the interest of a Co-joint supervision thesis between two leading institutions in food research from Mexico and France with the main objective of increase the knowledge in dairy science and develop a scientific collaboration between research teams.

The Tecnológico de Monterrey (Mexico) through its Food Biotechnology research group has for objective the study of biological properties in food, with special interests in the Mexican endemic products.

Meanwhile, the AgroParisTech-INRA through the Engineering and Microbiology Laboratory of Food Process, (LGMPA for its acronym in French) has been focusing on the effect of food composition and structure on the intestinal microbiota.

From the collaboration between both international research teams results this Co-joint supervision thesis named "Potential of cheese microorganisms ecosystems for the production of bioactive peptides, and effect of the dairy matrices in the survival of dairy microorganisms through digestion". That has as objective to study the presence of bioactive peptides with activities as antioxidants and inhibitors of angiotensin converting enzyme in Mexican cheeses; and to observe the effect of dairy matrices on the survival of dairy microorganisms through digestion.

Experimental work was realized in facilities of both institutions in Mexico and France, leading to the main results presented along this thesis. These results were presented as publication papers and are exhibited and discussed among the chapter 2.

In Chapter 2.1, we demonstrate the presence of peptides with activities as antioxidants and angiotensin converting enzyme (ACE) inhibitors in Cotija cheese. We observed an increase in activities throughout ripening time that was consistent with the proteolysis extent.

In Chapter 2.2 we demonstrate the presence of peptides with activities as antioxidant and ACE inhibitory activity in Fresh goat cheese, made with raw and pasteurized milk. And we observed the impact of pasteurization on biological activity.

In Chapter 2.3 we observed that the tested dairy matrixes did not exerted a protective effect in the selected microorganisms, but in contrast, some of them increased the stress suffered by the microorganisms in the intestinal phase.

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Publications & Conferences

A. Articles

Hernández Galán, L., Cardador Martínez, A., Picque, D., Spinnler, H. E., López del Castillo Lozano, M., & Martín del Campo, S. T. (2016). Angiotensin converting enzyme inhibitors and antioxidant peptides release during ripening of Mexican Cotija hard cheese. Journal of Food Research, 5(3). Doi 10.5539/jfr.v5n3p85.

Hernández Galán, L., Cardador Martínez, A., Picque, D., Spinnler, H. E., López del Castillo Lozano, M., & Martín del Camp, S. T. (2016). Antioxidant and angiotensin converting enzyme inhibitory activity in fresh goat cheese prepared without starter culture. A preliminary study. CyTA Journal of Food, 15(1). Doi 10.1080/19476337.2016.1202325.

Hernández Galán, L., Cattenoz, T., Spinnler, H. E., Canette, A., Briandet, R., Guedon, E., Bonnarme, P., Martín del Campo, S. T., Dugat Bony E., Castellote, J., Delettre, J., Le-Guin, S., Le Feunteun, S., Picque, D. Effect of dairy matrices on the survival of *Streptococcus thermophilus*, *Brevibacterium aurantiacum* and *Hafnia alvei* during *in vitro* and *in vivo* digestion (*in preparation*).

B. Oral communications

Martín del Campo Barba, S. T , **Hernández Galán, L.,** Cardador Martínez, A., López del Castillo Lozano, M., Picque, D., Spinnler, H. E. (2015). Potential of cheeses microorganism ecosystems for the production of bioactive peptides, and effect of the dairy matrices in the survival of dairy microorganism through digestion. VI Congreso Nacional de Biotecnología y Bioingeniería. June 21-26. Guadalajara, México.

C. Posters

Hernández Galán, L, Spinnler, H. E., Picque, D., López del Castillo Lozano, M., Cardador Martínez, A.; Martín-Del Campo, S. T. (2014). Bioactive Peptides in Cotija Mexican cheese. 14th IFT Annual Meeting and Food Expo. June 21-24. New Orleans, Louisiana, U.S.A.

Hernández Galán, L., Spinnler, H. E., Martín-del-Campo, S. T., Cattenoz, T., Le Feunteun, S., Bonnarme, P., Picque, D. (2014). Impact of the dairy matrices on the viability of cheese microorganisms during *in vitro* dynamic digestion. 3rd International Conference on Food Digestion. CoST INFOGEST. March 11-13. Wageningen, Netherlands.

Hernández Galán, L., Spinnler, H. E., Martín-del-Campo, S. T., Cattenoz, T., Le Feunteun, S., Bonnarme, P., Picque, D. (2014). Impact of the composition of the dairy matrix on the survival of cheese microorganisms through digestion. Journee des Doctorants ABIES. February 25-26. Paris, France.

Hernández Galán, L., Spinnler, H. E., Martín-del-Campo, S. T., Cattenoz, T., Le Feunteun, S., Bonnarme, P., Picque, D. (2014). Potential of cheese microbial ecosystems for the formation of peptides of interest and effects on the structure of the intestinal flora. Journee des Doctorants du GMPA. February 13. Thiverval-Grignon, France.

References

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Bezerra, V. S., Campos, J. F., da Silva, R. A., Porto, T. S., de Lima-Filho, J. S., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. *Food Science and Technology, 33*(3), 513-520. doi:10.1590/S0101-20612013005000074

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., & Vanacore, D. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258. doi:10.1002/psc.1093.

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C. (2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Sumeri, I., Adamberg, S., Uusna, R., Sarand, I., & Paalme, T. (2012). Survival of cheese bacteria in a gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41. doi:10.1016/j.idairyj.2011.12.016

LITERATURE REVIEW

1. Literature review

1.1 Functional Foods

In recent decades, the study of food has become a success due to its recognition as a major agent for prevention or cure in chronic diseases like cancer, cardiovascular diseases, insulin resistance and obesity (WHO & FAO, 2003). This has produced the development of new aliments that beyond a nutritional value, have components that provide health benefits to consumers, resulting in attractive and healthy foods called "functional foods" (Martínez Augustin & Martínez de Victoria, 2006).

Functional foods are those aliments capable to produce a beneficial effect on one or more specific functions in the body, improving the health and wellbeing, and/or reducing the risk of disease (Mollet & Rowland, 2002). They own their functionality to their components, that when exhibit a biological activity are called bioactive components. There is a wide variety of these bioactive components identified in foods, which vary in chemical structure and function (Kris-Etherton *et al.*, 2002). Among those components, bioactive peptides have been recently highlighted because of its wide variety of effects on human health.

1.2 Bioactive peptides

Bioactive peptides are defined as substances with hormone or drug-like activity that eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses that can have a significant impact on the course of some diseases (FitzGerald, Murray, & Walsh, 2004; Kitts & Weiler, 2003). After consumption, these peptides may affect the major body systems specifically, the cardiovascular, digestive, endocrine, immune and nervous systems.

Bioactive peptides owe their functionality to its amino acid composition and sequence, which generally consists of 3 to 20 amino acid residues per molecule, but in some cases, may consist of more than 20 amino acids (Hajirostamloo, 2010). Depending on the amino acids sequence, bioactive peptides can exhibit one or diverse activities, including opiate-like, mineral binding, immunomodulatory, antimicrobial, antithrombotic, hypocholesterolemic, antioxidant and antihypertensive activities (Korhonen & Pihlanto, 2003; Korhonen & Pihlanto, 2006). The specific role of bioactive peptides against target diseases depends

primarily on their structural properties such as chain length and physicochemical characteristics of the amino acid residues, for example, hydrophobicity, molecular charge, and side-chain bulkiness (El-Salam & El-Shibiny, 2012; Hajirostamloo, 2010; Pihlanto, 2006).

1.3 Milk proteins as source of bioactive peptides

Despite there are numerous sources like soy, and meat, milk and dairy products are the best precursors of bioactive peptides (Korhonen 2009). The functionality of these proteins is evident when we think that milk is the main aliment in newborns that are provided by an immature digestive system, and who therefore depend completely of the proteins present in breast milk (immunoglobulins, lysozyme, lactoperoxidase, lactoferrin, etc.) and the immunocompetent cells (macrophages, lymphocytes, T and B cells, etc.) to fight potential infections. In addition with the growth factors, that have an important role on the development of the intestinal and immune system (Martínez Augustin & Martínez de Victoria, 2006).

It is important at this point to make a distinction between the bioactive proteins naturally present in milk (like immunoglobulins) and the bioactive peptides that are released from these native proteins after digestion. Bioactive peptides are the resulting product of a breakdown of proteins by enzymes, which exert a specific bio-function only after release from the original protein (Kitts & Weiler, 2003).

Some of bioactive peptides derived from caseins reveal multifunctional properties; for instance, peptides from the sequence 60-70 of β-casein show immunostimulatory, opioid and inhibition of the angiotensin converting enzyme (ACE) activities at the same time, probably because these regions in the primary structure of caseins contain overlapping peptides sequences, which exert different biological effects. These regions have been considered as "strategic zones" which are partially protected from proteolytic breakdown because of its high hydrophobicity and the presence of proline residues (Korhonen & Pihlanto, 2003; Meisel, 2005).

In dairy products, the bioactive peptides can be released through the proteolytic action of the natural present enzymes in milk. However the most productive way to liberate these peptides is during food processing, via proteolysis by microbial enzymes from the lactic acid bacteria or secondary starters (Choi *et al.*, 2012).

1.4 Bioactive peptides in cheeses

Cheese is a very complex food matrix containing numerous peptides released by a proteolysis especially during a ripening period. Proteolysis is a complex and important biochemical event that occurs during cheese manufacture, especially during ripening. Despite the type of cheese, the main objective of proteolysis is the degradation of complex proteins into peptides and amino acids.

In most ripened cheeses, proteolysis can be summarized as follows: Initial hydrolysis of caseins is catalyzed by residual coagulant, plasmin, cathepsin D (in some cases) and somatic cell proteinases releasing large (water insoluble) and intermediate-sized peptides (water soluble). Those peptides are subsequently hydrolyzed by the coagulant and enzymes from the starter and nonstarter flora of the cheese releasing small peptides and free amino acids (Fox & McSweeney, 1996; McSweeney & Sousa, 2000). In this regard, numerous studies in different cheeses have identified the biological activity of a wide range of peptides released during proteolysis, establishing that the type and quantity of these peptides is dependent mostly of the starter culture used and the ripening conditions employed (Choi *et al.*, 2012; Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Gupta *et al.*, 2009; Gupta *et al*., 2013).

Some of the bioactivities identified include the antibacterial activity in water extracts of Mozzarella, Gouda, Swiss and Cheddar cheeses (Pritchard *et al.*, 2010; Theolier *et al.*, 2014); cytomodulatory properties in buffala Mozzarella cheeses (De Simone *et al.*, 2009) and lyophilized extracts from middle aged Gouda cheese (Meisel & Günther, 1998); antioxidant activity in water extracts of Cheddar cheeses (Pritchard *et al.*, 2010) or anti-hypertensive activity in Cheddar cheeses added with probiotics (Ong *et al.*, 2007) or in water extracts of Mexican fresh cheeses (Paul & Van Hekken, 2011; Torres-Llanez *et al.*, 2011) among others activities (Table 1.1).

Additionally, many studies have found that biological activity of these peptides is dependent on the stage of ripening; suggesting that concentration of bioactive peptides in cheeses increases with ripening time, until certain level of proteolysis (Ryhänen *et al.*, 2001), to later decrease (Gupta *et al.*, 2013). Most of these activities have been previously

summarized in different works (Hartmann & Meisel, 2007; Korhonen, 2009; Meisel, 2004). In these work we are only going to describe the antioxidant and ACE-inhibitory activities.

Table 1.1 Bioactive Peptides Identified in cheeses and their reported biological activities

Cheese	Example of identified bioactive peptide	Bioactivity	Reference
Mozzarella, Gouda, Swiss, Cheddar.	Non identified peptides found in WSE*	Antibacterial Antifungal	Theolier *et al.* (2014)
Mozzarela di Bufala Campana cheese	β-CN f (57-68), f (60-68)	Cytomomodulatory	De Simone *et al.* (2009)
Festivo (low-fat, ripened cheese)	α_{s1}-CN f (1-9), f (1-7), f (1-6)	ACE-inhibitory	Ryhänen *et al.* (2001)
Cheedar	Non identified, peptides found in WSE*	Antioxidant	Gupta *et al.* (2009)
Cheddar with *Lactobacillus casei*	α_{s1}-CN f (1–6), f (1–7), f (1–9), f (24–32) and f (102–110) β-CN f (47–52) and f (193–209)	ACE-inhibitory	Ong *et al.* (2007)
Gouda	Fragments from α_{s1}-CN, and β-CN	ACE-inhibitory	Saito *et al.* (2000)
Mexico Fresco cheese	α_{s1}-CN f (1–15), f (1–22), f (14–23), and f (24–34) β-CN f (193–205), f (193–207), and f (193–209)	ACE-inhibitory	Torres-Llanez *et al.* (2011)
Cottage with *Lactobacillus casei* and *Lactobacillus rhamnosus*	Non identified peptides found in WSE*	Antioxidant	Abadía-García *et al.* (2013)

*WSE: Water soluble extracts

1.4.1 Antioxidant

Oxidative metabolism is crucial for the survival of human cells, though, this metabolism produces free radicals and reactive oxygen species that cause oxidative changes. Cells maintain complex systems of multiple types of antioxidants such as glutathione, vitamin C, and vitamin E, as well as enzymes such as catalase, superoxide dismutase and various peroxidases. However, when free radicals and reactive oxygen species are present in higher

amounts than the endogenous antioxidants an imbalance in the redox state of the cell is produced causing toxic effects through the production of peroxide and free radicals than star chain reactions. This oxidative stress is involved in many diseases like Parkinson, Alzheimer or even cancer (Ames, Shigenaga, & Hagen, 1993). Antioxidants stop these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions through their own oxidation.

Antioxidant peptides derived from milk are composed of 5 to 11 amino acids including in the sequence hydrophobic amino acids, proline, tyrosine, tryptophan and histidine (Pihlanto, 2006). Some peptides with a Pro-His-His sequence showed the greatest antioxidant activity among all tested peptides and had synergistic effects with non-peptidic antioxidants. The hydrophobicity is an important factor for the antioxidant activity due to increased accessibility to hydrophobic targets (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998).

Histidine and proline have been described as the most important residues in the lipoprotein peroxidation inhibitory activity of peptides. Seven of the eight peptides identified in the highest antioxidant fraction contained at least one proline residue, and six of them had more than two residues of proline (Pihlanto, 2006). The properties of these amino acids may be explained by the special capability of phenolic and indol groups to serve as hydrogen donors (Gupta *et al.*, 2009), however there is evidence that the antioxidant effect of these amino acids is higher when they are incorporated in dipeptides than when the amino acid is alone (Erdmann *et al.*, 2008).

In addition, the antioxidant properties of these peptides have been suggested to be due to metal ion chelation, and singlet oxygen quenching. Additionally, the antioxidant activity of whey-derived peptides and whey itself has been correlated with the high amounts of cysteine present which promote the synthesis of glutathione (Erdmann *et al.*, 2008).

However, some studies demonstrated that none of these properties could be correlated exclusively with the antioxidant activity of the tested peptides. Therefore, overall antioxidant action is most likely attributed to the cooperative effects of the mechanisms mentioned (Chen

et al., 1998). At this moment, neither the structure-activity relationship nor the antioxidant mechanism of peptides is fully understood (Pihlanto, 2006).

Water soluble extract of Cheddar cheeses was tested by 2,2´-azinobis (3-ethyl benzothiazoline)-6-sulphonic acid, 2,2-diphenyl 1-picryl hydrazyl (ABTS) and superoxide radical scavenging activity to evaluate their antioxidant activity through ripening, and the obtained results indicated that antioxidant activity was highly correlated with the extent of proteolysis and the enzymatic action of the starter culture (*L. casei ssp casei* 300 and *L. paracasei ssp paracasei* 22) (Gupta *et al.*, 2009). Meanwhile in another study on water soluble extracts of Cottage, a fresh cheese, the release of antioxidant peptides was attributed to the use of probiotics strains (Abadía-García *et al.*, 2013).

Bioactive peptides with antioxidants activities have been previously reported in Cheddar (Pritchard *et al.*, 2010), Caciocavallo (Perna *et al.*, 2015), Parmigiano Regigiano (Bottesini *et al.*, 2013) and Cottage cheeses (Abadía-García *et al.*, 2013). For instance, Perna *et al.* (2015) observed that antioxidant activity in water soluble extracts of Caciocavallo cheeses increased through ripening. In contrast, Bottesini *et al.* (2013) observed that antioxidant activity of water soluble extracts of Parmigiano Reggiano, remained stable through ripening. Meanwhile Gupta *et al.* (2009) who observed that antioxidant activity of Cheddar cheese increased throug ripening, but only until certain level, because extensive proteolysis reduced the antioxidant activity. On the other hand, Timón *et al.* (2014) observed that the type of rennet used for Burgos-type cheeses coagulation influences the concentration of peptides with antioxidant activity. In these works are reported different methodologies to determine antioxidant capacity in cheese extracts, where 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, is one of the most widely used (Abadía-García *et al.*, 2013; Gupta *et al.*, 2009; Pritchard *et al.*, 2010; Timón *et al.*, 2014).

DPPH is a straightforward method that measures the radical scavenging activity of antioxidants against free radicals, represented by DPPH, an stable free radical (Prakash, Rigelhof, & MIller, 2001). The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants (Kedare & Singh, 2011). Reduction of DPPH by antioxidant effec, is represented by a loss of absorbance at 515 nm (Olvera-García, Cardador-Martínez, & Martín del Campo, 2015). It is considered to be a rapid, simple effective to

evaluate antioxidant activity in food or in complex biological systems for both solid and liquid samples. Some advantage of this method are that DPPH is stable and do not need to be generated immediately before the test; it can react with the whole sample and if enough time is given for this reaction DPPH can react slowly even with weak antioxidants. DPPH method can be used in aqueous and non-polar solutions to measure, both hydrophilic and lipophilic antioxidants(Kedare & Singh, 2011).

1.4.2 Antihypertensive

Hypertension is defined as a significant increase in blood pressure, which is associated with a greater risk for cardiovascular diseases (CVD) like strokes and coronary infarctions. This condition is one of the most common chronic medical conditions in the developing countries. Even small decreases in blood pressure could result in a decrease of the CVD risks (Vermeirssen, Van Camp, & Verstraete, 2004). Some milk-derived antihypertensive peptides have been shown to reduce hypertension *in vivo and in vitro* studies. Most of these peptides are derived from both casein and whey protein by fermentation or proteolysis by digestive enzymes (Korhonen, 2009).

Although bioactive peptides may have a wide range of mechanisms to decrease the blood pressure, the inhibition of the angiotensin-I converting enzyme (ACE) is the most studied one (Vermeirssen *et al.*, 2004). Angiotiensin I-converting enzyme (ACE) is a multifunctional enzyme with a crucial role in the regulation of blood pressure and of several endogenous peptides like enkepephalins, and bradykinin, that are inhibitory and competitive substrates for ACE (Meisel, 2005).

Peptides with ACE-inhibition activity prevent the formation of angiotensin II, a potent vasoconstrictor (hypertensive) and to potentiate the vasodilator (hypotensive) effect of bradykinin, leading to a decrease of the blood pressure (Erdmann *et al.*, 2008; Jäkälä & Vapaatalo, 2010; Meisel, 2004; Meisel, 2005).

ACE inhibitory peptides are generally short chain peptides, often carrying polar amino acid residues like proline. Structure-activity correlations among different peptide inhibitory of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. ACE appears to prefer substrates or competitive inhibitory

containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions (Espejo-Carpio *et al.*, 2013; Meisel, 2004) and many of the peptidic inhibitory contain proline at the C-terminus. It has been proposed that the C-terminal tripeptide residues may interact with the substitutes S1, S1´ and S2 at the active site of ACE, as shown in figure 1.1 (Hong *et al.*, 2008).

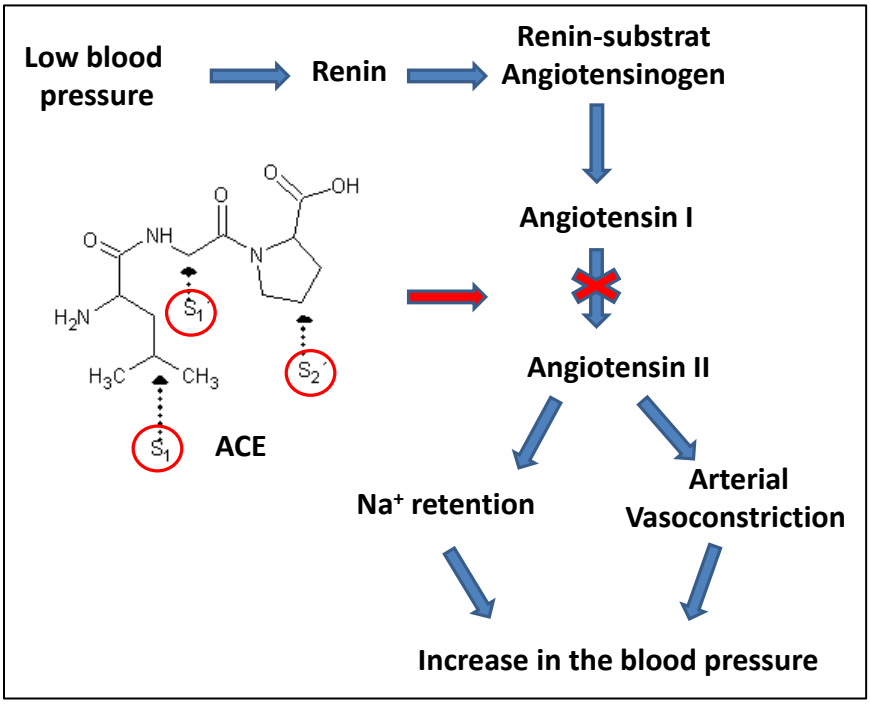

Figure 1.1. Role of angiotensin converting enzyme on the regulation of blood pressure.

When ACE inhibitors are di or tripeptides, they have a Tyr, Phe, Trp or Pro residue at the C terminal; from these, Trp seems to increase the most the ACE inhibitory potential. Also some structure-activity data suggest that the positive charge on this position contribute to the inhibitory potency (Meisel, 2005). Meanwhile, when peptides have a longer chain, is the peptide conformation, thus the structure adopted in the specific environment of the binding site, is contributing to the ACE inhibitory potency (Meisel, 2005).

A wide range of studies have proved the inhibition of ACE by bioactive peptides naturally found in cheeses, and some of these peptides have also been identified. According with Saito *et al.*, (2000), four peptides were purified from water soluble extracts of 8 months Gouda cheese and identified with the follow amino acid sequences: a) Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln, b) Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln, c) Tyr-Pro-Phe-

Pro-Gly-Pro-Ile-Pro-Asn, and d) Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Gln-Pro-Phe; the first two were derived from α_{s1}-caseine and the rest from β-caseins, they present a high inhibitory activity of ACE, and are able to decrease the systolic blood pressure in spontaneously hypertensive rats. Meanwhile, Ryhänen *et al.,* (2001) observed ACE inhibitory activity in the 80% ethanol soluble fraction of low-fat, ripened Festivo cheese produced with probiotic strains (*Lactobacillus acidophilus* and bifidobacteria). Both studies concluded that ACE inhibitory activity increased during ripening until a certain level, to later decrease, suggesting that bioactive peptides are naturally formed in cheeses and are active only for a limited period, before being hydrolyzed into other peptides and amino acids as ripening proceeds.

ACE inhibitory activity has also been found in the water extracts of Mexican Fresco cheeses manufactured with specific lactic acid bacteria (LAB); the release of these peptide was attributed to the activity of the LAB present on α_{s1}- and β-casein (Torres-Llanez *et al.*, 2011). Another study in water soluble extracts of a model Queso Fresco, made with pasteurized milk and without starter cultures, exhibited ACE inhibitory activity that was significantly higher in samples of eight weeks of ripening, when these cheeses are no longer suitable to consumption. This increase in activity was attributed to bacterial spoilage (Paul & Van Hekken, 2011).

Despite ACE inhibition, bioactive peptides might regulate blood pressure in through other mechanisms. It has been shown that the active sites on the somatic ACE also interact with endogenous opioid, explaining why some opioid peptides act as competitive inhibitory of ACE (Meisel, 2005).

Results obtained by *in vivo* studies on hypertensive rats and hypertensive human volunteers have probed the effectiveness of peptides derived from caseins and whey proteins on the control of blood pressure, either after intravenous or oral administration. Furthermore, human trails realized by FitzGerald *et al.,* (2004) suggested that these bioactive peptides (in fermented milk and milk protein hydrolysates) an evident decrease in both systolic and diastolic pressures after 2 weeks of consumption without any side effects.

Methods for in vitro measurement of ACE inhibitory activity in cheese extracts are generally performed by the Cushman and Cheung (1971) method based on the liberation of

hippuric acid (HA) from hippuryl- histidyl-leucine (HHL) catalyzed by the ACE. The concentration of HA liberated (representing the activity of ACE) is commonly measured through spectrophotometric assay that need the extraction of HA with a solvent, drying and subsequent resuspension (purification) (Gupta *et al.*, 2013; Lignitto *et al.*, 2010; Torres-Llanez *et al.*, 2011). However, Wang *et al.,* (2013) have recently developed a reversed-phase high-performance liquid chromatography method that eliminates the need of HA purification has proved to be easier, faster and veracious. This method uses isogradient elution conditions that allows the separation of HA and HHL at a wavelength of 226.5 nm.

1.5 The case of Mexican cheeses

Although several papers have described the biological activity of peptides generated during cheeses ripening, almost all of them are referred to European cheeses and only few data are available about the production of theses peptides in Latin American cheeses which have a very important market because of its specific sensorial characteristics that result very different to the European cheeses.

Cheese is a very important component in the Mexican cuisine, which has been recognized by the UNESCO as cultural heritage. In Mexico, most of the cheeses come from artisan cheese makers therefore, the geographical characteristic provides them with a strong link to their place of origin and describes somehow the history and culture of the communities that produce them (González-Córdova *et al.,* 2016).

There exist dozens of type of cheeses, each one with its own characteristics and particular uses (González-Córdova *et al.*, 2016; Hnosko, Clark, & Van Hekken, 2009), however despite its importance, very little is known about Mexican cheeses and their biological properties (Abadía-García *et al.*, 2013; Torres-Llanez *et al.*, 2011). Thus the finding of bioactive peptides in popular Mexican cheeses could provide them with an added value form a functional point of view.

1.5.1 Cotija cheese

Cotija cheese is an artisanal Mexican cheese made from whole cow´s milk that has recently obtained a Collective Trademark “Cotija Región de Origen” that protects it, and is on

its way to obtain a Protected Denomination of Origen (Hernández, Navarro, & Quirasco, 2009a).

It is an artisanal and seasonal cheese. It is produced during the months of July to October in the region of Jalmich, located in the Mexican states of Jalisco and Michoacán at 700-1700 meters above sea level; the specificity of the region of Cotija may have an important impact on the microbiota found in the milk (Flores-Magallón, Oliva-Hernández, & Narváez-Zapata, 2011; Hernández *et al.*, 2009a; Poméon, 2007).

Artisanal production of Cotija cheese (Figure 1.2) is with raw milk without the addition of starter cultures, the fermentation is performed by the autochthonous microbiota from the milk (Chombo-Morales, Kirchmayr, Gschaedler, Lugo-Cervantes, & Villanueva-Rodríguez, 2016). The process for Cotija production, such as cutting curd, salting, pressing and specially ripening (at least 3 months) vary between producers.

Cotija cheese is traditionally salted several times for preservation purposes, which provides it with its distinctive color and taste (Figure 1.3) (Flores-Magallón *et al.*, 2011). The produced Cotija cheese distinctive features are hardcover, high salt content, matured, firm or friable texture with a strong, sharp, or pungent aroma (Hernández *et al.*, 2009a) with 35-42% moisture, 23-30% fat, 28-31% protein, and 4% salt (Hnosko *et al.*, 2009). Because of the technology use to make it, Cotija cheese is considered as a hard or semi-hard cheese. It is comparable to Italian cheeses, especially with the Parmigiano-Reggiano cheese (Hnosko *et al.*, 2009), however there are main differences between both cheeses, for instance Cotija cheese is not cooked and the ripening time is shorter.

There are some works that described the microbiological and sensorial characteristic of Cotija cheese (Flores-Magallón *et al.*, 2011; Hnosko *et al.*, 2009), however until now there has not been a deeper study about its biochemical changes during ripening or its biological properties.

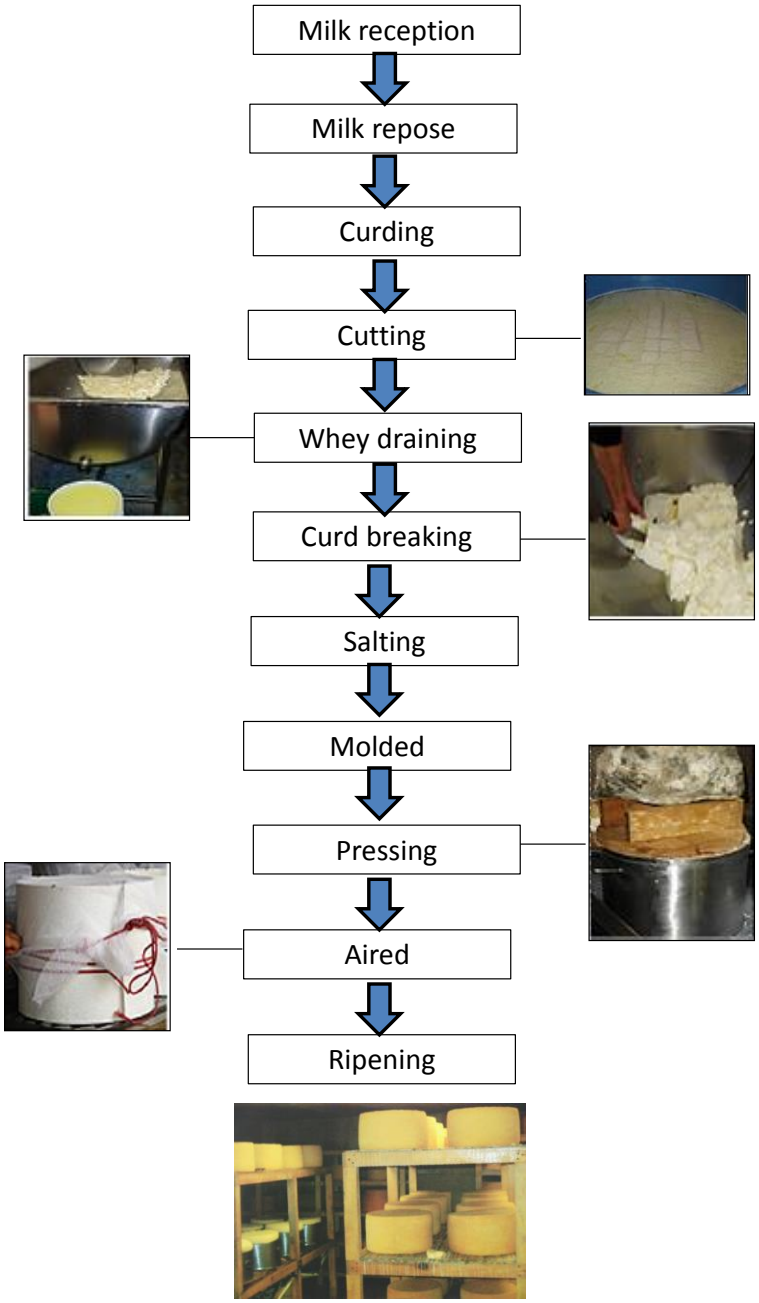

Figure 1.2. General diagram for Cotija cheese elaboration (Hernández, Quirasco, & Quintero, 2009b).

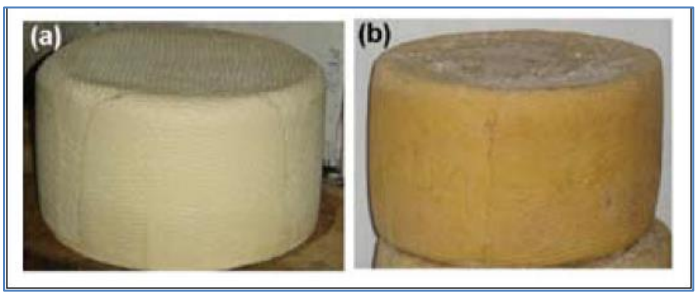

Figure 1.3. Cotija cheese. a) During early ripening, b) After three months of ripening (Hernández *et al.*, 2009a).

1.5.2 Fresh goat cheese

As previously described, most of the studies about the bioactive peptides in cheeses have been conducted with ripened cheeses; though some of the most popular Mexican cheeses are the soft cheeses, also called “Quesos frescos”. These cheeses are characterized by its high moisture content, mild milky flavor and a soft and creamy texture and short shelf-life (Torres-Llanez *et al.*, 2011).

Mexican fresh cheeses are mostly made from cow’s milk, however in the past decade there has been an increasing interest in goat milk products. Fresh goat cheese from Veracruz also called “Ranchero” or “de Rueda” has gained popularity in the latest years.

This cheese is made in the central region of Veracruz. It is artisanal made from raw milk from goat or a mix of goat and cow milk (Figure 1.4). The curd is soft and lightly pressed and the final shape can be cylindrical or prismatic-rectangular of between 250 g and 1 kg (González-Córdova *et al.*, 2016).

Figure 1.4. Artisanal conditions for fresh goat cheese production (Sangabriel Hernandez, 2011).

Following the example put by Cotija cheese, the Mexican government and the goats’ breeders from the region have jointed efforts to impulse the knowledge about this cheese and standardize its manufacture process with the final objective to protect the regionally of this cheese through a Collective Mark (Sangabriel Hernandez, 2011).

Still one of the main problems faced by the producer is given by sanitary issues. Ranchero fresh goat cheese is traditionally made with raw milk; however changes in the Mexican law demand all fresh cheeses to be made with pasteurized milk.

Nevertheless, in order to exert a real health benefit in the consumers, it is necessary that those bioactive peptides are released from the food matrix after the ingestion, resist the digestion process and reach intact to the target site of action where they are supposed to perform their bioactivity (Rein *et al.*, 2012).

However, the study of an aliment and its impact on health can be extremely complex, mainly because it depends first on its structure and composition (initial and/or during digestion) and then because it can act directly on the host or through modulation of the functional potential of the intestinal microbiota.

Cheese is not only an important source of bioactive peptides (Gupta *et al.*, 2013; Pritchard *et al.*, 2010; Torres-Llanez *et al.*, 2011), but also of microorganism. In cheeses the association of milk-microorganisms (lactic bacteria and ripening microorganisms) led to the development of the sensorial characteristics of each cheese. Moreover, the proteolytic system of lactic acid bacteria plays a key role during proteolysis and on the release of bioactive peptides. In the next paragraph, the role of specific microbiota during cheese ripening will be developed.

1.6 Cheese microbiota

Microorganisms live as part of cheeses ecosystems, are also known as cheese microbiota, and are composed of various types of bacteria, yeast and molds, which interact together forming a very complex ecosystem. This microbiota is more diverse in cheeses made with raw milk than in those made with pasteurized milk; and undergoes considerable modifications through ripening (Spinnler & Gripon, 2004). Ripening conditions as curd pH, temperature and humidity, promote the colonization of microorganisms as yeast and bacteria on the cheese surfaces. Thus surface microbiota comes from ripening cultures and indigenous flora from raw milk and environment (Irlinger *et al.*, 2012) .

During the first 24 h of cheese-making process, the dominant microbiota is composed of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* known as lactic acid bacteria (LAB). This bacterial growth generates lactate from lactose.

The succession of microorganisms is determined by the evolution of the chemical environment. Production of lactate reduces the pH and allows the development of acid tolerant microorganism, like *Debaryomyces hansenni*, *Geotrichum candidum* and other yeast or filamentous fungi on the cheese surface. In growing on the surface, these microorganisms ascend the pH through lactate uptake and casein proteolysis allowing a further development of less acid-tolerant, aerobic or facultative anaerobic, catalase-positive, halo-tolerant ripening bacteria known as Non-starter lactic acid bacteria (NSLAB). This group of bacteria is mainly represented by *Staphyloccus*, *Brevibacterium*, *Arthrobacter*, and *Corynebacterium* (Gripon, 1997; Irlinger *et al.*, 2012; Spinnler & Gripon, 2004). These bacteria play a key role in the development of sensorial characteristics of the cheese, mostly in terms of aroma compounds (Irlinger *et al.*, 2012).

Beyond NSLAB, other gram-negative bacteria have also been isolated from cheeses microbiota. These are aerobic or anaerobic bacteria that develop mainly during cheese manufacture and sometimes represent 40% of the total bacterial population. The most common genera are *Psychrobacter* that includes species highly resistant to salt and *Hafnia* which is able to produce flavor compounds (Irlinger *et al.*, 2012).and widely seeded in milk for camembert type cheeses manufacture.

1.7 Cheese as a vehicle for viable microorganisms

The total microbial population in a cheese ecosystem is in average 10^8 –10^9 viable cells/g of cheese, and that the annual consumption is 24 kg per French, it is traduced in an annual consumption of 2.4 x10^{12} and 2.4 x 10^{13} viable cells through the consumption of cheeses (Adouard *et al.*, 2015). This raises the interests of cheese as a dairy matrix that can act as a vehicle for viable cells to enter in the digestive tract.

In this field, there are many questions that actually remain about the fate of eaten microorganisms in the gastro-intestinal (GI) tract. When a food microorganism is capable to reach the epithelial surfaces in the gastrointestinal tract, it automatically interacts with the

intestinal microbiota. From this interaction results the beneficial effects exert by probiotics. However this interaction might not be exclusive for recognized probiotic microorganisms, cheese microorganisms could also exert some kind of effects on the intestinal microbiota as will be discussed in the next paragraph.

1.8 Potential effect of cheese microorganisms on human health

Interaction between cheese and intestinal microbiota could result in beneficial effects for the host. Lay *et al.,* (2004) tested the effect of the Camembert microbiota on rats, finding an important decrease of azoreductase activity and an increase in mucolytic activity. Ibrahim *et al.,* (2010) tested the immunomodulatory effect of commercial probiotic cheese in elderly patients and, further the effect of the probiotics on the host, they observed an increase in phagocytosis activity related to the consumption of control Gouda cheese. They attributed this immune response to the starter strains of the control cheese. Additionally, authors suggested that immune response of the host could be enhance according to the matrix used, founding higher response when cheese was used as probiotic carrier than previous studies from other authors using skim milk. Adouard *et al.,* (2015), tested the immunomodulatory effect of different cheese-ripening microorganisms. Authors found that some of those dairy microorganisms exert a significant production of interleukin 10 (IL-10) that resulted even higher than the one observed for a well-known probiotic strain identified as *B. longum Bb536*.

Thus, from this point of view and without attempting to give the name or characteristics of probiotic to these microorganisms, we can notice the potential of cheese microbiota on health; and the importance of cheese as a dairy matrix carrier of live microorganism.

However and, as we previously established, in order to exert any kind of biological activity, cheese microbiota (and bioactive peptides) need to pass through stomach and tolerate its high acidity, before going through an intestinal stress mainly related to the effects of bile salts (Sumeri *et al.,* 2012). It therefore appears essential to study their subsequent survival to the stress induced by the digestion.

1.9 Bacterial stress in the stomach

Bacterial stress can be defined as any change in the genome expression and proteome or the environment that decreases growth or affect the ability to survive. Such changes lead cells to try to restore a metabolic profile that would be favorable to survival, or to faster growth. In this definition, metabolism includes all stages of gene expression: transcription, translation and post-translational modifications (Baliarda, 2003).

The first obstacle faced by microorganisms after consumption is the low pH in the stomach because of the chlorhydric acid secretion. During digestion, pH varies between 4.5 and 2. These conditions are extremely severe for microorganisms. Although LAB may frequently be isolated from relative acid habitats (during cheese making LAB are in charge to descend pH), this group of bacteria is best characterized as neutrophil (Hutkins & Nannen, 1993). Also the NSLAB and the ripening bacteria have an optimal pH almost neutral or basic (Adouard, 2015).

In order to conserve cell viability, bacteria need to control a pH homeostasis inside the cell. The cytoplasm of most microorganisms has a relatively high buffering capacity of 50 to 100 nmol of H^+ pH unit per mg of cell protein (Hutkins & Nannen, 1993). Some authors postulated that the accumulation of histidine into bacterial cells may contribute to the buffering capacity within the cell enhancing the acid resistance (Mills, Stanton, Fitzgerald, & Ross, 2011). Additionally, the relative impermeability of the bacterial membrane to extracellular protons provides the cells with some protection against acidity (De Angelis & Gobbetti, 2004).

Further to these responses from the cells, some bacteria have additional mechanisms of response against pH stress that include protein and/or gene expression, transport mechanisms and metabolism modifications.

1.9.1 Protein expression

Some microorganisms are capable to produce specific proteins to face the acid response, named acid shock proteins (ASP). The genes involved in the resistance to stress are called the σ^s regulon. These genes are under the control of an alternative subunit of the RNA polymerase named σ^S coded by the gene RpoS (RNA polymerase sigma S) that is the main

controller of the expression of the genes involved in the stress response. Exposing log phase cells to acid stress provides cross protection against other type of stress (oxidative, heat shock, etc); however reciprocal cross protection does not occur (Mills *et al.*, 2011).

Studies also reveal that some heat shock proteins (HSPs) are produced during acid adaptation, and the identity of these proteins is dependent of the strain. In *Lb. delbrueckii subsp. bulgaricus*, the identified chaperon proteins were GroES, GroEL and DnaK (De Angelis & Gobbetti, 2004; Mills *et al.*, 2011) however these proteins are not involved in protein refolding, but they are necessary for normal cellular functions as growth, stability of DNA and RNA and they prevent the inclusion of bodies (Mills *et al.*, 2011).

1.9.2 Transport protein activation

Proton translocating ATPase (H^+-ATPase) is complex within the membrane that regulates the proton expel from cell through the proton motive force (PMF). PMF hydrolyses ATP, producing the necessary energy to move protons against gradient. In respiratory bacteria, the PMF is generated by the respiratory chain, meanwhile, in non-respiratory ones (i.e. *Streptococcus faecalis*) the only function of this complex is the extrusion of H^+ to regulate the pH homeostasis (Cotter & Hill, 2003).

The number of protons carried out the cell, is directly dependent on the amount H^+-ATPase. This enzyme has different optimal pH depending on the strain, in *Lactobacillus casei* and *Lactobacillus plantarum*, the optimal activity is at pH 5.0-5.5, meanwhile for *Streptococcus thermophilus*, the optimal pH is 7.0-7.5 (Hutkins & Nannen, 1993). The more H^+-ATPase content in a bacteria and a lower optimal pH, more acid resistant. Additionally, other cation transport ATPases as K^+-ATPases can also contribute to pH homeostasis interchanging K^+ for H^+ as observed for *S. mutans* and *L. lactis* (Cotter & Hill, 2003).

1.9.3 Metabolism modifications

1.9.3.1 Arginine Deiminase (ADI) pathway

This system has 4 components, ADI (EC 3.5.3.6), catabolic ornithine transcarbamoylase (cOTC, EC 2.1.3.3), carbamate kinase (CK, EC 2.7.2.2) and a membrane transport protein. It converts arginine to L-citrulline and ammonia. Citrulline is further degraded forming ATP and L-ornithine. The released ammonia gives protection to LAB

against acid damage by increasing the pH in the cytoplasm (Hutkins & Nannen, 1993). Additionally ADI pathway produces extra ATP that enhances the expulsion of protons by H^+-ATPase complex. *Lactobacillus sanfranciscensis* utilize this ADI pathway during sourdough fermentation resulting in in an increase in ornithine production (De Angelis & Gobbetti, 2004).

1.9.3.2 Metabolism of urea

Helicobacter pylori, a well-known acid resistant bacteria, capable to live in the human stomach and cause gastric ulcers, has the ability to use urease to produce ammonia and bicarbonate to maintain a pH of around 6 inside its periplasmic space, even when the external conditions are around pH 2 (Adouard *et al.*, 2015; Sachs, Weeks, Wen, Marcus, & Scott, 2005). This enzyme has been found in *S. termophilus* and the absence of it has been associated with the lack of ability of some strains to survive the *in vitro* digestive stress (Uriot *et al.*, 2016).

Those microorganisms that are capable to survive the acid conditions in stomach can reach the duodenum, where they need to face a new stress caused mostly by the bile acids.

1.10 Bacteria stress in the intestine

Bile acids are synthesized in the liver from cholesterol and secreted from the gall bladder into the duodenum in the conjugated form. Later in the colon these acids suffer modifications such as deconjugation, dehydroxylation, dehydrogenation and deglucuronidation, through microbial metabolism. Both conjugated and deconjugated bile acids exhibit antibacterial activity, but the deconjugated forms are more lethal (Dunne *et al.*, 2001).

Gram-positive bacteria are more sensitive to these salts than gram-negative. However, bile tolerance is also strain specific (Begley *et al.*, 2005; Li, 2012). In gram-negative bacteria, the outer membrane constitutes an excellent hydrophobic barrier against bile. Still bile salts can go through the membrane and disturb the membrane characteristics such as charge, hydrophobicity and lipid fluidity because of their detergent properties (Begley *et al.*, 2005). Bile salts can generate oxygen free radicals, alter RNA secondary structure, induce DNA damage and activate DNA repair related enzymes.(Li, 2012).

However, some bacteria are highly resistant to the stress caused by the bile salts/acid in the intestine. Some of the mechanisms observed by these bacteria are related to the expulsion of the bile by pumps, enzyme actions to metabolize the bile, production of specific proteins related to the membrane synthesis, etc.

1.10.1 Efflux pumps

Outflow of bile salts from the bacterial cytoplasm is without doubts the best-characterized and probably the most important mechanism of bile salt resistance.

Efflux pumps are enzymes active transporters localized in the cytoplasmic membrane of cells. The best characterized one it's identified as AcrB which gives the resistance to *E. coli* against solvents, detergents, antibiotics and bile salts (Gunn, 2000). AcrB is formed by: a transporter (efflux) protein (AcrB) an accessory protein (i.e. AcrA) and an outer-membrane (for example TolC), located in the outer membrane protein channel. AcrB captures its substrate either from within the phospholipid bilayer of the inner membrane, or from the cytoplasm, and then transport then to the extracellular medium through TolC, which forms a channel in the outer membrane. AcrA protein works as the intermediate between TolC and AcrB. This pump utilizes the energy of the proton motive force (PMF) (Piddock, 2006). In gram-positive bacteria there are other efflux pumps with only one component, instead of three which can realize the same function as AcrB., for instance MexAB-OprM in *P. aeruginousa* (Sun, Deng, & Yan, 2014).

1.10.2 Bile salt hydrolases

Bile salt hydrolases (BSH) confer protection against bile stress through deconjugation, a reaction that separates glycine/taurine moiety from the steroid core; and that is necessary before further bile degradation.

BSH are (mostly) intracellular, non-allosteric, oxygen insensitive enzymes that catalyze the hydrolysis of the amide bond between the posisition C-24 of the steroid part and the amino acid chain of bile acids. Their optimal pH is between 5–6 (Begley *et al.*, 2005). BSH catalyzes a reaction in which glycine and taurine are deconjugated from bile salts,

releasing unconjugated acids that can be expelled out from the cell and be further metabolized by the gut microbiota (Ruiz, Margolles, & Sanchez, 2013).

The real mechanism of these enzymes on the tolerance of bile salts has not been completely explained, but some hypotheses have been made: a) deconjugation, might confer a nutritional advantage as liberated amino acids could be used as carbon, nitrogen and energy sources. b) BSH helps the bacteria to incorporate the cholesterol or bile into the membrane, increasing its strength, changing their fluidity or charge (Begley *et al.*, 2005; Ruiz *et al.*, 2013). Penicillin amidases, are enzyme related to the BSH, they are in charge of hydrolyse penicillin. It has been suggested that these amidases might also have affinity for the bile acid substrates (Ruiz *et al.*, 2013).

1.10.3 Extracellular polysaccharides

LAB are able to produce extracellular polysaccharides (EPS) that can encapsulate the bacteria, and protect it against environmental stress. The studies of Boke, Aslim, and Alp (2010) and Uriot *et al.*, (2016) found that there is a high correlation between the ability of LAB to produce EPS and their survival on the intestine, concluding that EPS play a key role on the survival of these microorganisms during digestion protecting them from low pH and bile salts.

Thus, the ability of bacterial cells to survive the passage through the gastro intestinal tract is mostly due to its acid and bile tolerance. In order to face this stress, cells can follow different mechanism (Figure 1.5); for instance: 1) conjugated bile acids enter into the cytoplasm, 2) activating BSH that deconjugate the bile acid releasing amino acids that might be later used as C, N and energy sources; 3) deconjugated bile acid can also enter the cell by passive diffusion and 4) became deprotonated; 5) ionized bile salts are non-permeable and have to be excreted by efflux pumps, like AcrB (composed by AcrB, AcrA and TolC); 6) chaperons, like DnaK and GroEL are produced as response to low pH, can also act in response to bile stress (cross-protection); 7) cell membrane can suffer changes in its fatty acid composition, increasing its straight, through BSH action; 8) exposure to acid pH or bile salt deprotonation results in acidification of the cytoplasm; 9) this can be neutralized through the production of ammonia by the ADI pathway; or 10) through proton expel by the ATPase bomb; 11) the required ATP for this bomb can be generated through glycolysis.

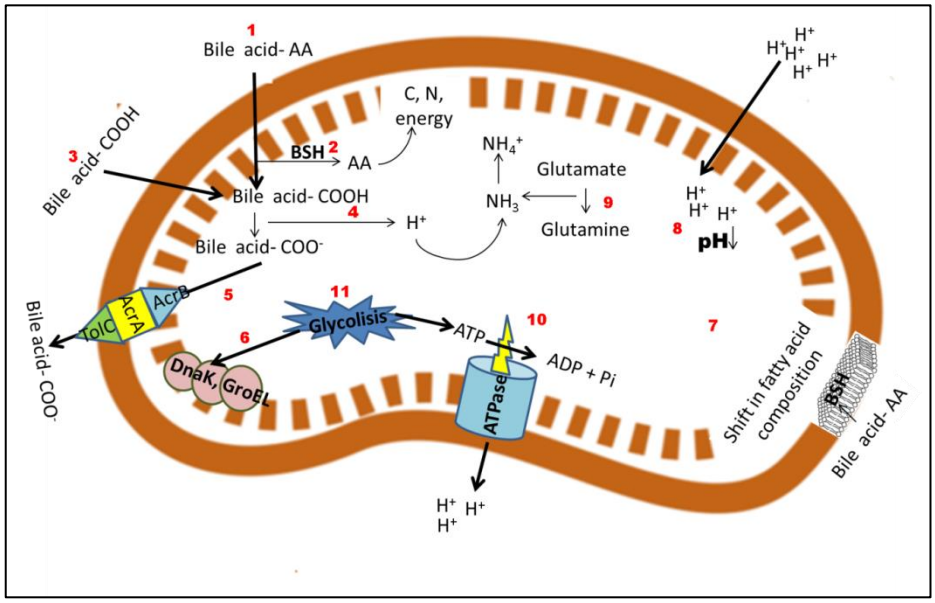

Figure 1.5. Bacterial main stress responses to digestive stress caused by low pH and bile salts. Modified from (Mills *et al.*, 2011).

Beyond the previously mentioned mechanisms of responses, that are inherent to each microorganism, some authors affirm that the presence of a food matrix could enhance the viability of those microorganisms through digestion (Do Espirito Santo *et al.*, 2011; Lay *et al.*, 2004; Saxelin *et al.*, 2010).

1.11 Effect of a food matrix in the microorganism survival

Food matrix refers to the composition of an aliment and the way those components are structured. Inside it, food components are located into spatial arrangements at a scale of 10 to 100 micrometers known (microstructure) where they interact at different levels between components and structures of the medium (Parada & Aguilera, 2007). This microstructure is produced by natural interactions in the raw material or due to the process followed for matrix manufacture, i.e. homogenization, pasteurization and coagulation (Rinaldi, Rioux, Britten, & Turgeon, 2014b; Turgeon & Rioux, 2011). Thus foods with the same components but with different manufacture process have not necessarily the same structural organization.

1.11.1 Dairy matrices

Dairy products are one of the most studied food matrices, mostly because of their nutritional importance and functional properties; but also because they can be eaten under a

great variety of structures (solutions versus gels, native versus denatured proteins, etc.), and constitute a natural source of microorganisms (as in cheese microbiota).

Dairy matrices have three major components: proteins (caseins) fat and water; organized in building blocks as fat globules, casein micelles and whey proteins, which through interaction between them contribute to the structure and texture of the food matrix. Caseins are held together through hydrophobic and electrostatic forces, largely driven by the pH and mineral content that entrap fat globules, while water binds to the minerals and proteins and fills the "empty" spaces. These compounds are carefully organized according to the process followed for matrix manufacture like homogenization (mechanical action), pasteurization (heat treatment) and coagulation (Rinaldi *et al.*, 2014b; Turgeon & Rioux, 2011) where they interact at different levels with the components and structures of the medium.

1.11.2 Dairy matrix effect on microorganism survival

Dairy products are considered the best carriers for live microorganism (Castro, Tornadijo, Fresno, & Sandoval, 2014). Milk is considered a good protective matrix mainly because of its buffer capacity and its cryoprotectant effect (Saarela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000). It has been suggested that the high concentration of amino acids like histidine could enhance acid resistance in bacteria (Begley *et al.*, 2005).

The authors that claim the protective effect of a food matrix suggest that the structure of the matrix and/or that the presence of certain ingredients, like fat, could modify the survival of the microorganisms in the gastric phase through direct or indirect interaction between microorganisms and food components (Do Espirito Santo *et al.*, 2011; Ranadheera *et al.*, 2012; Sanders & Marco, 2009).

However results reported in the literature are highly contradictory. For instance Faye *et al.*, (2012) observed that survival of *L. lactis ssp cremoris* during *in vitro* experiments was higher in fermented milk than in synthetic medium. In contrast, Sumeri *et al.*, (2012) observed that the same microorganisms did not survive during *in vitro* experiments when semi-hard cheese was used.

Lay *et al.,* (2004) observed that the viability of *S. thermophilus* during *in vivo* experiments was greater when eaten and grown in Camembert cheese than in yogurt. In contrast Adouard *et al.,* (2016) , observed higher survival of *B. auranticum* and *H. alvei* during *in vitro* experiments when grown in synthetic medium than when grown in cheese.

Meanwhile, Saxelin *et al.,* (2010) observed that the viability of *P. freudenreichii ssp. shermanii* JS, and *B. animalis ssp. lactis* Bb12 during *in vivo* experiments was higher when eaten in yogurt than in cheeses; however the food matrix did not affect the survival of *L. rhamnosus* GG and LC705.

1.12 Mechanisms for matrix protection effect

Thus the real effect of the food matrix in the survival of microorganisms has not been completely elucidated. The proposed mechanisms that may explain this protective effect refers to a) the effect of the microorganism´s preadaptation to the matrix b) Macrostructure effects (buffer capacity and/or effects on gastric emptying rate) and c) Microstructure effects (interactions between microorganisms and dairy components and/or microstructure itself).

1.12.1 Preadaptation effect

Preadaptation effect is related to the inherent mechanism of response to stress expressed during food manufacture that produces a cross-adaptation to further digestive stress (Begley *et al.,* 2005). In this regard, Pitino *et al.,* (2012) observed that survival of *L. rhamnosus* in cheese *during in vitro* experiments was related to the production of extracellular polysaccharide.

Uriot *et al.,* (2016) observed that viability of different strains of *S, thermophilus* to *in vitro* experiments was dependent on the activation of urease and small Heat Shock Proteins. Meanwhile Boke *et al.,* (2010) observed that survival of *S. thermophilus, L. delbrueckii ssp* and *L. bulgaricus* during *in vitro* experiments was depending on the production of exopolysaccharides. Monnet *et al.,* (2014) observed that halotolerance of *B. auranticum* 9174 during cheese ripening was due to transport mechanisms that might provide it with a cross-adaptation to other stresses (Begley *et al.,* 2005).

1.12.2 Macrostructure effects

This is mostly related to the buffer capacity of the matrix during stomach phase. In dairy, buffer capacity is given by the milk constituents (caseins, whey proteins, soluble minerals and colloidal calcium phosphate, CCP) (Salaün *et al.*, 2005). The role of each component on the buffer capacity is given by compound interactions, physicochemical conditions and technologic treatments. For instance, heat treatment produces changes in the structure and composition of micellar calcium phosphate; fermentation causes degradation of lactose, production of carbonic acid (by urea degradation), casein degradation, etc. All this phenomena produce variations in buffer capacity. Thus buffer capacity is depend of the type of dairy product (Al-Dabbas, Al-Ismail, & Al-Abdullah, 2011; Salaün *et al.*, 2005; Upreti, Bühlmann, & Metzger, 2006). In this field Gardiner *et al.,* (1998) suggested that the higher viability of *E. faecium* in Cheddar and yogurt during *in vitro* experiments was due to its higher buffer capacity compared to the synthetic medium.

On the other hand, food macrostructure impacts in the digestive process, for instance, coagulation of the milk increases the viscosity of the matrix, disturbing the gastric emptying and intestinal transit, caused by changes in the ratio liquid/solid in the chime (Turgeon & Rioux, 2011). The presence of certain components like proteins and fat affects the time course of stomach emptying (Turgeon & Rioux, 2011), and the bile secretion is related with the content of fat in the matrix (David *et al.*, 2014). These digestive chances could further affect the time and conditions of stress for microorganisms through digestion.

1.12.3 Microstructure effects

On the other hand microstructure role in microorganism's survival is maybe the less studied one. As previously said, food microstructure is dependent on the manufacture process. For instance, milk homogenization reduces the size of the fat globules changing the interface between proteins and fat globule membrane (Michalski, 2007; Turgeon & Rioux, 2011).

Sumeri *et al.,* (2012) observed that survival of LAB during *in vitro* experiments was higher in semi-hard cheeses than in synthetic medium. Suggesting that, high protein content and presence of fat globules provide a physical barrier, and a further interaction surface with the microorganisms, incorporated into the matrix.

Hannon *et al.*, (2006) observed that survival of *L. lactis* during *in vitro* experiments was higher when cheese was rennet at pH 5.2 than at 6.2, because at low pH the matrix was more homogeneous. Pitino *et al.*, (2012) observed the preference of *L. rhamnosus* to the casein network into a cheese matrix.

Microstructure also impacts the digestive process. Pasteurization of milk denatures the protein structure, affecting its enzymatic susceptibility thus changing the proteolysis patterns (Rinaldi *et al.*, 2014b; Turgeon & Rioux, 2011). Mullally, Mehra, and Fitzgerald (1998) observed that after thermal denaturation, β-lactoglobulin, was significantly more susceptible to hydrolysis by gastric enzymes. Meanwhile Rinaldi *et al,*. (2014b) observed that addition of polysaccharide into a yogurt matrix during *in vitro* digestion caused a microstructure disruption with a further increase in proteolysis rate.

Thus, the role of the food matrix is still unknown and the several proposed mechanisms makes difficult to determine its real contribution on microorganisms survival. Therefore, the study of the relationship between microorganism and food matrix components, before and during digestions could help to elucidate its mechanism.

References

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., . . . Amaya-Llano, S. L. (2013). Influence of probiotic strains added to Cottage Cheese on potentially-antioxidant peptides generation, anti-Listerial activity, and survival of probiotic microorganisms in simulated astrointestinal conditions. *International Dairy Journal, 33*(2), 191–197. doi:10.1016/j.idairyj.2013.04.005

Adouard, N. (2015). *Les micro-organismes du microbiote fromager survivent-ils à la digestion et peuvent-ils avoir un effet immunomodulateur sur l'hôte ?* (Docteur), AgroParisTech, Thiverval-Grignon.

Adouard, N., Foligné, B., Dewulf, J., Bouix, M., Picque, D., & Bonnarme, P. (2015). In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese. *International Journal of Food MIcrobiology, 197*, 98-107. doi:10.1016/j.ijfoodmicro.2014.12.011

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Al-Dabbas, M. M., Al-Ismail, K., & Al-Abdullah, B. M. (2011). Effect of Chemical Composition on the Buffering Capacity of Selected Dairy Products. *Jordan Journal of Agricultural Sciences, 7*, 690-700.

Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences, 90*, 7915-7922.

Baliarda, A. (2003). *Evaluation de la reponse au stress chez les bacteries lactiques partenant aux genres Pediococcus et Tetragenococcus. Approches physiologiques et genetiques.* (Docteur), Universite Bordeaux, Bordeaux. Retrieved from http://grenet.drimm.u-bordeaux1.fr/pdf/2003/BALIARDA_AURELIE_2003.pdf (2747)

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29*, 625-651. doi:10.1016/j.femsre.2004.09.003

Boke, H., Aslim, B., & Alp, G. (2010). The role of resistance to bile salts and acid tolerance of exopolysaccharides (EPSS) produced by yogurt starter bacteria. *Archives of biological sciences (Beograd), 62*(2), 323-328. doi:10.2298/abs1002323b

Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., . . . Sforza, S. (2013). Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. *Food Science and Nutrition, 68*(8), 963-968. doi:10.3109/09637486.2013.821696

Castro, J. M., Tornadijo, M. E., Fresno, J. M., & Sandoval, H. (2014). Biocheese: A Food Probiotic Carrier. *BioMed Research International, 2015*. doi:10.1155/2015/723056

Cotter, P. D., & Hill, C. (2003). Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbioology and Molecular Biology Reviews, 67*(3), 429-453. doi:10.1128/MMBR.67.3.429–453.2003

Cushman, D., & Cheung, H. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical pharmacology, 20*(7), 1637-1648.

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry, 46*, 49-53. doi:10.1021/jf970649w

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12. doi:10.1111/j.1471-0307.2011.00725.x

Chombo-Morales, P., Kirchmayr, M., Gschaedler, A., Lugo-Cervantes, E., & Villanueva-Rodríguez, S. (2016). Effects of controlling ripening conditions on the dynamics of the native microbial population of Mexican artisanal Cotija cheese assessed by PCR-DGGE. *LWT - Food Science and Technology, 65*, 1153-1161. doi:10.1016/j.lwt.2015.09.044

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Fischbach, M. A. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563. doi:10.1038/nature12820

De Angelis, M., & Gobbetti, M. (2004). Environmental stress responses in Lactobacillus: A review. *Proteomics, 4*, 106-122. doi:10.1002/pmic.200300497

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., & Vanacore, D. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258. doi:10.1002/psc.1093.

Do Espirito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2011). Influence of food matrices on probiotic viability - A review focusing on the fruity bases. *Trends in Food Science and Technology, 22*, 377-385. doi:10.1016/j.tifs.2011.04.008

Dunne, C., O´Mahony, L., Murphy, L., Thorton, G., Morrissey, D., O´Halloran, S., . . . Collins, J. K. (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *The American Journal of Clinical Nutrition, 73*(2), 386S-392S.

El-Salam, M. H. A., & El-Shibiny, S. (2012). Bioactive Peptides of Buffalo, Camel, Goat, Sheep, Mare, and Yak Milks and Milk Products. *Food Reviews International, 29*(1), 1-23. doi:10.1080/87559129.2012.692137

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654. doi:10.1016/j.jnutbio.2007.11.010

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183. doi:10.1016/j.idairyj.2013.04.002

Faye, T., Tamburello, A., Vegarud, G. E., & Skeie, S. (2012). Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human astric and duodenum juice. *Journal of dairy science, 95*, 558-566. doi:10.3168/jds.2011-4705

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *The Journal of nutrition, 134*(4), 980S-988S.

Flores-Magallón, R., Oliva-Hernández, A. A., & Narváez-Zapata, J. A., . (2011). Characterization of Microbial Traits Involved with the Elaboration of the Cotija Cheese. *Food Science and Biotechnology, 20*(4), 997-1003. doi:10.1007/s10068-011-0137-z

Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Reviews International, 12*(4), 457-509. doi:10.1080/87559129609541091

Gardiner, G., Ross, R. P., Collins, J. K., Fitzgerald, G., & Stanton, C. (1998). Development of a Probiotic Cheddar Cheese Containing Human-Derived *Lactobacillus paracasei* Strains. *Applied and Enviromental Microbiology, 64*(6), 2192-2199.

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., & Di Cagno, R. (2002). Latent

bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Critical Reviews in Food Science and Nutrition, 42*(3), 223-239. doi:10.1080/10408690290825538

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gripon, J. C. (1997). Flavour and textrure in soft cheese. In B. A. Law (Ed.), *Microbiology and Biochemistry of Cheese and Fermented Milk* (Vol. 1, pp. 193-203). Berkshire, U. K.: Blackie Academic and Professional

Gunn, J. S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes and Infection, 8*, 907-913. doi:10.1016/S1286-4579(00)00392-0

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Hajirostamloo, B. (2010). Bioactive component in milk and dairy product. *World Acad Sci Eng Technol, 72*, 162-166.

Hannon, J. A., Lopez, C., Madec, M. N., & Lortal, S. (2006). Altering Renneting pH Changes Microstructure, Cell Distribution, and Lysis of *Lactococcus lactis* AM2 in Cheese Made from Ultrafiltered Milk. *Journal of dairy science, 89*(3), 812-823.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current opinion in biotechnology, 18*(2), 163-169. doi:10.1016/j.copbio.2007.01.013

Hernández, V., Navarro, A., & Quirasco, M. (2009a). Antioxidant capacity in the Cotija "Región de Origen" Cheese, the case of an artisanal cheese. *Carnilac Industrial, 24*(1), 24-31.

Hernández, V., Quirasco, M., & Quintero, B. (2009b). Un acercamiento al mundo del queso Cotija Región de Origen MC: arte y tradición de México. *Culinaria, Revista Virtual Gastronómica 5,* 5-19.

Hnosko, J., Clark, S., & Van Hekken, D. (2009). Latin American Cheeses. In S. Clark, Costello, M., Drake, M., Bodyfelt, F. (Ed.), *The Sensory Evaluation of Dairy Products*

(pp. 489-504). Lincoln, New England: Springler

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*, 1062-1071. doi:10.1016/j.peptides.2008.02.005

Hutkins, R. W., & Nannen, N. L. (1993). pH Homeostasis in Lactic Acid Bacteria. *Journal of dairy science, 76*(8), 2354-2365. doi:10.3168/jds.S0022-0302(93)77573-6

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2010). Probiotics and immunosenescence: cheese as a carrier. *FEMS Immunology and Medical Microbiology, 59*, 53-59. doi:10.1111/j.1574-695X.2010.00658.x

Irlinger, F., Ah Yuen In Yung, S., Sarthou, A., Delbès-Paus, C., Montel, M., Coton, M., . . . Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food MIcrobiology, 153*, 332-338. doi:10.1016/j.ijfoodmicro.2011.11.022

Jäkälä, P., & Vapaatalo, H. (2010). Antihypertensive Peptides from Milk Proteins. *Pharmaceuticals, 3*(1), 251-272.

Kedare, S. B., & Singh, R. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of food science and technology, 48*(4), 412-422.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323. doi:10.2174/1381612033454883

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods, 1*(2), 177-187. doi:10.1016/j.jff.2009.01.007

Korhonen, H., & Pihlanto, A. (2003). Food-derived Bioactive Peptides – Opportunities for Designing Future Foods. *Current Pharmaceutical Design, 9*, 1297-1308. doi:10.1016/j.idairyj.2005.10.012

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945-960.

Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., . . . Etherton, T. D. (2002). Bioactive Compounds in Foods: Their Role in the Prevention of Cardiovascular Disease and Cancer. *The American Journal of Medicine, 113*(9B), 71-88. doi:10.1016/S0002-9343(01)00995-0

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C.

(2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Li, G. (2012). *Intestinal Probiotics: Interactions with Bile Salts and Reduction of Cholesterol*. Paper presented at the 2011 International Conference on Environmental Science and Engineering (ICESE 2011).

Lignitto, L., Cavatorta, V., Balzan, S., G., G., Galaverna, G., Novelli, E., . . . Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal, 20*, 11-17. doi:10.1016/j.idairyj.2009.07.001

Martínez Augustin, O., & Martínez de Victoria, E. (2006). Proteins and peptides in enteral nutrition. *Nutrición Hospitalaria, 21*(2), 1-14.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait, 80*, 293-324.

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Meisel, H. (2005). Biochemical Properties of Peptides Encrypted in Bovine Milk Proteins. *Current medicinal chemistry, 12*, 1623-1629. doi:10.2174/0929867054546618

Meisel, H., & Günther, S. (1998). Food proteins as precursors of peptides modulating human cell activity. *Food / Nahrung, 42*, 175-176.

Michalski, M. (2007). On the supposed influence of milk homogenization on the risk of CVD, diabetes and allergy. *British Journal of Nutrition, 97*, 598-610. doi:10.1017/S0007114507657900

Mills, S., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2011). Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories, 10*(1), 1-15. doi:10.1186/1475-2859-10-S1-S19

Mollet, B., & Rowland, I. (2002). Functional foods: at the frontier between food and pharma. *Current in Opinion and Biotechnology, 13*, 483-486. doi:10.1016/S0958-1669(02)00375-0

Monnet, C., Landaud, S., Bonnarme, P., & Swennen, D. (2014). Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters, 263*(1), 1-9. doi:10.1093/femsle/fnu025

Mullally, M. M., Mehra, R., & Fitzgerald, R. J. (1998). Thermal effects onthe conformation

and susceptibility of beta-lactoglobulin to hydrolysis by gastric and pancreatic endoproteinases. *Irish Journal of Agricultural and Food Research, 37*(1), 51-60.

Olvera-García, V., Cardador-Martínez, A., & Martín del Campo, S. T. (2015). Effect of Thermal Process and Filtration on the Antioxidant Activity and Physicochemical Parameters of Agave atrovirens Extracts. *Journal of Food Research, 4*, 155-167. doi:10.5539/jfr.v4n1p155

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Parada, J., & Aguilera, J. M. (2007). Food Microstructure Affects the Bioavailability of Several Nutrients. *Journal of Food Science, 72*(2), 21-32. doi:10.1111/j.1750-3841.2007.00274.x

Paul, M., & Van Hekken, D. L. (2011). Assessing antihypertensive activity in native and model Queso Fresco cheeses. *Journal of dairy science, 94*, 2280-2284. doi:10.3168/jds.2010-3852

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2015). Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening. *Journal of dairy science, 98*, 3690-3694. doi:10.3168/jds.2014-9097

Piddock, L. J. V. (2006). Multidrug-resistance efflux pumps, not just for resistance. *Nature Reviews Microbiology, 4*, 629-636. doi:10.1038/nrmicro1464

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63. doi:10.1016/j.fm.2012.02.013

Poméon, T. (2007). *El queso Cotija, México. Un producto con marca colectiva queso "Cotija Región de origen", en proceso de adquisición de una Denominación de Origen*. Retrieved from

Prakash, A., Rigelhof, F., & MIller, E. (2001). Antioxidant activity. *Medallion laboratories analytical progress, 19*(2), 1-4.

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548.

doi:10.1016/j.foodres.2010.03.007

Ranadheera, C. S., Evans, C. A., Adams, C. A., & Baines, S. K. (2012). In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International, 49*, 619-625. doi:10.1016/j.foodres.2012.09.007

Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K., & da Silva-Pinto, M. (2012). Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British Journal of Clinical Pharmacology, 75*(3), 588-602. doi:10.1111/j.1365-2125.2012.04425.x

Rinaldi, L., Rioux, L., Britten, M., & Turgeon, S. L. (2014b). In vitro bioaccessibility of peptides and amino acids from yogurt made with starch, pectin, or β-glucan. *International Dairy Journal*, 1-7. doi:10.1016/j.idairyj.2014.09.005

Ruiz, L., Margolles, A., & Sanchez, B. (2013). Bile resistence mechanisms in Lactobacillus and Bifidobacterium *Frontiers in Microbiology, 4*, 396. doi:10.3389/fmicb.2013.00396

Ryhänen, E.-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal, 11*, 441-447. doi:10.1016/S0958-6946(01)00079-6

Saarela, M., Mogensen, G., Fonden, R., Matto, J., & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology, 84*, 197-215. doi:10.1016/S0168-1656(00)00375-8

Sachs, G., Weeks, D. L., Wen, Y., Marcus, E. A., & Scott, D. R. (2005). Acid Acclimation by Helicobacter pylori. *American Physiological Society, 20*(6), 429-438. doi:10.1152/physiol.00032.2005

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese. *Journal of dairy science, 83*(7), 1434-1440. doi:10.3168/jds.S0022-0302(00)75013-2

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal, 15*, 95-109. doi:10.1016/j.idairyj.2004.06.007

Sanders, M. E., & Marco, M. L. (2009). Food Formats for Effective Delivery of Probiotics. *The Annual Reviews of Food Science and Technology, 1*, 65-85. doi:10.1146/annurev.food.080708.100743

Sangabriel Hernandez, H. (2011). *Red Regional de Microqueserías Artesanales con Leche de Cabra* Veracruz: Comite Nacional Sistema Producto Caprinos Retrieved from

http://www.cnsp.caprinos.org.mx/documentos/conferencias2011/RedRegionalMicroqueseriasVer.pdf.

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., . . . Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food MIcrobiology, 144*, 293-300. doi:10.1016/j.ijfoodmicro.2010.10.009

Spinnler, H. E., & Gripon, J. C. (2004). Surface Mould-ripened Cheeses. In P. F. Fox, P. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: Chemistry, Physics and Microbiology (1 ed., Vol. 2, pp. 157-174). Retrieved from http://www.sciencedirect.com/science/article/pii/S1874558X04800435. doi:10.1016/S1874-558X(04)80043-5

Sumeri, I., Adamberg, S., Uusna, R., Sarand, I., & Paalme, T. (2012). Survival of cheese bacteria in a gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41. doi:10.1016/j.idairyj.2011.12.016

Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications, 453*, 254-267. doi:10.1016/j.bbrc.2014.05.090

Theolier, J., Hammami, R., Fliss, I., & Jean, J. (2014). Antibacterial and antifungal activity of water-soluble extracts from Mozzarella, Gouda, wiss, and Cheddar commercial cheeses produced in Canada. *Dairy Science and Technology, 94*, 427-438. doi:10.1007/s13594-014-0170-9

Timón, M. L., Parra, V., Otte, J., Broncano, J. M., & Petrón, M. J. (2014). Identification of radical scavenging peptides (<3 kDa) from Burgos-type cheese. *LWT - Food Science and Technology, 57*(1), 359-365. doi:10.1016/j.lwt.2014.01.020

Torres-Llanez, M., González-Córdova, A., Hernandez-Mendoza, A., Garcia, H., & Vallejo-Cordoba, B. (2011). Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. *Journal of dairy science, 94*(8), 3794-3800. doi:10.3168/jds.2011-4237

Turgeon, S. L., & Rioux, L. (2011). Food matrix impact on macronutrients nutritional properties *Food Hydrocolloids, 25*, 1915-1924. doi:10.1016/j.foodhyd.2011.02.026

Upreti, P., Bühlmann, P., & Metzger, L. E. (2006). Influence of Calcium and Phosphorus, Lactose, and Salt-to-Moisture Ratio on Cheddar Cheese Quality: pH Buffering Properties of Cheese. *Journal of dairy science, 89*, 938-950.

Uriot, O., Galia, W., Awussi, A. A., Perrin, C., Denis, S., Chalancon, S., . . . Roussel, Y.

(2016). Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. *Food Microbiology, 53*, 18-29. doi:10.1016/j.fm.2015.05.007

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 357–366. doi:10.1079/BJN20041189

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910

WHO, & FAO. (2003). Diet, Nutrition and The prevention of chronic diseases (pp. 160). Geneva: WHO

RESULTS
AND
DISCUSSION

2. Results and Discussion

2.1: Angiotensin converting enzyme inhibitory and antioxidant peptides release during ripening of Mexican Cotija hard cheese.

2.1.1 Presentation of the first article published in the Journal of Food Research

Cheese is an important part of the Mexican culture. Since most of these cheeses come from artisan cheese makers, the geographical characteristic provides them with a strong link to their place of origin and describes somehow the history and culture of the communities that produce them (González-Córdova *et al.*, 2016).

Despite Cotija cheese is the only Mexican cheese with a trademark "Cotija Región de Origen" very little is known about this cheese with a special lack of information about its biological properties.

Numerous studies in different European cheeses have identified the biological activity of a wide range of peptides released during proteolysis, establishing that the type and quantity of these peptides is dependent mostly of the starter culture used and the ripening conditions employed (Choi *et al.*, 2012; Gobbetti *et al.*, 2002; Gupta *et al.*, 2009; Gupta *et al.*, 2013). Furthermore, most of those previous studies concluded that the biological activity increases as ripening advances but only until certain point. After this point is reached, biological activity decreased.

Cotija cheese has been always compared to the Italian cheeses (Parmigiano reggiano) however there are huge differences (ripening time, cooking curd, microbiota, etc.) in manufacture that makes of Cotija a very unique cheese. In this work we researched if bioactive peptides with antioxidant and angiotensin converting enzyme (ACE) inhibitory activity are present in Cotija cheese due to the specific technology used to make it. Since this is a cheese that should be commercialized between 3 to 6 months of ripening, it resulted important to measure the stability of both antioxidant and ACE inhibitory activity throughout ripening, to make sure that the commercialized cheese could exert those biological properties.

To answer these questions Cotija cheese was ripened during 6 months in conditions similar to the artisanal. During this time samples were taken to measure the main changes occurred. A special attention was dedicated to characterize the proteolysis, measured throughout the nitrogenous fractions. Based on the peptides size obtained, nitrogenous fractions were selected to measure a) antioxidant activity (ASN and NPN) and b) ACE inhibitory activity (EtOH-SN and EtOH-NSN).

Since most of the previous works on bioactive peptides are considering the importance of hydrophobicity to get the biological function, it resulted important to determine the type of peptides related to each biological activity. Thus in a second stage, the hydrophilic and hydrophobic peptides contents were measured in each fraction.

To our knowledge, there are no previous studies about the ripening of Cotija, thus this work would constituted the first approach into a deep characterization of this cheese to provide value added by its biological properties. The knowledge of it could help to get a Denomination of Origen, making it the first in Mexico.

Highlights

- We proved that nitrogenous fractions of Cotija cheese had biological activity as antioxidant and ACE inhibitors.
- We made a first approach in the characterization of Cotija proteolysis during its ripening.
- We followed the changes in nitrogenous fractions, antioxidant and ACE inhibitory y activity during Cotija ripening.
- We correlated the biological activity with the type of peptides in each nitrogenous fraction.

2.1.2 Article: Angiotensin converting enzyme inhibitors and antioxidant peptides release during ripening of Mexican Cotija hard cheese

Leticia Hernández-Galán[1,2,3], Anaberta Cardador-Martínez[1], Daniel Picque Picque[3], Henry Eric Spinnler[2], Micloth López-del-Castillo Lozano[4] & Sandra Teresita Martín del Campo[1]

[1] Escuela Nacional de Ingeniería y Ciencias, Tecnológico de Monterrey. Epigmenio González 500, Fracc. San Pablo. CP 76130, Santiago de Queretaro, Qro. México
[2] AgroParisTech, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Genie & Microbiologie des Procedes Alimentaires, F-78850 Thiverval Grignon, France
[3] INRA, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Genie & Microbiologie des Procèdes Alimentaires, F-78850 Thiverval Grignon, France
[4] Universidad Veracruzana, Instituto de Ciencias Básicas, Av. Dr. Luis Castelazo Ayala S/N, Col. Industrial Ánimas, C.P. 91192, Xalapa, Veracruz, México

Correspondence: Sandra Teresita Martín del Campo, Tecnológico de Monterrey, México. E-mail: smartinde@itesm.mx

Abstract

Cotija cheese is an artisanal Mexican cheese produced with raw cow´s milk. Our objective was to measure the antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of the peptides released during its ripening. For that, Cotija cheeses were ripened 6 months in a chamber at 25 ºC without humidity control. Weekly samples were taken to determine acid soluble nitrogen (ASN), non-protein nitrogen (NPN) and ethanol soluble nitrogen (EtOH-SN) indexes, by Kjeldahl method. Antioxidant and ACE inhibitory activities were measured by spectrophotometry and HPLC methods, respectively. Peptides in each nitrogen fraction were determined by HPLC. Our results showed that during ripening of Cotija cheeses peptides with antioxidant and ACE inhibitory activities were released and increased through ripening time reaching a maximum of 79.8 % of 2,2- diphenyl-1-picrylhydrazyl (DPPH) discoloration and 100 % of ACE inhibition at the end of ripening. Both activities were highly correlated with the types of peptides present in each fraction.

Keywords: Cotija cheese, ripening, bioactive peptides, antioxidant activity, ACE inhibitory y activity

Introduction

Cotija cheese is a ripened Mexican hard cheese made from raw cow's milk. It is produced seasonally according to traditional protocol from July to October in Jalmich region, located between Jalisco and Michoacán at 700-1700 meters above sea level (Flores-Magallón *et al.*, 2011). Cotija cheese is a 20 kg cylindrical cheese. Their distinctive characteristics are hard rind, high salt content, firm or friable texture with a strong, sharp, or pungent aroma (Hernández *et al.*, 2009a) with 35-42 % moisture, 23-30 % fat 28-31% protein and 4% salt (Hnosko *et al.*, 2009).

Cotija cheese production and process vary among producers in the curd cutting, salting, pressing and especially in ripening time which takes 3 months minimum. The microbiological and sensorial characteristics of Cotija cheese have been described before (Chombo-Morales *et al.*, 2016; Flores-Magallón *et al.*, 2011; Hnosko *et al.*, 2009), however little is known about its functional properties.

Proteolysis is the principal biochemical process during cheese ripening and it depends among other factors on the origin of milk, microbial populations, ripening time, etc. Proteolysis results in a unique peptide profile characteristic of each cheese variety. The peptides released during proteolysis may have biological activities such as antioxidants or inhibitors of angiotensin converting enzyme (ACE) (Ong *et al.*, 2007; Vermeirssen *et al.*, 2004). However, the biological activity depends on the state of cheese ripening (Gupta *et al.*, 2013).

Therefore, the present study was focused on the evaluation of the biological activity as antioxidants and ACE inhibitory of the peptides released during the ripening of Mexican Cotija cheese.

Material and Methods

Cheese production and sampling

Cotija cheeses were part of the regular production of an artisan dairy farm located in the Cotija region. Cheese elaboration was as follows: Cow's milk was standardized (3% fat) and heated to 30-35 °C for 1 h, followed by rennet addition (strength 1:10,000). Curd was cut into 1 cm^3 cubes and rested 5–10 min. Serum-free curd was salted (5 -6 %), and molded (35 kg molds) in cylindrical shape molds. After that, the curd was pressed 24 h in istle molds covered with muslin. Next day (1d), molds were open and cheeses were wrapped with a stainless steel sheet to protect them during transportation to the laboratory. Cheeses were kept wrapped during 5 days at room temperature, daily turn. On day 6, cheeses were unwrapped and placed at 25 °C in a chamber without humidity control for 6 months to be ripened. Fifty grams of cheese were sampled every week during 4 months (W1-W16), then every two weeks for the last 2 months (W18, W20, W22, W24 and W26).

Nitrogen fractions

Cotija cheese extracts for total nitrogen (TN), acid soluble nitrogen (ASN), non-protein nitrogen (NPN) and 70 % ethanol soluble (EtOH-SN) and insoluble nitrogen (EtOH-NSN) were determined according to Guerra-Martínez *et al.,* ., (2012). Where ASN is a mixture of long, medium and short size peptides, NPN is a mixture of medium and short size peptides. While EtOH-SN contains short size peptides and EtOH-NSN the long and medium

size peptides from ASN. Samples were stored at -80 °C until analyses. Nitrogen content was quantified using Kjeldahl and micro-Kjeldahl methods.

Nitrogenous fractions analysis by RP-HPLC

Nitrogenous fractions (ASN, NPN, EtOH-SN, and EtOH-NSN) were profiled according to the method of Abadía-García *et al.,* (2013). Resulting peaks were divided according to their retention time into hydrophilic (HI) and hydrophobic (HO) peptides following the criteria of Gonzalez de Llano *et al.,* (1995).

Determination of antioxidant activity

ASN and NPN fractions antioxidant activity of each ripening point were tested using the DPPH (2,2- diphenyl-1-picrylhydrazyl) radical scavenging activity, following the method of Abadía-García *et al.,* (2013). Samples were tested in triplicate and the results were expressed as DPPH·discoloration percentage.

***In vitro* determination of ACE inhibitory activity**

EtOH-SN and EtOH-NSN fractions ACE inhibitory activity of each ripening point were tested using angiotensin converting enzyme from porcine kidney (0.5 UN) following the method of Wang *et al.,* (2013). Hippuric acid (HA) was used as standard and hippuryl-histidyl-leucine (HHL) was used as equivalent of 100% of ACE activity. All reagents came from Sigma-Aldrich.

Results were calculated according with the equation 1:

$$\text{ACE inhibitory activity (\%)} = [(\%\ HHL) - (\%HA)] \quad (1)$$

Where:

$$\%\ HHL = [HHL\ area/(HHL\ area + HA\ area)]*100 \quad (2)$$

$$\%\ HA = [HA\ area/(HHL\ area + HA\ area)]*100 \quad (3)$$

Statistical analysis

Data analyses were carried out with Statistica v 12 (Statsoft, Inc., Tulsa, OK, USA).Antioxidant activity in the ASN and NPN; and ACEI activity in EtOH-SN and EtOH-

NSN were analyzed as a two different data sets to determinate significant differences in biological activities between fractions at each ripening point. Antioxidant and ACE inhibitory activities were correlated with the type of peptides of the corresponding fraction.

Results and Discussion

Nitrogenous fractions

Total nitrogen was stable throughout the ripening time (5.4 ± 0.4% dry base). On the other hand, during the first 5 weeks, ASN mean values through ripening, (15.5 ± 2.2 %TN), were higher than mean values of NPN during the same period (7.8 ± 4.4 %TN). These results are normal since ASN fraction is considered as an index of primary proteolysis in cheeses and in this fraction are contained all the peptides regardless the size, meanwhile NPN contains only medium and short size peptides (McSweeney & Fox, 1997). At 5 weeks, ASN and NPN showed similar values (13.66 % and 12.41% respectively) and behavior. From this point both fractions values increased slowly reaching the highest values (41.1 %TN and 36.2 %TN, respectively) at the end of ripening (24 weeks). EtOH-SN increased from 10 %TN in week 1 to a maximum of 31.7 %TN in week 16, to later decrease reaching almost the initial value at week 24. This decrease is due to a further metabolism of the peptides in this fraction (McSweeney, 2004).

Nitrogenous fractions analysis by RP-HPLC

Peptide profiles showed noticeable differences between ASN, NPN, EtOH-SN and EtOH-NSN fractions. Chromatograms showed that new peaks appeared during ripening, meanwhile, peaks that existed at the beginning increased or decreased through time. Thereby, the evolution of hydrophilic peptides (HI), hydrophobic peptides (HO) and HO/HI ratio were followed during ripening (Table 2.1).

Hydrophilic peptides (HI) are present in the all the nitrogenous fractions the first week. ASN showed the highest increase in HI mainly after 12 weeks (Table 2.1). On the other hand, during the first week NPN had the lowest number of peptides and then increased dramatically at week 17. In EtOH-SN, HI remained steady up to week 17 then the level almost doubled to 19736 at week 24. EtOH-NSN increased during the first 5 weeks and then remained steady until the end of ripening.

Hydrophobic peptides (HO) were present only in ASN and EtOH-SN in the first week. HO in ASN increased until week six, and then remained steady up to the end of the ripening. HO in NPN and EtOH-NSN appeared at week 6 and both fractions reached their highest HO concentration at the end of the ripening. Meanwhile, HO in EtOH-SN remained steady all the time.

Table 2.1. Total area of HI, HO, and HO/HI ratio in nitrogenous fractions of Mexican Cotija cheese during ripening

Week	HYDROPHILIC (HI) 10-34 min of retention time				HYDROPHOBIC (HO) 35-100 min of retention time				HO/HI ratio			
W	NPN	ASN	EtOH-SN	EtOH-NSN	NPN	ASN	EtOH-SN	EtOH-NSN	NPN	ASN	EtOH-SN	EtOH-NSN
1	1349	1671	11662	7178	0	8194	24699	0	0	4.9	2.1	0
2	1160	3600	8787	8258	0	12923	4343	0	0	3.6	0.5	0
3	1230	8281	10520	8351	0	22886	4332	0	0	2.8	0.4	0
4	540	2493	8698	10198	0	8842	4561	0	0	3.5	0.5	0
5	241	2151	10695	11538	0	12792	5202	0	0	5.9	0.5	0
6	788	5851	10524	9815	1854	21264	22216	1927	2.4	3.6	2.1	0.2
7	555	4159	9997	9012	263	13948	8666	0	0.5	3.4	0.9	0.0
8	576	9108	10362	9403	1296	22716	15336	635	2.3	2.5	1.5	0.1
9	1551	17632	12229	9183	1235	20110	9200	1414	0.8	1.1	0.8	0.2
10	385	2658	8475	9310	0	12298	1652	0	0	4.6	0.2	0.0
11	1020	7051	9360	11322	2145	20370	21808	1966	2.1	2.9	2.3	0.2
12	ND	6339	11997	9425	ND	21139	19661	2140	ND	3.3	1.6	0.2
14	ND	29294	12661	9060	ND	24253	11305	1773	ND	0.8	0.9	0.2
17	3783	12502	11941	8504	5123	28358	13339	1786	1.4	2.3	1.1	0.2
20	4306	26206	29210	9624	3859	25055	57563	502	0.9	1.0	2.0	0.1
24	12698	38761	19736	12569	8682	14060	24137	4082	0.7	0.4	1.2	0.3

*ND= Non Determined.

**The amount of HI and HO peptides was expressed as units of chromatogram area.

The evolution of HO/HI ratio was different in each nitrogenous fraction (Table 2.1). For ASN HO/HI ratio oscillated until week 12 to later decrease until the end of the ripening. In NPN the HO/HI ratio increased after week 6, to later decrease after week 17. HO/HI ratio in EtOH-SN fluctuated during all ripening. Meanwhile, EtOH-NSN fraction had a very low proteolysis as it showed by a constant HO/HI ratio throughout all ripening time. The

variations observed in HO and HI peptides of both ASN and NPN fractions between week 4 and 10 could be attributed to dynamic mechanisms related with changes in the microbiota of Cotija cheese. No reference were found in the bibliography to compare our results, most of the HO/HI ratio analysis in cheeses are reported only in water soluble extracts, that are only comparable with the ASN fractions, because of the size of peptides content (McSweeney & Fox, 1997). However the observed decrease in ASN HO/HI ratio is similar to the results reported by Gonzalez de Llano *et al.*, (1995) in cheddar cheeses.

Determination of antioxidant activity

ASN and NPN fractions, which exhibited a content of medium and short-chain peptides, were tested for antioxidant activity (Figure 2.1A). Antioxidant activity showed statistically significant differences between nitrogenous fractions ($p < 0.05$) until week 23, at the end of the ripening (week 24) both fractions were similar.

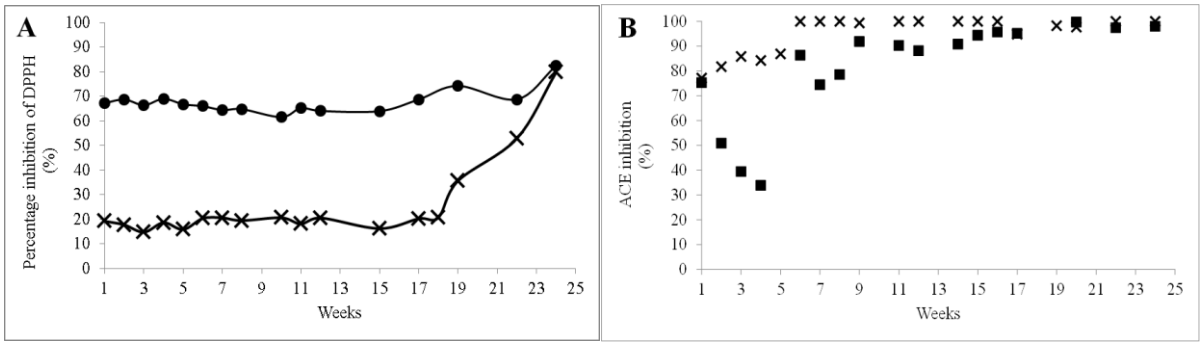

Figure 2.1. Biological activity evolution of nitrogenous fractions obtained during ripening of Mexican Cotija cheese. A) Antioxidant activity: ● NPN, X ASN; B) ACE inhibitory activity: X EtOH-SN, ■ EtOH-NSN

NPN showed higher antioxidant activity during all ripening time at a constant level (67.7 ± 4.9 % discolorations). While ASN remained steady (18.8 ± 1.6 % discoloration) during the first 18 weeks of ripening. Then, it increased significantly ($p < 0.05$) until week 24 (79.8% discoloration), reaching similar values to those of NPN (Figure 2.1A).

Our results indicated that Cotija cheese exhibited important antioxidant activity. Differences between ASN and NPN at the beginning of ripening can be explained by differences in the peptide content. At the beginning, NPN had shorter size peptides, which are

the holders of biological activity (Pihlanto, 2006) but as the ripening goes on, the bigger peptides in ASN go through higher proteolysis, releasing smaller peptides with antioxidant activity. Similar results were observed in cheddar cheeses by Gupta *et al.*, (2009) who observed that changes in the antioxidant activity where related to the rate of formation of soluble peptides.

Cotija cheese exhibited high antioxidant activity and ACE inhibitory activity from the beginning of ripening, in NPN, EtOH-SN and EtOH-NSN, however, it's at the end of the ripening that the highest values of activity in ASN fraction are reached. Still is after 12 weeks, that this cheese is commercialized and is after this point when its biological activity is most important and reaches its maximum.

As shown in Table 2.2, NPN antioxidant activity exhibited a higher correlation with HI (0.92) but also significant correlation with HO peptides (0.79). ASN antioxidant activity was positive correlated with the HI peptides (0.81) but there was no significant correlation with HO peptides.

Table 2.2. Correlation coefficients of nitrogenous fraction, peptide profile and biological activity of Mexican Cotija cheese during ripening.

		ACEI†		**Antioxidant activity**	
		EtOH-SN	**EtOH-NSN**	**NPN**	**ASN**
NPN	HI			0.92*	
	HO			0.79*	
ASN	HI				0.81*
	HO				-0.18
EtOH-NSN	HI		0.33		
	HO		0.60*		
EtOH-SN	HI	0.23			
	HO	0.28			

Bold values are significant at *p*-value< 0.05 (*)

†ACEI: Angiotensin converter enzyme inhibitory activity

In vitro determination of ACE inhibitory activity

ACE inhibitory activity of peptides was measured in the fractions with the lowest molecular weight peptides (EtOH-SN and EtOH-NSN) since these peptides are the holders of

this biological activity (Espejo-Carpio *et al.*, 2013; Meisel, 2004). Results were expressed as percentage of ACE inhibition compared to a blank sample (Figure 2.1B).

ACE inhibitory activity showed significant differences ($p < 0.05$) between fractions where EtOH-SN exhibited the highest activity during the first 16 weeks (Figure 2.1B). Peptides in EtOH-SN and ETOH-NSN are small and medium size peptides, respectively (McSweeney & Fox, 1997). ACE inhibitory are generally short chain peptides, which explains the differences observed between fractions (Meisel, 2004). In EtOH-SN ACE inhibitory activity increased steeply during the first 5 weeks of ripening and at week 6 reached the 100%. Whereas, in EtOH-NSN ACE inhibitory activity increased continuously after week 6, reaching a steady state after week 9 (94.1 ± 3.8). After 17 weeks ACE inhibitory activity in both fractions was similar, presumably because at this point most of the peptides in EtOH-SN and EtOH-NSN are short chain with similar activity.

ACE inhibitory activity correlations between fractions and peptide profile (HO, HI and HO/HI ratio) are shown in Table 2.2. In EtOH-NSN activity was highly correlated with HO and HO/HI ratio of this fraction (0.60 and 0.64, respectively). EtOH-SN activity did not show significant correlation with the peptides types in its fraction.

As expected, biological activities were highly correlated with the peptide profile. ACE inhibitory activity has been always related to the hydrophobicity of the peptides (Espejo-Carpio *et al.*, 2013; Meisel, 2004), which explains the significant correlation between HO peptides and activity in EtOH-NSN. However, the lack of correlation with EtOH-SN fraction couldn't be fully explained, and it could be only attributed to other characteristics of the peptides present like the electrostatic charge of the amino acids or the peptide conformation (Meisel, 2004).

On the other hand, antioxidant is also attributed to hydrophobic peptides, however in our results we found that this activity was mostly correlated with the HI peptide content, this could be explained by the effect that antioxidant activity can be attributed to the metal ion chelation properties of the amino acids in the peptide, or that despite the hydrophobicity or hydrophilic nature of the peptides, the antioxidant activity is furthermore attributed to chelation characteristics of the amino acids presents, or to the high amounts of cysteine in this

ASN and NPN fractions that promote the synthesis of peptides that of glutathione, a potent antioxidant. Furthermore, some authors support the hypothesis that antioxidant action is most likely attributed to the cooperative effects of the mechanisms mentioned (Erdmann *et al.*, 2008).

The increases of biological activities during ripening of Cotija cheese are in agreement with other authors (Gupta *et al.*, 2009; Pritchard *et al.*, 2010; Ryhänen *et al.*, 2001). Gupta *et al.*, (2009) reported that antioxidant activity and Pritchard *et al.*, (2010) reported that antioxidant and ACEI activities in the water soluble extract of cheddar cheeses depended of the stage of ripening. Ryhänen *et al.*, (2001) also showed that ACEI activity in an experimental low fat cheeses increased during ripening and after decreased after 4 months when proteolysis exceeded certain level due to catabolism of peptides.

Conclusions

Ripening of Mexican Cotija cheese released peptides with high antioxidant and ACE inhibitory activity which increased during ripening time. Both biological activities of the analyzed nitrogenous fractions were highly correlated with the type of peptides in the fractions. Despite the maximal antioxidant activity is reached at 24 weeks, the ACE inhibitory activity exerts its maximal levels after 13 weeks of ripening. Thus if we consider that Cotija cheese is ripened at least for 3 months (12 weeks) we can conclude that the commercialized product possess a significant content of bioactive peptides with potential health effect that makes of Cotija a functional cheese and making interesting future studies to evaluate the biological activity of peptides present in long-ripened Cotija cheese, as to evaluate other biological activities.

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2.1.3 Complementary results

During this study, we analyzed the main changes occurred during ripening of Cotija cheese to relate them with the changes in the biological activities and contribute to a deeper characterization of Cotija cheese.

2.1.3.1 Evolution of pH

The pH value of Cotija remained almost stable through all ripening time, falling slightly from pH 4.86 ± 0.48 to 4.80 ±0.06. This pH value was similar to Canestrato Pugliese cheese (pH 5.0 – 5.2) (Gobbetti, 2004) and Cheshire cheese (pH 5.0) (Lucey & Fox, 1993), but lower than Parmigiano Reggiano (pH 5.4-5.5) and Grana cheeses (pH 5.5-5.6) (Gobbetti, 2004). The average of pH for the evaluated Cotija cheese (pH 4.84 ± 0.48) was slightly lower to that reported by Flores-Magallón *et al.,* (2011) for artisanal (pH 5.0) and semi-industrial (pH 5.3) Cotija cheeses.

When compared to original pH of the milk (pH ~ 6.7), Cotija followed an important fall of pH during its manufacture and early ripening (pH 4.86). It is known that at the first stage of cheese manufacture, low pH is due to the production of lactic acid by the starter cultures (McSweeney, 2004). However, since Cotija cheese is produced without the addition of starters, the pH drop could be attributed to the action of the autochthonous microflora present in milk. Similarly, it is known that the increment of pH during cheese ripening could be due in part to the lactic acid metabolism by fungi and yeasts part of the secondary flora (Franco, Prieto, Urdiales, Fresno, & Carballo, 2001). However, for Cotija cheese´s ripening, yeast and fungi were not inoculated, therefore lactic acid metabolism could not be expected to occur and consequently the pH remains almost stable throughout ripening.

2.1.3.2 Moisture evolution

On the other hand, Cotija cheese had a steeply loss of moisture through ripening from 39.31 ± 3.70 % at the beginning to 25.27 ± 1.99 % after six months, with a rate of moisture loss of 2.81 % per month (Figure 2.2).

In a similar manner to Cotija cheese artisanal conditions, in our study, the moisture was not controlled by any means (coat, humidity control) during ripening. Under this conditions loss of moisture can be attributed to surface water evaporation. Average moisture

of Cotija cheese through ripening (33.33± 5.1 %) was between the observed in artisanal (30.0 %) and semi-industrial Cotija cheeses (33.7%) (Flores-Magallón *et al.*, 2011). Furthermore, this parameter was in accordance with the established by the Collective Trademark normativity which demand a maximum of 36 % of moisture (Hernández *et al.*, 2009b; Poméon, 2007). Moreover, moisture content of Cotija cheese was similar to the observed in Italian cheeses as Grana Padano (32 %) and Parmigiano Reggiano (30.8 %) (Gobbetti, 2004).

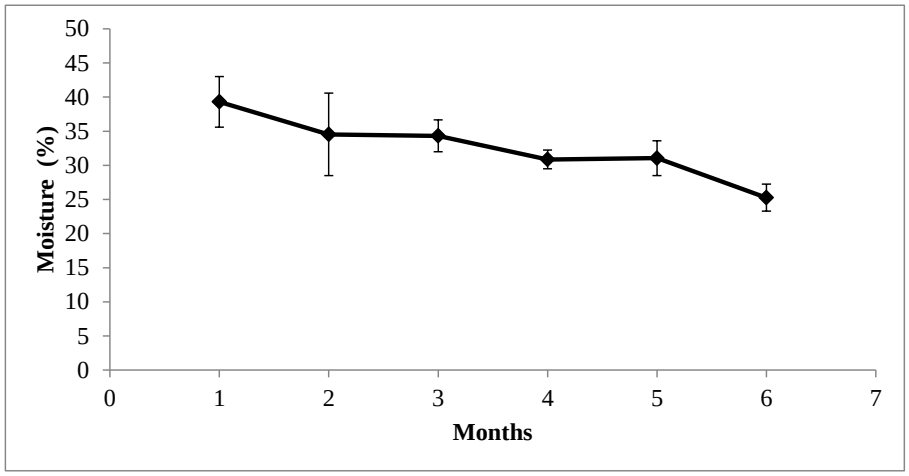

Figure 2.2. Moisture evolution during ripening of Cotija cheese.

2.1.3.3 Protein evolution

Meanwhile, protein content in Cotija (34.50 ± 2.55 %) remained stable throughout ripening. Our results were slight higher that the previously reported by Flores-Magallón *et al.*, (2011) who found values of 30.1 % and 24.6 % of protein content in artisanal and semi-industrial Cotija cheeses respectively. Differences observed in our study might be attributed to initial milk composition and differences in cheese manufacture processes, since Cotija cheese production has not been completely standardized. Hernández *et al.*, (2009a) found important differences in protein content (39.1 - 47.5%) between different Cotija cheeses manufacturers. This variability can also be observed in other type of cheeses, such as Mexican Añejo cheeses (Hernández-Morales, Hernández-Montes, Aguirre-Mandujano, & Villegas, 2010).

Compared with other cheeses, protein content in Cotija cheese was similar to some hard Italian varieties as Grana Padano (33 %) and Parmigiano Reggiano (33 %), and higher than Canestrato Pungliese (26.5 %) (Gobbetti, 2004), or English cheeses as Cheshire (24 %) or Cheddar (25.4 %) (Lucey & Fox, 1993).

2.1.3.4 Evolution of nitrogenous fractions during ripening

Proteolysis suffered by Cotija cheese during ripening was monitored by measuring the percentage of soluble nitrogen at pH 4.6 (ASN), 12 % TCA (NPN) and 70 % ethanol soluble (EtOH-SN) and total nitrogen (TN). The rate of each fraction over total nitrogen content was used as comparison (Figure 2.3).

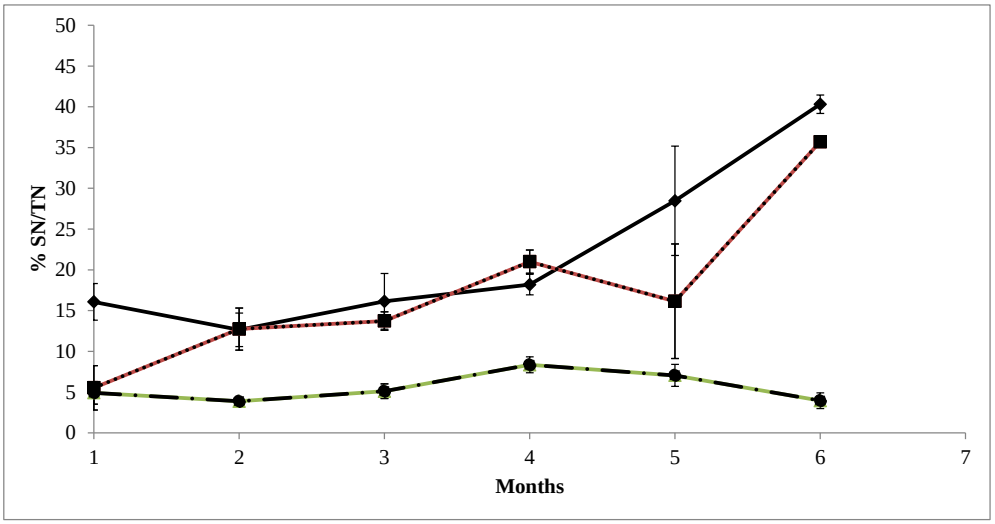

Figure 2.3. Evolution of nitrogenous fractions during ripening of Cotija cheese. Soluble nitrogen (%SN) at pH 4.6 (ASN) (♦,) 12 % TCA (NPN) (■) and 70 % ethanol soluble (EtOH-SN) (●) during ripening of Cotija cheese. Results are expressed in proportion to the total nitrogen.

ASN was the fraction correspondent to nitrogenous compounds soluble at pH 4.6. Because of the pH of Cotija cheese (4.81 ± 0.41), ASN fraction and water soluble extracts of cheese samples should not differ significantly as suggested by Gobbetti (2004).

ASN fraction contains proteins, large peptides, small peptides and free amino acids (McSweeney & Fox, 1997; Panizzolo *et al.*, 2011). During the first month, mean ASN values were 16.06 ± 2.25 %. After a slight decrease of 3.4 % at second month, ASN increased in a rate of 2.77 % per month until 18.19 ± 1.25 % at the fourth month. However, during the last two months the increase in ASN fraction was more evident, with a proteolysis rate of 11.06 % per month reaching a final value of 40.31 ± 1.13 %.

The EtOH-SN fraction contained the smallest and hydrophilic peptides obtained from the ASN fraction. The value of EtOH-SN fraction during the first months was 4.92 ± 1.39 %.

Values remained stable with a slight increase at the fourth month, 8.35 ± 0.98 %. At the end of ripening values decreased to 3.95 ± 0.97 %.

The NPN fraction regularly contains small peptides (1 to 7 amino acids) and amino acids soluble at 12 % of TCA (McSweeney & Fox, 1997; Panizzolo *et al.*, 2011). Initial percentage for this fraction was 5.52 ± 2.72 %, increasing to 12.73 ± 2.60 % for the second month. Subsequently, NPN fraction remained stable at this value for the third month to finally rise during the fourth month to a percentage of 21.00 ± 1.40 %. Towards the end of the ripening period a decline in the fifth month was observed (16.14 ± 7.03 %), to once again be increased to a value of 35.71 ± 0.59 % in the sixth month.

Increase of both ASN and NPN were consistent with the increase of proteolysis during ripening of Cotija cheese. However, when compared with other cheeses, values were higher than the reported by Gobbetti (2004) and Mayer, Rockenbauer, and Mlcak (1998) in 22 months Parmesan (30 % for ASN and 27 % for NPN); and lower than the reported by Bertola, Califano, Bevilacqua, and Zaritzky (2000) and Choi, Yang, Choi, and Bae (2015) for 2-3 months Gouda cheeses (40.58 and 35 % respectively).

Initial values of ASN, 16.06 ± 2.25 %, were higher that the observed in fresh cheeses as "Panela", 9.17 % after 15 day of storage (Guerra-Martínez *et al.*, 2012). Furthermore ASN values during the first month were in accordance with the 15 % observed in two months Parmesan cheeses (Mayer *et al.*, 1998). Thus suggesting, that from the beginning Cotija cheese, had already suffered an extensive proteolysis during the cheese manufacture and early ripening. These results could be correlated with the low pH values observed at the beginning of our study.

Increase in ASN could be attributed to the proteolysis, probably by rennet action (McSweeney, 2004), released mostly proteins and large peptides that were later hydrolyzed producing small peptides and amino acids causing the increase in NPN fraction. Furthermore, the observed decrease in EtOH-SN after the fourth month of ripening could due to a further metabolism of the small peptides and amino acids that implied the consumption of these compounds (McSweeney, 2004).

Additionally, ASN, NPN and EtOH-SN evolutions suggests that Cotija suffers a relative low rate of proteolysis during the first 4 months of ripening. However after four months, a phenomenon occurs that increases dramatically the proteolysis rate, indicated by the substantial increase in ASN and NPN (between the fifth and sixth month) and the further degradation of small peptides. This behavior could be associated to variations in the microbiota of Cotija cheese occurred during ripening as previously observed by Chombo-Morales *et al.*, (2016).

2.1.3.5 Nitrogenous fractions analysis by RP-HPLC

To follow the evolution of peptides in each fraction throughout ripening, nitrogenous fractions ASN, NPN and EtOH-SN and EtOH-NSN (the non-soluble fraction in 70 % ethanol) were further fractionated by RP-HPLC. The resulting chromatograms showed that the area of peaks increased with increasing ripening time regardless of the nitrogenous fraction. Likewise, chromatograms showed a very complex peptide profile with a wide range of polarity.

According to Gonzalez de Llano *et al.*, (1995), the polarity of the peptides could be established in the chromatograms following its retention time. The eluted peaks between 10 and 35 min could be considered as hydrophilic peptides, whereas peptides eluted between 36 and 100 min could be considered as hydrophobic peptides, while peaks eluted before 10 min could be assumed as free amino acids and not further taken into account in the peptide profile determination. Evolution of peptides in each nitrogenous fraction is shown in figure 2.4 (A-D), results are expressed as units of area under de peak. Regardless of the nitrogenous fraction the proportion of HI and HO peptides varied with respect to time of ripening.

For NPN, HI peptides were slightly variable during the first four months. Initial content was 1070.0 ± 361.6 and decreased to 832.9 ± 242.8 at the fourth month. Meanwhile HO peptides appeared after the second month, 1050.0 ± 951.3, and increased slightly till the fourth month to 1402.0 ± 8.1. During fifth month both HI and HO peptides increased to 4044.3 ± 369.8 and 6358 ± 1746.4 respectively. This increase continued until the end of ripening time reaching final values of 12230.9 ± 660.8 and 7654.3 ± 1453.0 for HI and HO peptides respectively.

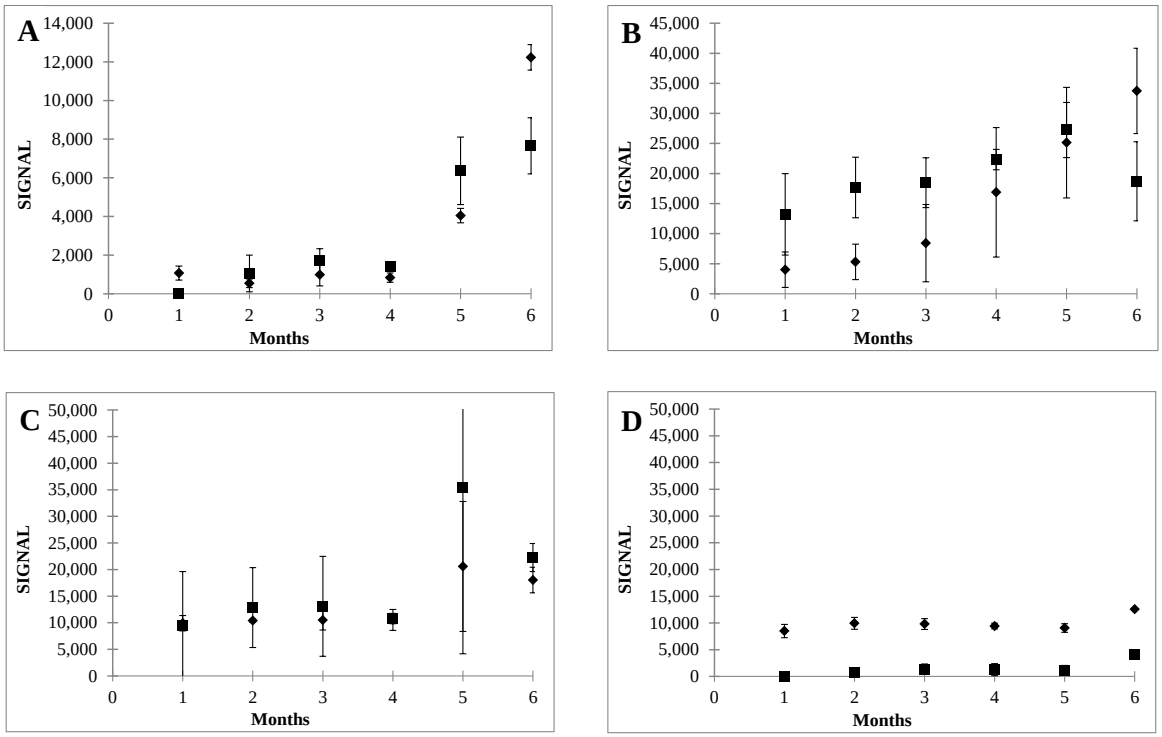

Figure 2.4. Evolution of (♦) HI and (■) HO peptides in A) NPN, B) ASN C) EtOH-SN and D) EtOH-NSN during ripening of Cotija. *Results are expressed as units of area under de peak.

On the other hand, ASN was the fraction with the highest amount and variety of peptides . HI peptides increased linearly from 4011.2 ± 2954.1 until 333748.5 ± 7088.6 at the end of ripening in a rate of 5947.5 per month. HO peptides, increased constantly from initial 13211.6 ± 6781 to 27236 ± 4572.7 at the fifht month in a rate of 3506.1 per month, to later decreased during the sixth month till 18711.1 ± 6577.7. The constant increase in HI and HO througout ripening is consistent with the increase in nitrogenous content of the same fractions.

Meanwhile, in the EtOH-SN fraction, HI peptides initial content was 9916.6 ± 1434.7. Values increased very sligthly throuhoutout fourth months untill 10534.8 ± 1951. However, at the end of ripening the increase give almost twice the inicitial value (18036.5 ± 2227.1). During the first month (four weeks) HO peptides, variated considerably and content was 9438.6 ± 10143.8 and they increased slightly untill 13080.4 ± 9401.0 at the third month, to later decrease to 10789.1 ± 971.5 at the fourth month. To fifth month, HO peptides increased again in a rate of 5741.2 per month, reaching final values of 22271.6 ± 2637.7 at the end of ripening time.

In EtOH-NSN, HI peptides were slightly variable througout five months (9349.2 ± 587.1), to later increased to 12569.0 at the end of ripening. Similar behaviour was observed in HO peptides. They apperead until the second month and were slightly variable until the fifth month (1112.1 ± 329). At the end of ripening HO peptides increased almost four times its initial content reaching 4082. Contrary to the observed in the other fractions, in EtOH-NSN peptides were mostly HI.

Generally, in ASN and NPN fractions HO peptides were present in higher amounts that HI peptides. However during the last month, HI peptides increased in higher amounts than the HO. This could be due to that despite both type of peptides increased throughout ripening, at late stages, HO peptides were consumed in higher amounts that HI. This behaviour has been attributed by other authors to the degradation of HO peptides and the formation of HI peptides, altogether with the loss of solubility of the HO peptides (Vivar-Quintana *et al.*, 2009). Meanwhile, the higher amounts of HO observed in late stages of ripening in EtOH-SN fraction could be further attributed to a higher consumption of HI peptides that were probaly introduced in another metabolic pathways (McSweeney, 2004). Furthermore, the evolution of HI and HO peptides in all fractions were consistent with the evolution of nitrogenous content of the same fraction, where a major change in the proteolysis rate ocurred after four months. Thus suggesting that proteolysi in Cotija was relatively slow at the beginning, but increased considarable at late stages of ripening.

Several authors have suggested that the expression of HO peptides in relation to HI peptides in the water soluble extracts could be a validated way to study the proteolysis throughout ripening. Ratio of HO/HI in Cheddar, Manchego-type, Spanish hard cheeses and Semi-hard ovine cheeses is influenced by cheese age (Agboola, Chen, & Zhao, 2004; Gonzalez de Llano *et al.*, 1995; Pavia, Trujillo, Guamis, & Ferragut, 2000; Ruas-Madiedo, Bada-Gancedo, Delgado, Gueimonde, & De los Reyes-Gavilán, 2003). For Cotija cheese, HO/HI ratio of the ASN fraction was used to measure proteolysis, because pH of the cheese was close to 4.6 (4.81 ± 0.41) making ASN equivalent to water soluble extracts (Gobbetti, 2004). Results are shown in table 2.3.

HO/HI ratio remained stable during the first two months (3.30 ± 0.02), to later decreased sharply until the end of ripening (0.55). This decrease in petide ratio has been

previously observed in Cheedar, Manchego-type, and Spanish-hard cheeses (Gonzalez de Llano *et al.*, 1995; Pavia *et al.*, 2000; Ruas-Madiedo *et al.*, 2003). Authors have suggested that this decrease is due to hydrolysis of HO peptides with the further release of HI peptides. Meanwhile Vivar-Quintana *et al.*, (2009) have previously reportet that HO peptides are basically stable troughout ripening of goat, cow, and sheep cheeses, Whereas Katsiari, Alichanidis, Voutsinas, and Roussis (2000) observed that HO peptides decreased throughout ripening of Feta cheese.

Table 2.3. Evolution of hydrophobic: hydrophilic ratio (HO/HI) in ASN fraction during ripening of Cotija.

ASN PEPTIDE FRACTION					
TIME	**HI PEPTIDES**		**HO PEPTIDES**		**HO/HI ratio**
MONTH	AVERAGE	STD	AVERAGE	STD	
1	4011.18	2954.07	13211.63	6780.98	3.29
2	5317.30	2945.17	17679.90	5034.44	3.33
3	8420.15	6436.16	18479.18	4143.73	2.19
4	16878.63	10768.21	22322.47	1680.40	1.32
5	25138.48	9185.87	27235.95	4572.65	1.08
6	33748.50	7088.60	18711.10	6577.65	0.55

*HO/HI = hydrophobic: hydrophilic ratio.

**The amount of HI and HO peptides was expressed as units of area under de peak.

However, the results in Cotija cheese suggest that HO and HI peptides increased paralelly through ripening, and that the consumption of HO was given at late stages of ripening. Differences observed between cheeses could be due to differences in manufacture process. In this regard Hayaloglu, Ozer, and Fox (2008) reported that changes in the microbiota produced further variotions in the peptide profile of the pH 4.6 soluble fraction (ASN) of brine Turkish cheeses.

2.1.3.6 Antioxidant activity

The nitrogenous fractions of Cotija cheese were further analyzed to test its biological activity attributed to its peptide content. ASN and NPN fraction were selected to measure antioxidant activity by DPPH method (Figure 2.5).

Antioxidant activity in NPN fraction was slightly variable throughout ripening. Activity during the first month mean values were 67.86 ± 1.24 % and at the end of ripening it increased to 75.66 ± 9.70 %. On the other hand, in ASN, antioxidant activity was significant lower that the observed in NPN ($p<0.05$). Initial value was 17.59 ± 1.98 % and it remained constant (19.74 ± 2.00 %) until the fourth month, but at the fifith mont it increased slightly to 24.89 ± 7.26 %; and at the end of ripening antioxidant activity increased dramatically to 66.35 ± 19.05 %, reaching similar values as the observed in NPN. Higher antixidant activity in NPN fraction than in ASN could be attributet to the type of peptides content in each fractions. It is possible that smalller peptides in NPN had higher activity that bigger peptides in ASN (Pihlanto, 2006; Pritchard *et al.*, 2010).

However, increase in the value in ASN fraction activity at the end of ripening could be due to a continuos hydrolysis of the bigger peptides releasing smaller peptides. Increase of antioxidant activity throught ripening of Cheddar and Caciocavallo cheeses has been previously reported by Gupta *et al.*, (2009) and Perna *et al,*. (2015). Gupta *et al,*. (2009) suggested that this increase was related to the formation of soluble peptides, but only until certain level because peptides with antioxidant activity can not resist further proteolysis.

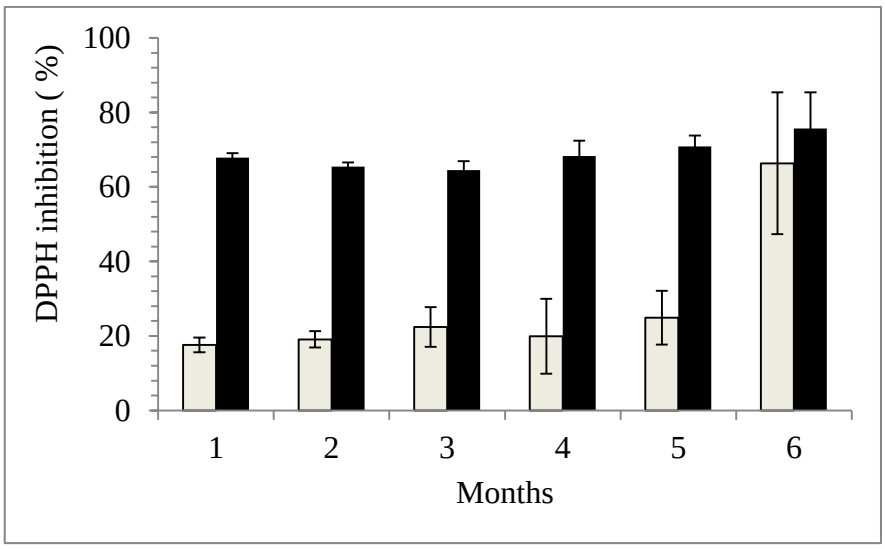

Figure 2.5. Antioxidant activity in ■ ASN and ■ NPN fractions during ripening of Cotija cheese.

On the other hand, increase in antioxidant activity in both ASN and NPN at the end of ripening were consistent with the increase in nitrogenous content observed in figure 2.3. This

could be partially comparable with the previous results of (Pritchard *et al.*, 2010). In their work, authors found that antioxidant activiy of water extracts of commercial Cheddar cheeses increased with higher concentration of peptide content. However according to our results, increase in antioxidant activity could be related not only to the concentration of peptides but to the type of peptides. As observed in table 2.2, antioxidant activity in ASN was highly correlated to the HI peptides (0.81) that, as observed in figure 2.4, increased throughout all ripening time, reaching its highest at the end of ripening at the same time as highest activity was observed. Meanwhile antioxidant actitity in NPN was highly correlated with both HI and HO peptides (0.92 and 0.79 respectively).

2.1.3.7 ACE inhibitory activity

The method for ACE inhibitory activity quantification (Wang *et al.*, 2013) is based on the production of hippuric acid (HA) from hippuryl-histidyl-leucine (HHL) catalyzed by the ACE. Thus decrease in HA production was related with inhibitory activity of ACE. Samples were compared with the HA produced by a blank without ACE inhibitors.

Considering the size distribution of peptides among different nitrogen fractions, the ETOH-SN and ETOH-NSN fractions contain small and medium size peptides which could interact with the active sites of ACE and thereby inhibit its activity on the angiotensin system (Espejo-Carpio *et al.*, 2013; Meisel, 2004). Therefore, ETOH-SN and ETOH-NSN fractions were chosen for testing the ACE inhibitory activity. Results are shown in figure 2.6.

During the first month ACE inhibitory activity in EtOH-NSN was 49.85 ± 18.36 % (Figure 2.6). After the first month activity in EtOH-NSN increased steply. From the first to the third month the increase was in a rate of 20.09 % per month. From the third to the fifht month the increased rate descended dramatically to 3.69 %, reaching final values of 97.41 ± 3.27 % that remainend constant until the end of ripening (97.68 ± 0.38 %).

Meanwhile in EtOH-SN initial values were 82.20 ± 3.79 %. At the second month activity increased to 96.71 ± 6.58 %. During the fourth month ACE inhibitory activity reached 100 % and remained stady until the end of ripening (Figure 2.6).

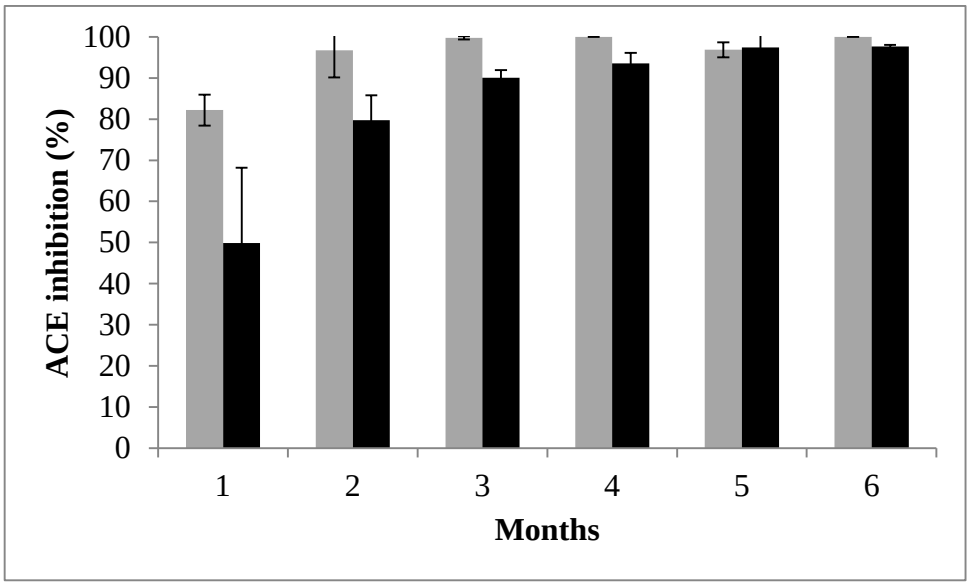

Figure 2.6. ACE inhibitory activity evolution during ripening of Cotija. ■EtOH-SN, ■ EtOH-NSN.

In general, both EtOH-SN and EtOH-NSN exhibited high ACE inhibitory activtiy, from very early stages of ripening. However EtOH-SN fraction was significantly ($p < 0.05$) more active than EtOH-NSN throughout ripening (Figure 2.6 and 2.7). This was probably due to the peptide content since EtOH-SN contained smaller peptides, wich, in accord with the litterature, are the holders of this activity (Espejo-Carpio *et al.*, 2013). However, one unexpected result was observed for the EtOH-NSN fraction wich also exhibited ACE inhibitory activity, suggesting that bigger peptides could also exert important inhibitory activity over ACE.

As observed in table 2.2, ACE inhibitory activity was highly correlated with HO peptides in EtOH-NSN fraction (0.60). However in EtOH-SN activity was not significantly correlated either with HI or HO peptides (0.23 and 0.28, respectively). ACE inhibitory activity has been always related to the hydrophobicity of the peptides (Espejo-Carpio *et al.*, 2013; Meisel, 2004), which explains the significant correlation between HO peptides and activity in EtOH-NSN (table 2.2). However, the lack of correlation with EtOH-SN fraction could not be explained, and it could be only attributed to other characteristics of the peptides present like the electrostatic charge of the amino acids or the peptide conformation (Meisel, 2004). However further studies need to be carried out to understand the precise relationship between the type of peptides in a determined nitrogenous fractions and its biological activity.

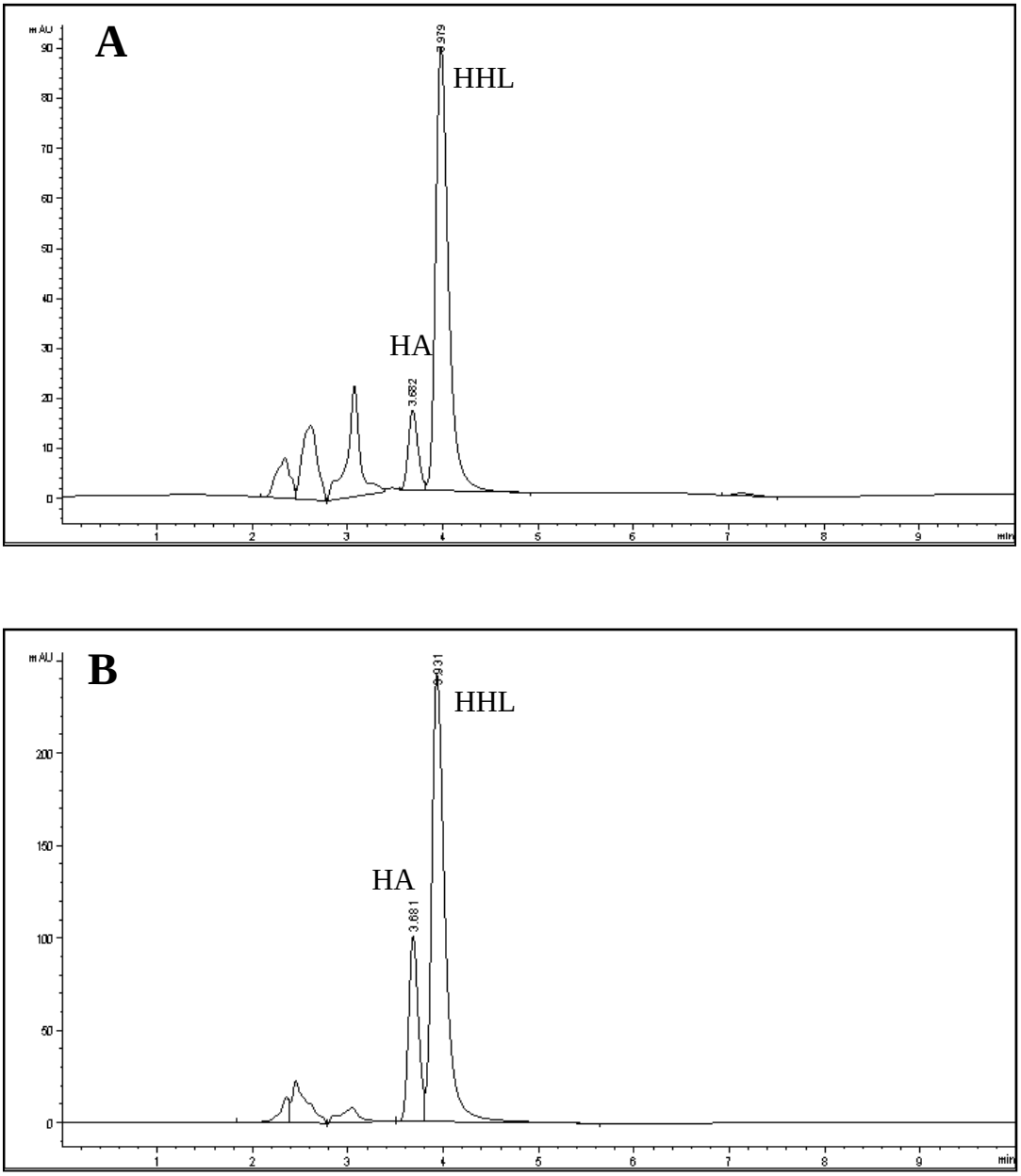

Figure 2.7. ACE inhibitory activity by RP-HPLC during the first month. A) EtOH-SN, B) EtOH-NSN. HA: Hippuric acid, HHL: Hippuryl-histidyl-leucine.

ACE inhibitory activity incresead during ripening (in both EtOH-SN and EtOH-NSN). Our results are partially in agreement with previous works of Gupta *et al.*, (2013), Lignitto *et al.*, (2010), and (2010) in water extracts of Asiago d´allevo and Cheddar cheeses. Authors observed that ACE inhibitory activity increased as ripening avanced, but only until some extent, to later decrease. However, inhibitory activity in Cotija cheese increased through all ripening time. Differences with our resulst might be due to manufacture differences, and to the time of ripening. While Cotija cheese was ripenend for 6 months, Asiago d´allevo cheese was ripened 18 months.

References

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., . . . Amaya-Llano, S. L. (2013). Influence of probiotic strains added to Cottage Cheese on potentially-antioxidant peptides generation, anti-Listerial activity, and survival of probiotic microorganisms in simulated astrointestinal conditions. *International Dairy Journal, 33*(2), 191–197. doi:10.1016/j.idairyj.2013.04.005

Agboola, S., Chen, S., & Zhao, J. (2004). Formation of bitter peptides during ripening of ovine milk cheese made with dierent coagulants. *Le Lait, 84*(6), 567-578. doi:10.1051/lait:2004032

Bertola, N. C., Califano, A. N., Bevilacqua, A. E., & Zaritzky, N. E. (2000). Effects of ripening conditions on the texture of Gouda cheese. *International Journal of Food Science and Technology, 35*, 207-214. doi:10.1046/j.1365-2621.2000.00347.x

Choi, H., Yang, C., Choi, K., & Bae, I. (2015). Characteristics of Gouda cheese supplemented with fruit liquors. *Journal of Animal Science and Technology, 57*(15). doi:10.5851/kosfa.2011.31.6.872

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12. doi:10.1111/j.1471-0307.2011.00725.x

Chombo-Morales, P., Kirchmayr, M., Gschaedler, A., Lugo-Cervantes, E., & Villanueva-Rodríguez, S. (2016). Effects of controlling ripening conditions on the dynamics of the native microbial population of Mexican artisanal Cotija cheese assessed by PCR-DGGE. *LWT - Food Science and Technology, 65*, 1153-1161. doi:10.1016/j.lwt.2015.09.044

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654. doi:10.1016/j.jnutbio.2007.11.010

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183. doi:10.1016/j.idairyj.2013.04.002

Flores-Magallón, R., Oliva-Hernández, A. A., & Narváez-Zapata, J. A., . (2011). Characterization of Microbial Traits Involved with the Elaboration of the Cotija

Cheese. *Food Science and Biotechnology, 20*(4), 997-1003. doi:10.1007/s10068-011-0137-z

Franco, I., Prieto, B., Urdiales, R., Fresno, J. M., & Carballo, J. (2001). Study of the biochemical changes during ripening of Ahumado de Áliva cheese: a Spanish traditional variety. *Food Chemistry, 74*, 463-469. doi:10.1016/S0308-8146(01)00164-9

Gobbetti, M. (2004). Extra-hard varieties. *Cheese: Chemistry, Physics and Microbiology, 2*, 51-70. doi:10.1016/S1874-558X(04)80039-3

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., & Di Cagno, R. (2002). Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Critical Reviews in Food Science and Nutrition, 42*(3), 223-239. doi:10.1080/10408690290825538

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gonzalez de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of Proteolysis in Liquid Chromatography Artisanal Cheeses: High Performance of Peptides. *Journal of dairy science, 78*, 1018-1024. doi:10.3168/jds.S0022-0302(95)76717-0

Guerra-Martínez, J. A., Montejano, J. G., & Martín-del-Campo, S. T. (2012). Evaluation of proteolytic and physicochemical changes during storage of fresh Panela cheese from Queretaro, Mexico and its impact in texture. *CyTA - Journal of Food, 10*(4), 296-305. doi:10.1080/19476337.2011.653791

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Hayaloglu, A. A., Ozer, B. H., & Fox, P. F. (2008). Cheeses of Turkey: 2. Varieties ripened under brine. *Dairy Science and Technology, 88*, 225-244. doi:10.1051/dst:2007014

Hernández-Morales, C., Hernández-Montes, A., Aguirre-Mandujano, E., & Villegas, A. (2010). Physicochemical, microbiological, textural and sensory characterisation of Mexican Añejo cheese. *International Journal of Dairy Technology, 63*(4), 552-560. doi:10.1111/j.1471-0307.2010.00615.x

Hernández, V., Navarro, A., & Quirasco, M. (2009a). Antioxidant capacity in the Cotija "Región de Origen" Cheese, the case of an artisanal cheese. *Carnilac Industrial, 24*(1), 24-31.

Hernández, V., Quirasco, M., & Quintero, B. (2009b). Un acercamiento al mundo del queso Cotija Región de Origen MC: arte y tradición de México. *Culinaria, Revista Virtual Gastronómica 5,* 5-19.

Hnosko, J., Clark, S., & Van Hekken, D. (2009). Latin American Cheeses. In S. Clark, Costello, M., Drake, M., Bodyfelt, F. (Ed.), *The Sensory Evaluation of Dairy Products* (pp. 489-504). Lincoln, New England: Springler

Katsiari, M. C., Alichanidis, E., Voutsinas, L. P., & Roussis, I. G. (2000). Proteolysis in reduced sodium Feta cheese made by partial substitution of NaCl by KCl. *International Dairy Journal, 10*, 635 - 646. doi:10.1016/S0958-6946(00)00097-2

Lignitto, L., Cavatorta, V., Balzan, S., G., G., Galaverna, G., Novelli, E., . . . Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal, 20*, 11-17. doi:10.1016/j.idairyj.2009.07.001

Lucey, J. A., & Fox, P. F. (1993). Importance of Calcium and Phosphate in Cheese Manufacture: A Review. *Journal of dairy science, 76*(6), 1714-1724. doi:10.3168/jds.S0022-0302(93)77504-9

Mayer, H. K., Rockenbauer, C., & Mlcak, H. (1998). Evaluation of proteolysis in Parmesan cheese using electrophoresis and HPLC. *Dairy Science and Technology, 78*(4), 425-438. doi:10.1051/lait:1998441

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127-144. doi:10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H., & Fox, P. F. (1997). Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait, 77*, 41-76.

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Panizzolo, L. A., Arujo, A. C., Taroco, L. V., Rodriguez, A., & Schopf, G. (2011). Evolución de la proteólisis durante la maduración de quesos Danbo elaborados con distintos

cultivos iniciadores. *REVISTA DEL LABORATORIO TECNOLÓGICO DEL URUGUAY, 6*, 24-27.

Pavia, M., Trujillo, A. J., Guamis, B., & Ferragut, V. (2000). Ripening control of salt-reduced Manchego-type cheeseobtained by brine vacuum-mpregnation. *Food Chemistry, 70*, 155-162. doi:10.1016/S0308-8146(99)00249-6

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2015). Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening. *Journal of dairy science, 98*, 3690-3694. doi:10.3168/jds.2014-9097

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Poméon, T. (2007). *El queso Cotija, México. Un producto con marca colectiva queso "Cotija Región de origen", en proceso de adquisición de una Denominación de Origen*. Retrieved from

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548. doi:10.1016/j.foodres.2010.03.007

Ruas-Madiedo, P., Bada-Gancedo, J. C., Delgado, T., Gueimonde, M., & De los Reyes-Gavilán, C. G. (2003). Proteolysis in rennet-coagulated Spanish hard cheeses made from milk preserved by refrigeration and addition of carbon dioxide. *Journal of Dairy Research, 79*, 115-122. doi:10.1017/S0022029902005952

Ryhänen, E.-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal, 11*, 441-447. doi:10.1016/S0958-6946(01)00079-6

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 357–366. doi:10.1079/BJN20041189

Vivar-Quintana, A. M., Blanco López, M. A., Revilla, I., González-Martín, I., Hernández-Hierro, J. M., & González-Pérez, C. (2009). Seasonal Evolution of Hydrophilic and Hydrophobic Peptide Contents in Cheeses Made from Ewe's Goat's or Cow's Milk. *Czech Journal of Food Science, 27*(special issue), 5106-5108.

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910.

2.2: Antioxidant and angiotensin converting enzyme inhibitory activity in fresh goat cheese prepared without starter culture. A preliminary study.

2.2.1 Presentation of the second article published in the CyTA Journal of Food

It is known that cheese contains numerous bioactive peptides released by a proteolysis process especially during a ripening period (Gupta *et al.*, 2013; Pritchard *et al.*, 2010). However as we previously observed in section 2.1, some of those peptides are present at very early stages of ripening. This observation raised the question if fresh unripened cheeses could also exert biological activities throughout the bioactive peptides released during cheese manufacture.

From the total cheese production around 70 % comes from artisanal production mainly from raw milk. However recent modifications in the Mexican law demands that all the milk used for cheese-making must be pasteurized. This situation is resulting in the loss of some of the Mexican cheeses made regionally with raw milk (González-Córdova *et al.*, 2016). From this exposed situation surges the interest to observe the effect of pasteurization on the biological activities of these cheeses.

The few works done about fresh cheeses (Abadía-García *et al.*, 2013; Torres-Llanez *et al.*, 2011) attributed the presence of bioactive peptides to the enzymatic action of the starter cultures or probiotic bacteria addition. However there exists a wide range of cheeses made only with the milk autochthonous microbiota as in the present study.

Some questions raised from these observations: does fresh goat cheese made without starter cultures would release peptides with activities as antioxidant and ACE inhibitors Is there an effect of milk pasteurization on these antioxidant and ACE inhibitors activities.

To answer these questions we worked together with the Universidad Veracruzana that manufactured the fresh goat cheeses imitating the artisanal conditions used by the regional producers. Cheeses were made with raw and pasteurized milk from three different milking periods of the year.

Nitrogenous fractions: acid soluble nitrogen (ASN), non protein nitrogen (NPN) and ethanol soluble nitrogen (EtOH-SN) were used to measure the proteolysis and the antioxidant and ACE inhibitory activities. These biological activities were further correlated to the type of peptides present in each fraction. Cheeses manufactured with raw and pasteurized milk were compared to observe the impact of heat treatment on the antioxidant and ACE inhibitory activities.

The obtained results are specially interesting, when we consider that almost 80 % of the cheeses consumed in Mexico are fresh (González-Córdova *et al.*, 2016). Thus, the identification of antioxidant and ACEI activities in this type of cheeses could provide them with an added value because of its health benefits.

Highlights

- We probed that nitrogenous fractions of fresh goat chesses made without starter cultures had biological activity as antioxidant and ACE inhibitory.
- We observed that pasteurization affects slightly the ACE inhibitory activity, but not the antioxidant activity.
- We observed that pasteurization affects the type of peptides present in ASN fraction
- We correlated the biological activity with the type of peptides in each nitrogenous fraction.

2.2.2 Article: Antioxidant and angiotensin converting enzyme inhibitory activity in fresh goat cheese prepared without starter culture. A preliminary study

Leticia Hernández-Galán[a,b,c], Anaberta Cardador-Martínez[a], Micloth López-del-Castillo[d], Daniel Picque[c], Henry Eric Spinnler[b], Sandra T. Martín del Campo[a].

[a] Escuela Nacional de Ingeniería y Ciencias, Tecnológico de Monterrey. Epigmenio González 500, Fracc. San Pablo. CP 76130, Santiago de Querétaro, Qro. México.
[b] AgroParisTech, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Génie & Microbiologie des Procédés Alimentaires, F-78850 Thiverval Grignon, France.
[c] INRA, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Génie & Microbiologie des Procédés Alimentaires, F-78850 Thiverval Grignon, France.
[d] Universidad Veracruzana, Instituto de Ciencias Básicas, Av. Dr. Luis Castelazo Ayala S/N, Carr. Xalapa-Las Trancas km 3.5, Col. Industrial Animas, C.P. 91192, Xalapa, Veracruz, México.

Correspondence: Sandra Teresita Martín del Campo, Tecnológico de Monterrey, México. E-mail: smartinde@itesm.mx

Abstract

Proteolysis is specific for each type of cheese and might be affected by heat treatment. Studies have shown the ability of cheeses to produce bioactive peptides with antioxidant or anti-hypertension bioactivities, which have been related to ripening time or activity of starter cultures, however little is known about their production in fresh cheeses. Our objective was to evaluate antioxidant and angiotensin converting enzyme inhibitory (ACEI) activities in fresh goat cheeses elaborated without starters using pasteurized and raw milk from different seasons. Proteolysis was measured by acid-soluble nitrogen, non-protein nitrogen and ethanol-soluble nitrogen indexes, using the Kjeldahl method, and reverse phase high performance liquid chromatography (RP-HPLC). Each fraction's antioxidant and ACEI activities were measured. Analysed cheeses showed high biological activities and slight differences between them were associated with heat treatment. Our results suggested that fresh goat cheese had important biological activity due to peptides present originally in the milk or released by rennet action during cheese manufacture.

Keywords: Fresh goat cheese, bioactive peptides, antioxidant activity, ACE inhibitory activity

Introduction

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and could ultimately influence health (Kitts & Weiler, 2003). Their functionality is due to its amino acid composition and sequence, which generally consists of 3 to 20 amino acid residues per molecule (Hajirostamloo, 2010). Depending on the amino acids sequence, these peptides can exhibit one or diverse activities, including immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, and antihypertensive activity (Korhonen & Pihlanto, 2006).

Cheese contains numerous bioactive peptides released during proteolysis occurred in fermentation, ripening or even during digestion (Gupta *et al.*, 2009; Gupta *et al.*, 2013; Hernández-Ledesma *et al.*, 2011; Theolier *et al.*, 2014).

The proteolysis process is different depending on the type of cheese due to differences in manufacturing practices, ripening time, etc., resulting in a unique peptide profile

characteristic of each variety of cheese. However, in general, proteolysis can be summarized as follows: Initial hydrolysis of caseins catalysed primarily by residual coagulant, and to a lesser extent by plasmin and perhaps cathepsin D and other somatic cell proteinases, resulting in the formation of large and intermediate-sized peptides which are subsequently degraded by the coagulant and enzymes from the starter and nonstarter flora of the cheese. The secondary proteolysis (in ripened cheeses) generates small peptides and free amino acids resulting from the action of bacterial proteinases and peptidases (Fox & McSweeney, 1996). Some of this peptides exhibit biological activities as antioxidants (Abadía-García *et al.*, 2013; Ahmed, El-Bassiony, Elmalt, & Ibrahim, 2015; Timón *et al.*, 2014), and anti-hypertensive (Gupta *et al.*, 2013; Ong *et al.*, 2007; Torres-Llanez *et al.*, 2011) between others.

Antioxidant peptides derived from milk are composed of 5 to11 amino acids including in the sequence hydrophobic amino acids, proline, histidine, tyrosine or tryptophan. Antioxidant activity of these peptides seems to be inherent to protease specificity (Pihlanto, 2006).

On the other hand, anti-hypertensive activity of peptides is given through the inhibition of angiotensin converting enzyme (ACE), preventing the conversion of angiotensin I to angiotensin II, which induces the release of aldosterone, increasing sodium concentration and blood pressure; and inactivating the vasodilator bradykinin protecting the body against cardiovascular disease (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Jäkälä & Vapaatalo, 2010). ACE inhibitory peptides are generally short chain peptides, often carrying polar amino acid residues like proline (Espejo-Carpio *et al.*, 2013; Meisel, 2004).

Bioactivity of peptides might be susceptible to structural and chemical modifications of proteins during food processing like heat treatment (Hayaloglu & Brechany, 2007; Korhonen & Pihlanto, 2003) and by geoclimatic conditions such as environmental temperature and wheatear (Lacroix, Verret, & Paquin, 1996; Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1984).

In recent decades, the interest of food as agents for prevention or cure in chronic diseases had increased. Although many studies have been conducted for the determination of peptides biological activity generated during cheeses ripening, little work has been done about

the production of theses peptides in fresh cheeses. Since fresh cheeses represent a high market especially in the Latin American countries, identifying antioxidant and ACE inhibitory (ACEI) activity in these types of cheeses could provide a potential additional value for consumers because of its health benefits. Consequently, the current study aimed to evaluate the production of bioactive peptides in experimental fresh goat cheeses prepared without starters, evaluating at the same time, the impact of milk pasteurization.

Materials and Methods

Cheese manufacture

Goat milk was provided by artisanal producers from the region of Coatepec, Veracruz, Mexico (at 1200 meters above sea level). From a herd of 28 goats, 10 healthy animals were selected according to their lactation period. From the whole morning milking batch (without colostrum), 4 l samples were taken and transported immediately in order to make cheeses. Sampling was carried out in three different periods of the year, November, January and March (N, J, and M, respectively), 2012-2013. Thus, a total of 12 l of milk were used to cheese manufacture. The selected period of sampling had medium values for higher temperatures (~22 °C to 27 °C), lower temperatures (~9 °C to 12 °C), and total precipitation (~55 mm to 90 mm) of the year, that let us control the goats feeding and living conditions. Milk samples (4 l for period of the year) were split in two, 2 l were kept raw and 2 l were pasteurized at 63 °C for 30 min. Cheeses were made with the two kinds of milks to obtain, on average, eight raw (NP) and eight pasteurized cheeses (P) for each one of the three milking periods. Thus a total of 48 cheeses were produced.

Experimental mini cheese production was performed at the Universidad Veracruzana according with the method reported by Scholz (1997) with slight modifications. Milk was heated to achieve 30 - 33 °C followed by the addition of 10 ml/l rennet (Coagulmex, strength 1:10 000, Grupo Maphsa, Xalapa, Mexico). Coagulation took place at 38 ± 1 °C for 50 min. Curd was cut into 1 cm^3 cubes and whey separation was held for 20 - 40 min. Afterward, cheeses were moulded in plastic moulds (119 mm x 64 mm x 34 mm) allowing natural whey drainage by gravity at 4 °C during 24 h. Cheeses of 9.9 -16.5 g were obtained and stored at 4 °C. After six days of storage, 4 raw and 4 pasteurized fresh cheeses of each milking period (November, January and March), in a total amount of 12 raw and 12 pasteurized cheeses,

were randomly selected to be transported and analysed in the Tecnológico de Monterrey facilities.

Proteolysis assessment

Samples were separated in raw and pasteurized cheeses. Raw cheeses from the same season were mixed, to obtain three samples of raw cheeses corresponding to different periods of milking (November, January and March). Same was made for pasteurized cheeses. Finally, a total of six samples of raw and pasteurized fresh goat cheeses produced in Veracruz at different periods of the year were analysed. Proteolysis of each sample was measured by following different nitrogenous fractions characteristics. For that, 20 g of each sample were homogenized with 20 ml of distilled water 5 min at 15500 rpm using an Ultraturrax IKA T18 Basic (IKA instruments, Germay). Then, samples were incubated 1 h in a water bath at 40 °C and centrifuged (Hermle, Z 383K Wehingen, Germany) at 4 °C for 50 min and 4000 x g. The fat upper layer was removed and samples were re-suspended to obtain a non-fat cheese homogenate (NFCH). An aliquot of NFCH was used to quantify the total nitrogen with Kjeldahl method. The sample was kept at -80 °C until fractionation.

Crude nitrogenous fractions: Acid soluble nitrogen (ASN) at pH 4.6, non-protein nitrogen (NPN) and 70 % ethanol soluble nitrogen (EtOH-SN) were prepared as described by Guerra-Martínez *et al.*, (2012). NFCH was diluted with a solution of NaCl (9 g/l) to achieve a final concentration of 0.1 g cheese m/l (CMS).

For NPN, 20 ml of CMS were mixed with 20 ml of trichloroacetic acid (240 g/l) and incubated 1 h at 25 °C. The soluble fraction was separated by centrifugation 10 min at 7 °C and 4000 x g. The supernatants were stored at -80 °C until nitrogen content analyses.

For the determination of ASN, 10 ml of the 0.1 g/ml of CMS were adjusted to pH 4.6 with HCl 1 M and incubated in a water bath for 20 min at 25 °C. The soluble fraction was separated by centrifugation for 45 min at 7 °C and 4000 x g. The supernatants were stored at -80 °C until nitrogen content analyses.

EtOH-SN was prepared by adding ethanol (KARAL, Leon, Gto, Mexico) to 2 ml of ASN to achieve a 700 ml/l EtOH final concentration, and incubated in a water bath 24 h at 25

°C, the soluble fraction, was separated by centrifugation for 10 min at 6 °C and 4000 x g. The precipitate, corresponding to the high molecular weight peptides (EtOH-NSN) and the supernatant (EtOH-SN), were stored at -80 °C until nitrogen content analyses.

Nitrogen content in all the obtained fractions was evaluated with Kjeldahl method according to the AOAC. Proteolysis index for each nitrogenous fraction was obtained by dividing the respective nitrogen content over total nitrogen content. All analyses were performed by duplicate.

Determination of biological activity

Antioxidant activity evaluation

The method described by Olvera-García *et al.,* (2015) was used to assess the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of ASN and NPN fractions. Briefly, 0.02 ml of each fraction (ASN and NPN) were added to a 96-well flat-bottom plates containing 0.22 ml of DPPH• solution (125 µM DPPH• in 800 ml/l methanol). Samples were prepared in triplicate. The plate was covered, left in the dark at room temperature 90 min before reading in a visible-UV microplate reader (X Mark Microplate Reader, Bio-Rad Laboratories, Inc. Japan) using a 520 nm filter. Resulting data are expressed as a percentage of DPPH•·discoloration.

Determination *in vitro* of ACEI activity

ACEI activity was determined by the reverse phase high performance liquid chromatography (RP-HPLC) method of Wang *et al.*, (2013) based on the liberation of hippuric acid (HA) from hippuryl-histidyl-leucine (HHL) catalysed by ACE.

Briefly, HHL and ACE (from porcine kidney, 0.5 U) were dissolved in 100 mM borate buffer (pH 8.3) supplemented with 300 mM NaCl, at a concentration of 3 mM and 0.1 U/ml, respectively. All reagents came from Sigma-Aldrich, St. Louis, MO, USA.

Aliquots of 25 µl of the EtOH-SN and EtOH-NSN fractions, were pre-incubated with 25 µl of ACE at 37 °C for 10 min, and then 25 µl of HHL were added and incubated at 30 °C for 30 min. Reaction was stopped by adding 83.5 µl of HCl 0.1 M.

Separations were conducted by RP-HPLC using an Agilent Technologies 1200 series (Palo Alto, CA, USA) coupled with a diode array detector (DAD), at 25 °C using a flow rate of 0.5 ml/min in a column Zorbax Eclipse XDB-C18 (5 µm, 4.6 µm i.d. x 150 mm, Agilent, USA). Injected volume was 5 µl. Solvents were as follows: (A) 0.5 ml/l TFA in HPLC-grade water. (B) Acetonitrile. The ratio of solvents A/ B was 7:3. Detection was set at 226 nm in the DAD and data were acquired with Chemstation software. HA and HHL were used as reference standards to identify the peaks in the chromatogram.

The extent of ACE inhibition in the samples was calculated with the equation (Eq. 1):

$$\text{ACEI activity (\%)} = [(\%\ HHL) - (\%\text{HA})] \quad (1)$$

Where:

$$\%\ \text{HHL} = [\text{HHL area}/(\text{HHL area} + \text{HA area})]*100 \quad (2)$$

$$\%\ \text{HA} = [\text{HA area}/(\text{HHL area} + \text{HA area})]*100 \quad (3)$$

A blank of HHL, treated as previously specified but with buffer instead of sample and without ACE (thus with the maximum HLL peak), was used as equivalent of 100% ACEI activity.

Nitrogenous fractions analysis by reverse phase HPLC

Nitrogenous fractions ASN, NPN, EtOH-SN, and EtOH-NSN were analysed by RP-HPLC. ASN and NPN were analysed directly. For EtOH-SN, 2 ml were concentrated until dryness in a speed-vac (Thermo, Asheville, USA) and then solubilized in 1 ml of 700 ml/l ethanol. EtOH-NSN precipitated was solubilized in 0.5 ml of 50 µM of Tris-HCl (Sigma-Aldrich, St Louis, MO, USA) and 1 µM of EDTA (Sigma-Aldrich, St Louis, MO, USA). All samples where filtered through a 0.45 µm filter (PTFE Syringe Filter, Agilent Technologies, Germany) before injection.

RP-HPLC was performed using an Agilent Technologies 1200 series (Palo Alto, CA, USA) coupled with a diode array detector (DAD). Separations were conducted at 25 °C using a flow rate of 0.75 ml/min in a column Zorbax Eclipse XDB-C18 (5 µm, 4.6 µm i.d. x 150 mm, Agilent, USA).

RP-HPLC system and the column used were previously described. Separations were conducted at 25 °C using a flow rate of 0.75 ml/min. Solvents used were: (A) 100 ml/l acetonitrile and 0.5 ml/l TFA in HPLC-grade water and (B) 600 ml/l acetonitrile and 0.5 ml/l TFA in HPLC-grade water. Samples were eluted initially with 100 % A for 10 min, then with a linear gradient from 0 % to 49 % B over 98 min and 50 % to 80 % B until 108 min, followed by a linear gradient from 80 % to 100 % B over 5 min and maintained at 100 % B for 5 min. Volume injected was 10 µl. The DAD was set at 215 nm. Data acquisition was performed with Chemstation software (B.04.03, Agilent, USA). From the obtained chromatograms all the peaks were integrated and codified according to their retention time for data analysis.

The chromatograms were analysed following the same criteria of Gonzalez de Llano *et al.,* (1995), who separated the peaks integrated area in two groups, hydrophilic (HI) and hydrophobic (HO) peptides and evaluated its ratio (HO/HI). Considering that most of the free AA (except Phe and Trp) eluted in the first 10 min, the HI portion consisted in those peaks between 10 to 35 min, and the group of HO where those from 35 to 120 min. The HO/HI ratio was obtained by dividing the total area of the peaks in the HO portion by the total area of the peaks in the HI portion of the chromatogram.

Statistical analysis

All statistical analyses were performed by using the Statistica software v 12 (Statsoft, Inc., Tulsa, OK, USA). Data were grouped in sets as follows: a) nitrogenous indexes, b) antioxidant activity (AA) in the ASN and NPN fractions, c) ACEI activity in EtOH-SN and EtOH-NSN, d) ASN peptides peaks area, e) NPN peptides peaks area, f) EtOH-SN peptides peaks area, g) EtOH-NSN peptides peaks area, and h) each nitrogenous fraction of HI and HO peptides.

For each fraction's peptide profile, peaks were coded according with the fraction and retention time, and percentage of individual area recorded. Then for the global analysis of HO, HI peptides and HO/HI ratio, the total of peaks areas of each group were used directly as variables.

All the data sets mentioned above were subject to analysis of variance (ANOVA) to evaluate if there were significant differences ($p < 0.05$) between cheeses. In addition, for biological activities, the fraction was also considered as a factor. After, the Fisher least square difference (LSD) test was conducted for all the variables that showed differences between cheeses. In addition, principal components analysis (PCA) was applied to b) and c) data sets together as well as for h) data set (mentioned earlier).

Results and Discussion

Proteolysis assessment

Total nitrogen and crude nitrogenous fractions (ASN, NPN, and EtOH-SN) were used to measure the primary proteolysis in fresh goat cheeses. The mean values obtained were as follow: total nitrogen, 20.5 ± 9.07 g/kg dry matter, ASN 0.28 ± 0.22; NPN 0.20 ± 0.24 and EtOH-SN 0.12 ± 0.08. These variation observed in nitrogen index where given by a combination of season and heat treatment, and can be attributed to differences in the milk from the different batches. However, analysis of variance (ANOVA) showed no significant differences between seasons or process for any of the evaluated index ($p >0.05$). These results suggest that tested fresh goat cheeses were not different between them, and could be considered as a homogenous batch.

Low NPN values could be explained by the lack of starter culture in our cheeses, which are considered the main agents for NPN production (Fox & McSweeney, 1996). The ASN values obtained were characteristic of fresh cheese because according to El Galiou, Zantar, Bakkali, and Laglaoui (2013), ASN reflects the extent of proteolysis, that increases during ripening.

McSweeney *et al.*, (1993) found no significant differences in primary proteolysis, between fresh cheeses made with raw milk or pasteurized milk, instead, they found that these differences in proteolysis appear and increase as ripening progressed. However, to our knowledge, no works have been done about the primary proteolysis in fresh cheeses; all the studies of proteolysis and the release of bioactive peptides have focused on maturated cheeses. Furthermore the few works about fresh cheeses in this field have been done on cheeses prepared with starter cultures (Abadía-García *et al.*, 2013; Guerra-Martínez *et al.*,

2012; Sánchez-Macías *et al.*, 2011; Torres-Llanez *et al.*, 2011), thus no comparison could be made with our results.

Biological activity of nitrogenous fractions

Antioxidant activity measure

NPN and ASN fractions were chosen to determinate the antioxidant activity, since they represent the medium and short chain peptide content together. NPN and ASN fractions of fresh goat cheese exhibited an important antioxidant activity. Significant differences were observed between fractions, where NPN presented a significant higher antioxidant activity (62.89 % discoloration) compared to the ASN fraction (22.11 %). No significant differences in antioxidant activity were found between cheeses in any of the evaluated fractions ($p > 0.05$).

It has been reported that this activity in cheeses depends mainly on ripening time (Gupta *et al.*, 2009), however, our results suggest that the release of antioxidant peptides occurs not only during ripening but also during cheese making.

NPN and ASN differences in antioxidant activity might be related to the type of peptides present in each fraction. However, lower antioxidant activity in ASN fraction could also be due to the features of DPPH radical which performance is highly dependent on the characteristics of the tested sample that limits its use for hydrophilic antioxidants, as previously observed by Aloğlu and Öner (2011) and Meira *et al.*, (2012). Considering our results, further analyses with different radical scavenging methods are recommended to clarify them.

***In vitro* ACEI activity**

EtOH-SN and EtOH-NSN fractions were chosen to evaluate the ACEI activity, since these fractions contain respectively the small and medium sized peptides fractioned from ASN. These, according to previous studies are the holders of ACEI activity (Korhonen & Pihlanto, 2003).

EtOH-SN and EtOH-NSN fractions showed ACEI activities ranging between 73.03 % and 100 %. ANOVA showed no significant differences between fractions, or cheeses (p

>0.05). Our results are consistent with those reported by Torres-Llanez *et al.*, (2011) who found values between 95.3 % and 99.8 % of ACEI activity in water extracts of Mexican fresh cheese. RP-HPLC obtained to quantify ACEI activity is shown in figure 2.8 (A and B).

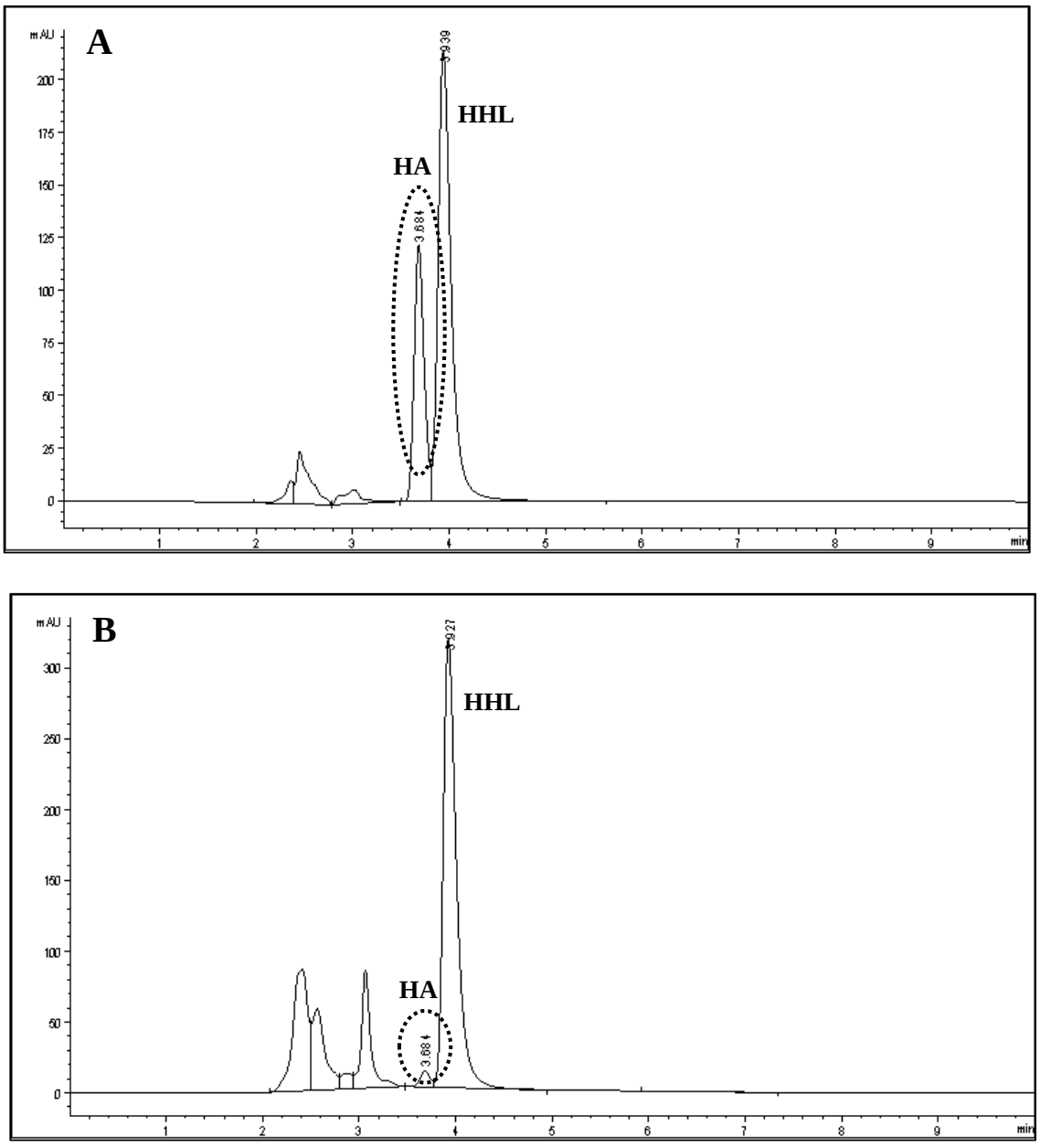

Figure 2.8. ACEI activity measurement by RP-HPLC. (A) Sample without ACE inhibitors. (B) EtOH-SN fraction of pasteurized fresh goat cheese. Decrease in HA production was related with ACEI activity. HA: Hippuric acid, HHL: Hippuryl-histidyl-leucine.

Although many studies have been carried out to measure the biological activity of cheeses with different ripening degrees, only few studies have focused on the evaluation of these activities in fresh cheeses (Paul & Van Hekken, 2011; Torres-Llanez *et al.*, 2011).

However the few works done focused on this type of cheeses attribute the release of ACEI peptides to the starter cultures (Torres-Llanez *et al.*, 2011). Nevertheless our fresh goats cheeses were fabricated without starter culture, we assume that the inhibitory compounds found in our cheeses were either present in the goat milk (Bezerra *et al.*, 2013) or generated during cheese making by the action of coagulant (Silva *et al.*, 2006).

Further PCA were applied to both antioxidant and ACEI activities as a unique data set. The factorial map shown in figure 2.9 A, is defined by PC1 and PC2 which explain 61.85 % and 29.99 %, respectively, of total variance. PC1 grouped samples according to the heat treatment. Samples from pasteurized cheeses showed positive values while samples from raw cheeses showed negative values.

In factor loading plots (Figure 2.9 B) we observe that only ACEI in the EtOH-NSN fraction shows a strong negative correlation with PC1 (R = -0.93), whereas ACEI in the EtOH-SN fractions shows a positive correlation with PC2 (R = 0.76).

Differences observed between PCA and ANOVA could be attributed to the fact that PCA evaluate a global response and not individual factors.

Thus, our results reveal that tested fresh goat cheeses exerted important ACEI and antioxidant activity. This is in accordance with previous works of Silva *et al.*, (2006), where authors observed that water soluble extracts of 45 days raw and sterilized goat cheeses made with plant coagulant exhibited both biological activities. However, authors found higher ACEI activity in cheeses made from raw milk than in those made with sterilized one. The lack of differences observed in our results, can be due to our tested cheeses were fresh and differences between raw and pasteurized cheeses are mainly observed throughout ripening, as previously discussed.

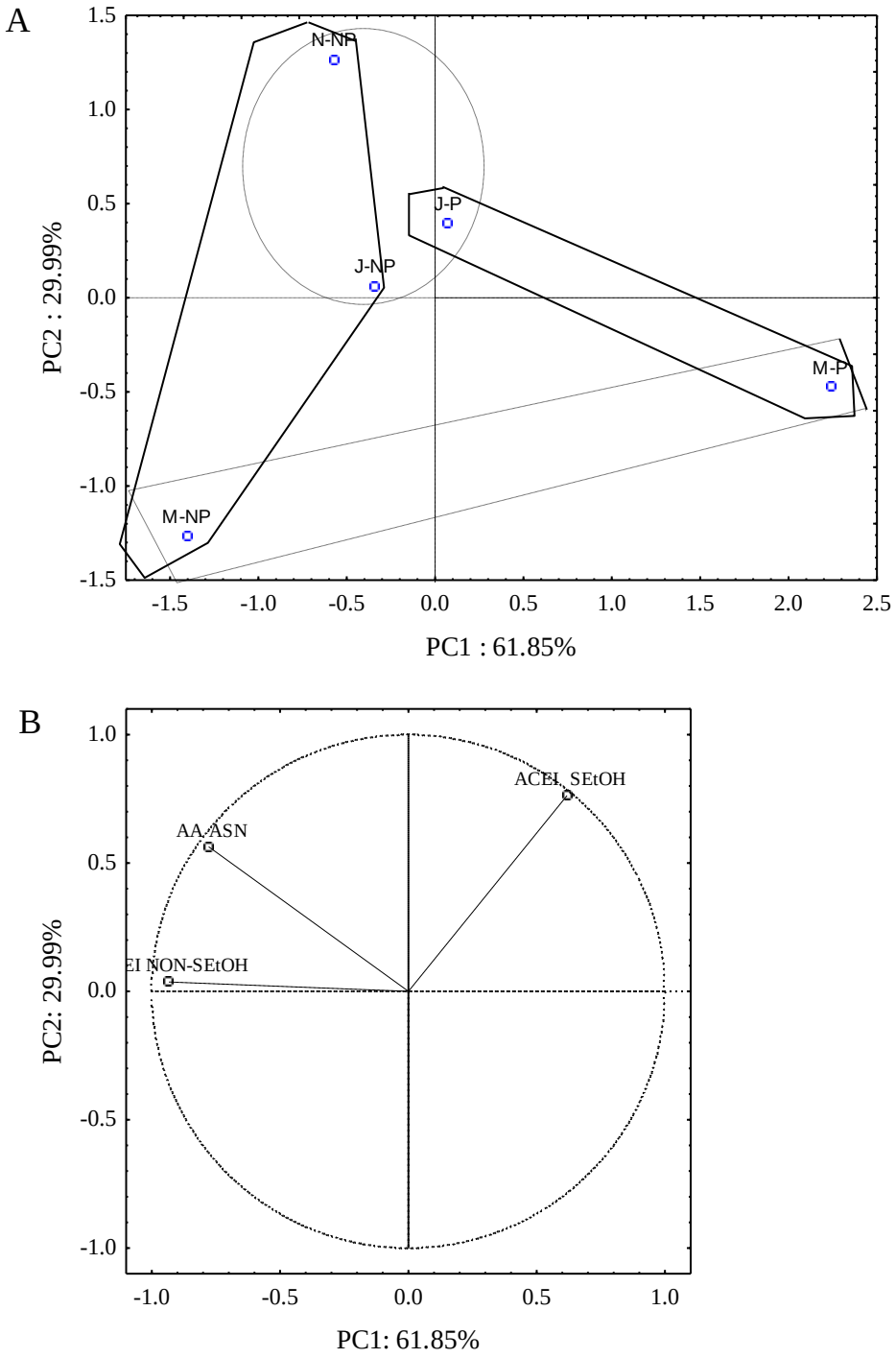

Figure 2.9. Antioxidant and ACEI activity. PCA plots of the first two principal components PC1 and PC2. (A) Factorial map. (B) Factor loading plots (PC1 and PC2). N: November, J: January, M: March. NP: Raw, P: Pasteurized.

Nitrogenous fractions analysis by RP-HPLC

In order to observe the impact of pasteurization over the obtained PCA results, RP-HPLC of ASN, NPN, EtOH-SN and EtOH-NSN fractions were performed. For data analysis we considered each peak as one compound. It is well known that pasteurization of milk prior to cheese making can affects the proteolysis, its effect over the proteolysis and the type of peptides produced in cheese has been previously reported (Albenzio *et al.*, 2001; Kırmacı *et al.*, 2014).

As a first approach, the nitrogenous fractions of each cheese were compared and noticeable differences were found between them. ASN and NPN fractions presented the most complexes profiles (207 and 120 peaks, respectively) while EtOH-SN and EtOH-NSN were simpler (43 and 22 peaks, respectively).

ANOVA and LSD tests showed that some peaks were statistically different between cheeses according to heat treatment. For ASN, 3 peptides with retention time (r.t.) at 2.05, 18.36 and 50.11 min showed significant differences (p= 0.048, 0.045 and 0.011 respectively) according to the heat treatment. NPN showed significant differences for one peptide r.t. at 49.27 min (p= 0.008). On the other hand, EtOH-SN and EtOH-NSN showed significant differences for two (r.t.72.55 and 80.26 min) and one peptides (r.t. 18.86), respectively, depending on the heat treatment (p= 0.00013, 0.012 and 0.0004 respectively).

After each run, chromatograms were divided in HO and HI peptides to calculate the HO/HI ratio and compare more globally the peptide profiles between cheeses. Results are shown in table 2.4. ASN was significantly different (p = 0.004) between pasteurized and raw cheeses only for HO peptides. Heat treatment increased the content of HO peptides (from an average of 11821.9 to 28214.5), due probably to the effect of heat on the caseins, making them more fragile for a breakdown therefore increasing the hydrophobic peptides. Statistical analysis to the HO and HI peptides in NPN, EtOH-SN and EtOH-NSN profiles did not differentiate between cheeses. The breakdown of caseins with the increase of hydrophobic peptides had been reported by McSweeney *et al.*, (1993) in Cheddar cheeses.

Table 2.4. Proportion of hydrophobic, hydrophilic peptides and ratio (HO/HI) in nitrogenous fractions of fresh goat cheese prepared with raw and pasteurized milk.

Fraction	Treatment	Hydrophilic peptides (HI)‡	Hydrophobic peptides (HO)‡	HO/HI Ratio‡
NPN	NP	6276.7 [a]	5893.1 [a]	3.65 [a]
	P	6369.1[a]	8677.8[a]	3.30 [a]
ASN	NP	9783.6 [a]	11821.9 [a]	4.84 [a]
	P	16314.7 [a]	28214.5 [b]	4.30 [a]
EtOH-SN	NP	7791.2 [a]	1776.0 [a]	0.23 [a]
	P	11659.1 [a]	11769.3 [a]	1.50 [a]
EtOH-NSN	NP	104435.3 [a]	642.1 [a]	0.01 [a]
	P	106586.3 [a]	2005.8 [a]	0.02 [a]

‡ Values in the same fraction with different letter within the same column are significantly different ($P < 0.05$).

§ NP: Raw, P: Pasteurized.

Further PCA was applied to ASN fraction (Figure 2.10 A and B). PCA factorial map (Figure 2.10 A) of the HO and HI peptides in the ASN fraction is defined by PC1 and PC2, explaining 67.61 % and 29.07 %, respectively of the total variance. In Figure 2.10 A, samples were classified in 2 groups according to heat treatment, where raw samples were in the positive values of the PC2 and pasteurized ones in the negative values.

As shown in factor loading plot (Figure 2.10 B) the studied variables HI and Ratio HO/HI were strongly correlated with PC1 (R = -0.96 and 0.94 respectively).Whereas HO was the only variable correlated (R = -0.88) with PC2.

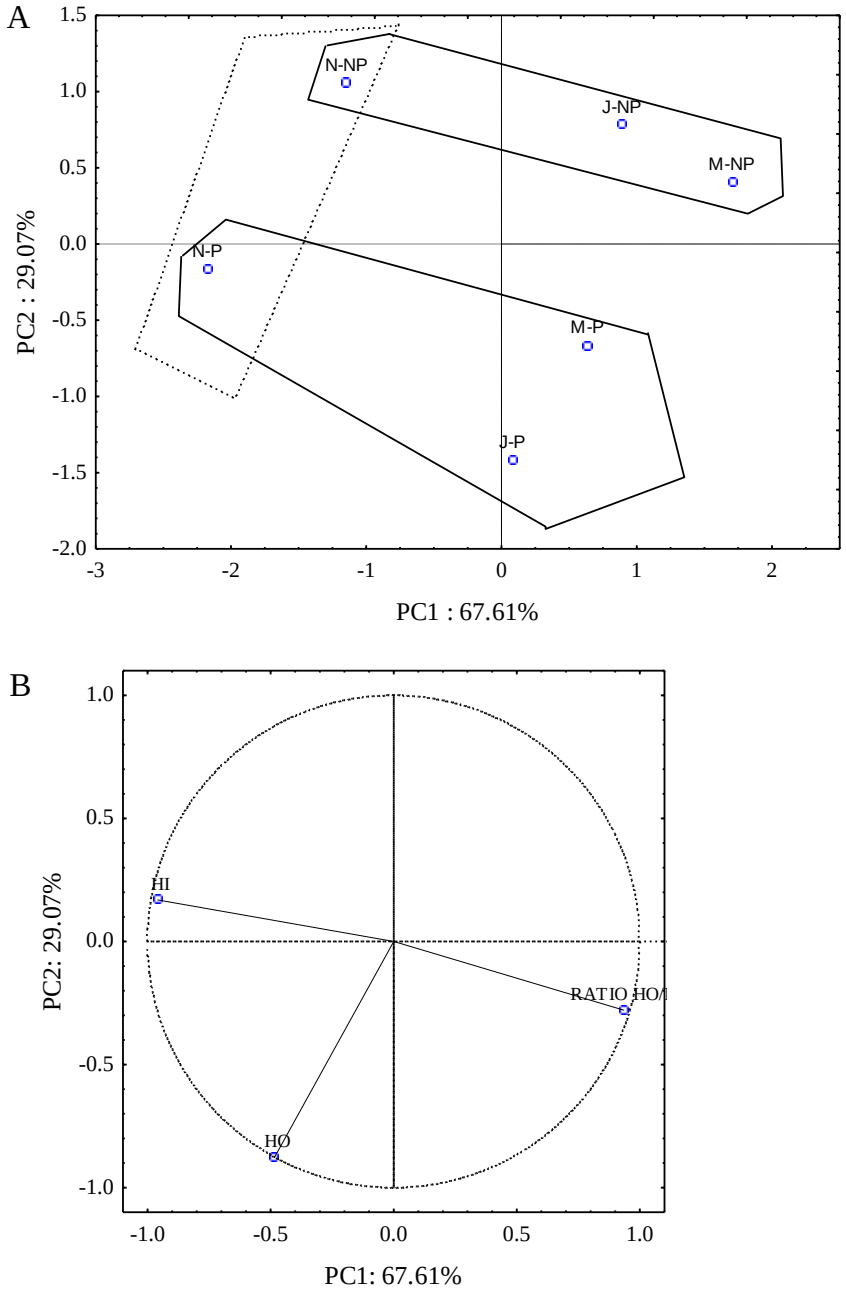

Figure 2.10. HO and HI peptides in the ASN profile. PCA plots of the first two principal components PC1 and PC2. (A) Factorial map. (B) Factor loading plots (PC1 and PC2). N: November, J: January, M: March. NP: Raw, P: Pasteurized.

These results suggest that heat treatment influence significantly the type of peptides in the ASN fraction. This can be explained by the effect of pasteurization over the tertiary structure of caseins, producing denaturation through destabilization of hydrogen bonding and electrostatic interactions, while hydrophobic interactions are stabilized. This denaturation

might further affect their enzymatic susceptibility, increasing the number of sites exposed to enzymatic attack, producing thus different peptides from a non-pasteurized milk (Hayaloglu & Brechany, 2007; Turgeon & Rioux, 2011). Despite some studies have been made to characterize the proteolysis by HPLC methods in ripened cheese, and even when heat treatment has already been probed to affect the proteolysis in cheeses, barely nothing has been published on the effect of heat treatment on the peptide profile of fresh cheeses. Therefore the lack of published results makes it difficult to compare our findings with the literature. However our results can be partially compared with previous works in the field of dairy protein degradation. Mullally *et al.,* (1998) observed that after thermal denaturation of β-lactoglobulin, this protein was significantly more susceptible to enzymes. In addition, Rinaldi *et al.,* (2014b) showed that high temperatures used during sterilization of the milk increases the pepsin susceptibility of β-lactoglobulin and α-lactoalbumin. Similar results were obtained by Dupont *et al.,* (2010) who observed that high temperatures used in sterilization of the milk produce extensive protein denaturation.

Thus, as observed in figure 2.10, heat treatment produced a change in the hydrolysis of the caseins affecting the production of HO peptides (Figure 2.10 B). This HO variation further affects the ACEI activity in the EtOH-SN fraction that differentiates between NP and P cheeses (Figure 2.9 B). Meanwhile, biological activity in NP cheeses seems to be more related to the HI peptides of the ASN fraction (Fig 2.9 B and 2.10 B). This is in agreement with previous results of our same research group for Cotija cheese (ripened cheese), where a high correlation was observed between HO peptides and ACEI activity; and HI peptides with the AA activity (Hernández Galán *et al.,* 2016). However, further studies need to be carried out to understand the precise relationship between the type of peptides in a determined nitrogenous fractions and its biological activity.

Conclusions

Although the studied goat cheese was fresh and fabricated without starter culture, antioxidant and ACE inhibitory activities, associated with peptides were observed, implying that nitrogenous fractions of fresh goat cheese had biological activity derived from the naturally present milk proteins or released by the rennet used during cheese manufacture.

PCA results of biological activity groped cheeses according with heat treatment. These could be related with the observed variation in the type of peptides (HO and HI) and the ratio

between them in the ASN fraction. Further studies need to be carried out to identify the peptides associated with each biological activity.

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References

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., . . . Amaya-Llano, S. L. (2013). Influence of probiotic strains added to Cottage Cheese on potentially-antioxidant peptides generation, anti-Listerial activity, and survival of probiotic microorganisms in simulated astrointestinal conditions. *International Dairy Journal, 33*(2), 191–197. doi:10.1016/j.idairyj.2013.04.005

Ahmed, A. S., El-Bassiony, T., Elmalt, L. M., & Ibrahim, H. R. (2015). Identification of potent antioxidant bioactive peptides from goat milk proteins. *Food Research International, 74*, 80-88. doi:10.1016/j.foodres.2015.04.032

Albenzio, M., Corbo, M. R., Rehman , S. U., Fox, P. F., De Angelis, M., & A. Corsetti , A. S., M. Gobbetti. (2001). Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food MIcrobiology, 67*. doi:10.1016/S0168-1605(00)00533-X

Aloğlu, H. Ş., & Öner, Z. (2011). Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. *Journal of dairy science, 94*, 5305-5314. doi:10.3168/jds.2011-4285

Bezerra, V. S., Campos, J. F., da Silva, R. A., Porto, T. S., de Lima-Filho, J. S., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. *Food Science and Technology, 33*(3), 513-520. doi:10.1590/S0101-20612013005000074

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Rolet-Repecaud, O., Duboz, G., . . . Mackie, A. R. (2010). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition and Food Research, 54*, 1677-1689. doi:10.1002/mnfr.200900582

El Galiou, O., Zantar, S., Bakkali, M., & Laglaoui, A. (2013). Lipolysis and Proteolysis During the Ripening of Fresh Moroccan Goats' Milk Cheese. *World Journal of Dairy & Food Sciences, 8*, 201-206. doi:10.5829/idosi.wjdfs.2013.8.2.8140

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183.

doi:10.1016/j.idairyj.2013.04.002

Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Reviews International, 12*(4), 457-509. doi:10.1080/87559129609541091

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gonzalez de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of Proteolysis in Liquid Chromatography Artisanal Cheeses: High Performance of Peptides. *Journal of dairy science, 78*, 1018-1024. doi:10.3168/jds.S0022-0302(95)76717-0

Guerra-Martínez, J. A., Montejano, J. G., & Martín-del-Campo, S. T. (2012). Evaluation of proteolytic and physicochemical changes during storage of fresh Panela cheese from Queretaro, Mexico and its impact in texture. *CyTA - Journal of Food, 10*(4), 296-305. doi:10.1080/19476337.2011.653791

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Hajirostamloo, B. (2010). Bioactive component in milk and dairy product. *World Acad Sci Eng Technol, 72*, 162-166.

Hayaloglu, A. A., & Brechany, E. Y. (2007). Influence of milk pasteurization and scalding temperature on the volatile compounds of Malatya, a farmhouse Halloumi-type cheese. *Lait, 87*, 39-57. doi:10.1051/lait:2006025

Hernández-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Angiotensin Converting Enzyme Inhibitory Activity in Commercial Fermented Products. Formation of Peptides under Simulated Gastrointestinal Digestion. *Journal of Agricultural and Food Chemistry, 52*(6), 1504-1510. doi:10.1021/jf034997b

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*(1), 23-35. doi:10.1016/j.cis.2010.11.001

Hernández Galán, L., Cardador Martínez, A., Picque, D., Spinnler, H. E., López del Castillo Lozano, M., & Martín del Campo Barba, S. T. (2016). Angiotensin converting enzyme

inhibitors and antioxidant peptides release during ripening of Mexican Cotija hard cheese. *Journal of Food Research, 5*(3).

Jäkälä, P., & Vapaatalo, H. (2010). Antihypertensive Peptides from Milk Proteins. *Pharmaceuticals, 3*(1), 251-272.

Kırmacı, H. A., Hayaloglu, A. A., Özer, H. B., Atasoy, A. F., & Türkoglud, H. (2014). Evolution of proteolysis in Urfa cheese made from ewe's milkby wild type starter culture systems. *Small Ruminant Research, 119*, 120-129. doi:10.1016/j.smallrumres.2014.01.006

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323. doi:10.2174/1381612033454883

Korhonen, H., & Pihlanto, A. (2003). Food-derived Bioactive Peptides – Opportunities for Designing Future Foods. *Current Pharmaceutical Design, 9*, 1297-1308. doi:10.1016/j.idairyj.2005.10.012

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945-960.

Lacroix, C., Verret, P., & Paquin, P. (1996). Regional and Seasonal Variations of Nitrogen Fractions in Commingled Milk. *International Dairy Journal, 6*, 947-961. doi:10.1016/S0958-6946(96)00022-2

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N., & Cogan, T. M. (1993). Contribution of the Indigenous Microflora to the Maturation of Cheddar Cheese. *International Dairy Journal, 3*, 613-634. doi:10.1016/0958-6946(93)90104-8

Meira, S. M. M., Daroit, D. J., Helfer, V. E., Corrêa, A. P. F., Segalin, J., Carro, S., & Brandelli, A. (2012). Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and Uruguay. *Food Research International, 48*(1), 322-329. doi:10.1016/j.foodres.2012.05.009

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Mullally, M. M., Mehra, R., & Fitzgerald, R. J. (1998). Thermal effects onthe conformation and susceptibility of beta-lactoglobulin to hydrolysis by gastric and pancreatic endoproteinases. *Irish Journal of Agricultural and Food Research, 37*(1), 51-60.

Ng-Kwai-Hang, K. F., Hayes, J. F., Moxley, J. E., & Monardes, H. G. (1984). Variability of Test-Day Milk Production and Composition and Relation of Somatic Cell Counts with

Yield and Compositional Changes of Bovine Milk. *Journal of dairy science, 67*, 361-366. doi:10.3168/jds.S0022-0302(84)81309-0

Olvera-García, V., Cardador-Martínez, A., & Martín del Campo, S. T. (2015). Effect of Thermal Process and Filtration on the Antioxidant Activity and Physicochemical Parameters of Agave atrovirens Extracts. *Journal of Food Research, 4*, 155-167. doi:10.5539/jfr.v4n1p155

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Paul, M., & Van Hekken, D. L. (2011). Assessing antihypertensive activity in native and model Queso Fresco cheeses. *Journal of dairy science, 94*, 2280-2284. doi:10.3168/jds.2010-3852

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548. doi:10.1016/j.foodres.2010.03.007

Rinaldi, L., Rioux, L., Britten, M., & Turgeon, S. L. (2014b). In vitro bioaccessibility of peptides and amino acids from yogurt made with starch, pectin, or β-glucan. *International Dairy Journal*, 1-7. doi:10.1016/j.idairyj.2014.09.005

Sánchez-Macías, D., Morales-delaNuez, A., Moreno-Indias, I., Hernández-Castellano, L. E., Mendoza-Grimón, V., Castro, N., & Argüello, A. (2011). Lipolysis and proteolysis profiles of fresh artisanal goat cheese made with raw milk with 3 different fat contents. *Journal of dairy science, 94*, 5786-5793. doi:10.3168/jds.2011-4423

Scholz, W. (1997). Elaboración de quesos de oveja y de cabra (pp. 145). Zaragoza, Spain: Acribia.

Silva, S., Pihlanto, A., & Malcata, F. (2006). Bioactive Peptides in Ovine and Caprine Cheeselike Systems Prepared with Proteases from< i> Cynara cardunculus</i>. *Journal of dairy science, 89*(9), 3336-3344. doi:10.3168/jds.S0022-0302(06)72370-0

Theolier, J., Hammami, R., Fliss, I., & Jean, J. (2014). Antibacterial and antifungal activity of water-soluble extracts from Mozzarella, Gouda, wiss, and Cheddar commercial cheeses produced in Canada. *Dairy Science and Technology, 94*, 427-438. doi:10.1007/s13594-014-0170-9

Timón, M. L., Parra, V., Otte, J., Broncano, J. M., & Petrón, M. J. (2014). Identification of radical scavenging peptides (<3 kDa) from Burgos-type cheese. *LWT - Food Science and Technology, 57*(1), 359-365. doi:10.1016/j.lwt.2014.01.020

Torres-Llanez, M., González-Córdova, A., Hernandez-Mendoza, A., Garcia, H., & Vallejo-Cordoba, B. (2011). Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. *Journal of dairy science, 94*(8), 3794-3800. doi:10.3168/jds.2011-4237

Turgeon, S. L., & Rioux, L. (2011). Food matrix impact on macronutrients nutritional properties *Food Hydrocolloids, 25*, 1915-1924. doi:10.1016/j.foodhyd.2011.02.026

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910

2.3 Final remarks of section 2.1 and 2.2

The main objective of this part of the work was to evaluate if Mexican cheeses as Cotija and Fresh goat cheese could exert biological properties by the production of bioactive peptides; with activities as antioxidants and ACE inhibitors; analyzing their behavior throughout ripening (Cotija) and milk pasteurization influence in production (Fresh goat cheese).

For that, nitrogen in cheeses was fractionated into ASN, NPN, EtOH-SN and EtOH-NSN fractions. Based on their size of peptide content, ASN and NPN fraction were selected to measure antioxidant activity, meanwhile EtOH-SN and EtOH-NSN were selected to measure ACE inhibitory activity.

Our results probe that both cheeses had important antioxidant activity that was higher in NPN than in ASN. At early stages of ripening, Cotija cheese exerted similar values than Fresh goat cheese. However, activity in ASN increased throughout ripening and older Cotija cheese had higher antioxidant activity that Fresh goat cheese. Meanwhile, pasteurization of the milk did not exert a visible effect on the antioxidant activity in Fresh goat cheeses.

On the other hand, both cheeses exerted important ACE inhibitor activity. In Cotija cheese activity was higher in EtOH-SN than EtOH-NSN, while in Fresh goat cheese, activity was similar in both fractions. ACE inhibitor activity in Fresh goat cheese was higher than the observed in young Cotija cheese.

An interesting change in proteolysis rate (probably associated with a cheese microbiota evolution) was observed after four months of Cotija cheese ripening that was associated with the increase of biological activity. Therefore biological activity seems to be related to the formation of soluble peptides.

Biological activities were highly correlated with the hydrophobicity of the peptides contained in each fraction analyzed through HPLC methods. Antioxidant activity was correlated with the hydrophilic peptides meanwhile ACE inhibitor activity was more correlated with the

hydrophobic peptides. However, further studies need to be carried out to understand the precise relationship between the type of peptides in a determined nitrogenous fractions and its biological activity. Pasteurization increased the production of hydrophobic peptides therefore affecting the ACEI activity in the EtOH-SN fraction and differentiating between NP and P cheeses.

The release of bioactive peptides has always been considered as dependent of the proteolysis degree during cheese ripening mainly because of microorganism action. However our results suggest that these peptides could be also present in the milk used for cheese making and released by endogenous enzymes or by coagulant during casein breakdown. Furthermore, our results suggest that the fractions with the smallest peptides exerted the highest biological activity; however, fractions with bigger peptides also exert important activity.

2.4 Effect of dairy matrices on the survival of Streptococcus thermophilus, Brevibacterium aurantiacum and Hafnia alvei during *in vitro* and *in vivo* digestion.

2.4.1 Presentation of the third article in preparation

The association between milk- microorganisms in cheeses (cheese microbiota) led not only to the development of characteristics of each kind of cheese but also to the release of bioactive peptides. However some of these microorganisms are capable to survive digestion and produce changes on human health through interaction with the intestinal microbiota. In this regard, some authors affirm that the presence of a dairy matrix enhance the survival of microorganisms during digestive stress.

The current hypothesis that try to explain this protective effect are related to a) the effect of the microorganism´s preadaptation to the matrix b) macrostructure effects (buffer capacity and/or effects on gastric emptying rate) and c) microstructure effects (interactions between microorganisms and dairy components and/or microstructure itself).

Therefore the aim of this study was to evaluate the ability of four dairy matrices to enhance diary microorganism´s survival through digestion through microstructure effects and buffer capacity.

For this, we measured the viability of three microorganisms of dairy interest grown in synthetic medium, *Streptococcus thermophilus, Brevibacterium aurantiacum* and *Hafnia alvei* through digestion; to later observed the effect of inclusion into four dairy matrices different in composition (with and without fat) and structure (liquid and gel) on their survival through *in vitro* digestion in a recently developed dynamic digester (DIDGI). To later perform a series of experiments with mice to observe the effect of dairy matrices on microorganism´s survival during full digestive transit in an *in vivo* model. Degradation of the matrices during in vitro digestion

was measured through methods of SDS electrophoreses (protein degradation) and gas chromatography (free fatty acid production).

1. Highlights

- *S. thermophilus* was highly sensitive to gastric stress, and was not capable to reach duodenal phase. *B. auranticum* was moderately sensitive to gastric stress but resistant to duodenal stress; meanwhile *H. alvei* was highly resistant to both stresses.
- Dairy matrices had high buffer capacity but they did not enhance microorganisms survival
- Fat addition had a negative effect on *H. alvei´*s survival.
- Protein degradation was similar for all tested matrices

2.4.2 Article: Effect of dairy matrices on the survival of *Streptococcus thermophilus, Brevibacterium aurantiacum* and *Hafnia alvei* during digestion. *In preparation.*

Leticia Hernández-Galán[a,b], Thomas Cattenoz[b], Steven Le Feunteun[b], Alexis Canette[c], Romain Briandet[c] ,Sylvia Le-Guin[c], Eric Guedon[d], Jessie Castellote[b], Jerome Delettre[b], Eric Dugat Bony[b], Pascal Bonnarme[b], Henry Eric Spinnler[b], Sandra T. Martín del Campo[a], Daniel Picque[b1].

[a] Escuela de Ingeniería y Ciencias, Tecnologico de Monterrey. Epigmenio González 500, Fracc. San Pablo. CP 76130, Santiago de Querétaro, Qro. Mexico.

[b] INRA-AgroParisTech, UMR 0782 GMPA Génie et Microbiologie des Procédés Alimentaires. Centre de recherche de Versailles-Grignon, Thiverval-Grignon, France.

[c] INRA-AgroParisTech, UMR 1319 MICALIS MICrobiologie de l'ALImentation au Service de la Santé Humaine. Centre de recherche Jouy-en-Josas, Jouy en Josas, France.

[d] INRA-AGROCAMPUS OUEST, UMR 1253 STLO Science et Technologie du Lait et de l'Oeuf. Centre de recherche Rennes, Rennes, France.

[1]Correspondence: Daniel Picque, INRA, UMR 782 Genie & Microbiologie des Procèdes Alimentaires, France. E-mail: picque@grignon.inra.fr

Abstract

This study evaluated the ability of dairy matrices, different in composition (with and without fat) and structure (liquid and gel), to enhance microorganism survival through digestion. The viability of three dairy microorganisms *Streptococcus thermophilus, Brevibacterium aurantiacum* and *Hafnia alvei* was measured during *in vitro* and *in vivo* digestion. During *in vitro* digestion, the microorganisms had different survival rates; however despite its buffer capacity, we did not observe any effect of the dairy matrices. *S. thermophilus* was highly sensitive to gastric stress, and was not found in the duodenal compartment. *B. auranticum* was moderately sensitive to gastric stress but resistant to duodenal stress. *H. alvei* was highly resistant to both stresses. LIVE/DEAD confocal microscopy´s images, probed the effect of low pH on microorganisms survival. However, *in vivo* analyses (16S rRNA gene metabarcoding) failed to confirm *in vitro* observations and tested microorganisms were not detected. Therefore, interactions between microorganisms and dairy matrices (components and microstructure) did not enhance microorganism´s survival during digestion, suggesting that protective effect of the dairy matrices is mostly strain-dependent or related to changes on the digestive parameters associated to the matrix.

Keywords: Dairy matrix, microorganism survival, digestion, buffer capacity.

Introduction

In recent years, there has been an increasing interest in studying food digestion and on the possible influence of their structure and composition on human health and wellbeing. Dairy products are the most studied foods, mostly because of their nutritional importance, but also because they can be eaten under a great variety of structures (solutions versus gels, native versus denatured proteins, etc.), and constitute a possible source of microorganisms (fermented products).

In this field, many questions actually remain regarding the effect of those eaten microorganisms, especially since some of them are capable to survive during its pass through the gastrointestinal tract and interact with the intestinal microbiota.

However, before reaching the colon, microorganisms need to pass through stomach and tolerate its high acidity, to later suffer the intestinal stress caused mainly by the bile salts (Sumeri *et al.*, 2012).

Some authors affirm that the presence of a food matrix (structure) and/or the addition of certain ingredients, like fat (composition), could modify the survival of these microorganisms during digestion through direct or indirect interaction between microorganisms and food components (Do Espirito Santo *et al.*, 2011; Ranadheera *et al.*, 2012; Sanders & Marco, 2009).

However, results reported in the literature are contradictory. For instance Faye *et al.*, (2012) observed that survival of *L. lactis ssp cremoris* during *in vitro* experiments was higher in fermented milk than in synthetic medium. In contrast, Sumeri *et al.*, (2012) observed that the same microorganisms did not survive during *in vitro* experiments when semi-hard cheese was used.

Lay *et al.*, (2004) observed that the viability of *S. thermophilus* during in vivo experiments was greater when eaten and grown in Camembert cheese than in yogurt. In contrast, Adouard *et al.*, (2016) observed higher survival of *B. auranticum* and *H. alvei* during *in vitro* experiments when grown in synthetic medium than when grown in cheese.

Meanwhile, Saxelin *et al.*, (2010) observed that the viability of *P. freudenreichii ssp. shermanii* JS, and *B. animalis ssp. lactis* Bb12 during *in vivo* experiments was higher when eaten in yogurt than in cheeses; however the food matrix did not affect the survival of *L. rhamnosus* GG and LC705.

Thus, the real effect of the food matrix in the survival of microorganisms has not been completely elucidated. The proposed mechanisms that may explain this protective effect refers to a) the effect of the microorganism´s preadaptation to the matrix b) macrostructure effects (buffer capacity and/or effects on gastric emptying rate) and c) microstructure effects (interactions between microorganisms and dairy components and/or microstructure itself).

Preadaptation effect is related to the inherent mechanism of response to stress expressed during food manufacture that produces a cross-adaptation to further digestive stress (Begley *et al.*, 2005). Pitino *et al.,* (2012) observed that survival of *L. rhamnosus* in cheese during *in vitro* experiments was related to the production of extracellular polysaccharide. Meanwhile, Uriot *et al.,* (2016) observed that viability of different strains of *S. thermophilus* to *in vitro* experiments was dependent on the activation of urease and small Heat Shock Proteins.

Meanwhile macrostructure effects are mostly related to the buffer capacity of the matrix during stomach phase. Gardiner *et al.,* (1998) suggested that the higher viability of *E. faecium* in Cheddar and yogurt during *in vitro* experiments was due to its higher buffer capacity compared to the synthetic medium.

Additionally, changes in the food macrostructure impact the digestion process, for instance, coagulation of the milk increases the viscosity of the matrix, disturbing the gastric emptying and intestinal transit, caused by changes in the ratio liquid/solid in the chime (Turgeon & Rioux, 2011).

On the other hand microstructure also plays a key role in microorganism's survival. Sumeri *et al.*, (2012) observed that survival of lactic acid bacteria during *in vitro* experiments was higher in semi-hard cheeses than in synthetic medium. Suggesting that, high protein content and presence of fat globules provide a physical barrier, and a further interaction surface with the microorganisms, incorporated into the matrix.

Hannon *et al.*, (2006) observed that survival of *L. lactis* during *in vitro* experiments was higher when cheese was rennet at pH 5.2 than at 6.2, because at low pH the matrix was more homogeneous. Pitino *et al.,* (2012) observed the preference of *L. rhamnosus* to the casein network into a cheese matrix.

Additionally, matrix components affect the digestive conditions, for instance, content of fat in the matrix, increase the bile secretion (David *et al.*, 2014). Meanwhile, polysaccharide addition changes the patters of protein degradation during digestion (Rinaldi *et al.*, 2014b).

Thus, the role of the food matrix is still unknown and the several proposed mechanisms makes difficult to determine its contribution on microorganisms survival.

It's in this context that this study was developed with the purposed objective of observe if microorganisms from dairy origin, represented by *S. thermophilus*, *H. alvei* and *B. auranticum*, were capable to survive to *in vitro* gastro-intestinal dynamic digestions. To later determine the ability of four dairy matrices, different in composition and structure, to enhance their viability beyond preadaptation or macrostructure effects.

Hence the used microorganisms were growth in pure culture and further included into the matrix previously to the digestion. Meanwhile, the *in vitro* digestive model (DIDGI) was set using the same digestion parameters of pH, gastric emptying and intestinal transit for all matrixes to avoid changes in the digestive process; focusing in the protective effect of the matrix beyond digestive changes between matrices. Additionally, a series of experiments with BALB/c mice were performed to observe the effect of dairy matrices on microorganisms survival during full digestive transit in an *in vivo* model

Material and Methods

Bacterial Strains

Streptococcus thermophilus TIL 257 (*ST*), *Brevibacterium aurantiacum* ATCC9174 (*BA*) and *Hafnia alvei* GB01 (*HA*) were selected by its role during manufacture of smear-ripened cheese. The strains were obtained from the culture collection of INRA, UMR 782 Genie & Microbiologie des Procédés Alimentaires (F-78850 Thiverval-Grignon, France).

Working cultures were reactivated from frozen stocks stored at -80 °C by transfer *B. aurantiacum* and *H. alvei* to 100 mL of Brain Heart Infusion broth (BHI), in 500 mL Erlenmeyer flask, at 25 °C and put on a shaking table at 250 and 200 rpm respectively. *S. thermophilus* was cultivated anaerobically in M17 in 100 mL Schott bottles at 37 °C. After 48 hours, 50 mL of each culture was mixed with 50 mL of new medium and incubated 24 h for *B. aurantiacum* and *H. alvei* and 18 h for *S. thermophilus*, in the previously described conditions, until reaching the

stationary phase, according to prior growth kinetics experiments (data not shown) that allows a final concentration of 10^7 cells of each strain introduced into the DIDGI system.

Viability of microorganisms during digestion was determined after dilution in Maximum Recovery Diluent (MRD, containing NaCl 8.5 g/L and peptones 1g/L) by plating on the same agar based medium used for liquid culture. The same temperatures of incubation were used as for broth cultures. All growth media were purchased from Biokar Diagnosis (Beauvais, France), MRD was purchased from Difco (Pessac, France).

Dairy matrices

Four dairy matrices with different composition and structures, skimmed milk (SM), whole milk (WM), rennet gel from skimmed milk (GSM) and rennet gel from whole milk (GWM)), were used to compare its protective effect in the survival of microorganisms during dynamic *in vitro* digestion. Synthetic medium (S) was used as control and was prepared with Maximum Recovery Diluent.

All dairy matrices were made from skimmed milk (SM) prepared with 10.9 g of skimmed milk powder (36.2% protein, 1.5% fat, 56% lactose, 8.5% minerals; Low heat powder, Lactalis, France) diluted in 110.1 mL of sterile $CaCl_2$ (0.01 M) and stirred for 30 min. Whole milk (WM) was prepared adding 4.4 g of anhydrous milk fat (Lactalis, France), previously melted, to 121 mL of SM and mixed 5 min at 3000 rpm using a Thermomix (TM31, Vorwerk, Nantes) at 50 ºC. Then, the whole milk (3.8 % fat) was cooled rapidly and kept at 4 °C until analysis.

Rennet gel from skimmed milk (GSM) and rennet gel from whole milk (GWM) were prepared by mixing 121 mL of SM or WM with 2.4 mL of rennet (1:50, Naturen 450, 555 mg/L of active chymosine, 145 IMCU/mL; Chr. Hansen, Arpajon, France), the mix was gently stirred for a few seconds before being placed at 30 °C for 40 min. Rheological characteristics of the gels were determined with a rotational rheometer (MCR301, Anton Paar, Ulis, France), operating in the oscillatory mode. A mix of 18 mL of milk (skimmed milk or whole milk) and 1.8 mL of microorganisms was introduced in the coaxial cylinder (CC27/T200/SS, Anton Paar, Ulis, France) and warmed at 30 °C. Then 400 µL of rennet were added and the evolution of the storage

modulus G' and the loss modulus G" recorded for 60 minutes. The deformation and frequency used were 1% and 1 Hz respectively, permitting to work in the linear viscoelastic region. After 40 min of rennet addition, G' and G" were stable and raised 28.7 Pa ± 1.25 and 7.2 Pa ± 0.13 respectively. For the digestion's experiments, the inclusion of the microorganisms in the matrix (4 mL of each microorganism) was made immediately before the *in vitro* digestion for liquid matrix (S, SM and WM) and just before the addition of rennet for gel matrices (GSM, GWM). Previous to the introduction of the matrix in the DIDGI®, artificial saliva (250 mg of mucine and 100 mg α-amylase in 100 mL of MRD) was added to the matrix to complete 200 mL.

The *in-vitro* dynamic model of the gastrointestinal tract

The DIgesteur Dynamique Gastro-Intestinal (DIDGI®, INRA Versailles-Grignon, France) was developed to simulate monogastric digestion (Ménard *et al.,* 2014). The DIDGI® consisted of three separated compartments simulating the stomach, the duodenum and the small intestine (Figure 2.11), as previously described by Adouard *et al.,* (2016) with some modification (Table 2.5).

The digestion's parameters were controlled by the software SToRM for Stomach Regulation and Monitoring (Guillemin *et al.,* 2010).All digestive juice components were purchased from Sigma (Saint-Quentin-Fallavier, France) and diluted in MRD. Digestion experiments for each food matrix were performed at least by duplicate. Samples from each compartment were collected at different stages of the digestion and kept a –20 °C to be later analyzed.

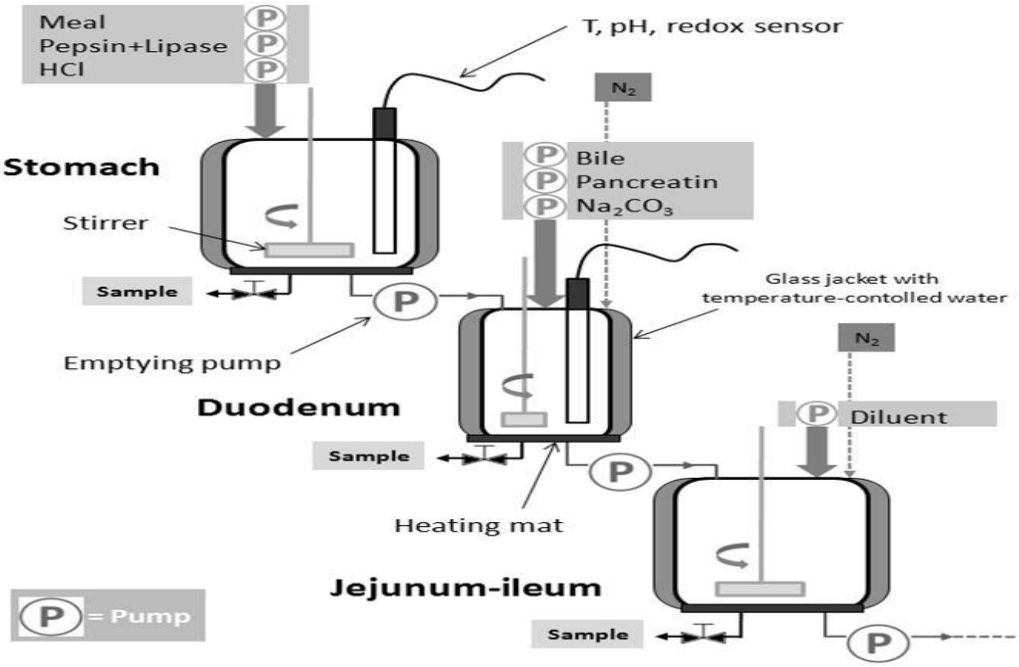


Figure 2.11. Diagram of the Digesteur Dynamique Gastro-Intestinal DIDGI®. P: peristaltic pump, N_2: Nitrogen.

Table 2.5. Parameters used for in-vitro dynamic digestion.

	Input	Concentration	Quantity or flow rate	Time lapse (min)
Stomach 37°C	Matrix + Artificial saliva HCl Pepsin Gastric Lipase	 0.5 M 0.5 g/L (~2000 U/.mL) 6.5 g/L (~200 U/.mL^{1})	200 mL pH regulation 0.4 mL/.min 0.4 mL/.min	0 [0;70] [0;90] [0;90]
Duodenum 37°C	 Na_2CO_3 Bile 40 Bile 20 Pancreatin	 1 M 40 g/L 20 g/L 20 g/L^{-}	pH regulation 10 mL 0.5 mL/.min 0.15mL/.min 0.2mL/.min 5 mL 0.25mL/.min 0.1mL/.min	 0 [0;80] [80;100] [80;220] 0 [0;50] [50;220]
Intestine (Jejunum and ileum) 37°C	Diluent	9.5 g/L	0.8mL./min 0.5mL/.min	[10;100] [100;250]

In vivo digestion

24 male mice (CH3/HeN) five weeks old were placed in a controlled room (21 °C) with a 12-h light/dark cycle. Mice had free access to standard food (SAFE RO3, Augy, France, www.safe-diets.com) and sterilized water. The 24 mice were separated in 3 groups which were feed differently during the five first days of the experiment. The controlled group (number 116) had only the standard feeding. The matrix group (number 152) and the matrix+microorganism group (number 131) were respectively intragastric inoculated in the morning with 200 µL of WM and WM + microorganism. This inoculating period was followed by a seven-day standard feeding period. The feces were collected at days 1, 5 and 12, in the morning, just before inoculation. Samples were placed in liquid nitrogen and stored at -80 °C.

Characterization of microorganisms and matrices

Metagenomic and metatranscriptomic analysis

DNA extraction was performed from 30 mg of feces as previously described (Godon *et al.*, 1997). The V3-V4 region of the 16S rRNA gene was amplified from 10 ng of purified genomic DNA using the primers F343-TACGGRAGGCAGCAG and R784-TACCAGGGTATCTAATCCT as previously described (Lazuka *et al.*, 2015). Then, library preparation and sequencing were performed at the GeTPlaGe platform (Toulouse, France) using the Illumina MiSeq technology. Paired-end reads were merged using Flash (Magoč & Salzberg, 2011) and FASTQ files were then quality-filtered and analyzed using QIIME v.1.9.0 (Caporaso *et al.*, 2010). Chimeric sequences were identified using USEARCH (Edgar, 2010) and operational taxonomic units (OTUs) were clustered at 97% identity using UCLUST (Edgar, 2010). Any OTU comprising less than 200 reads were removed, as recommended by Bokulich *et al.*, (2013). Representative sequences for each OTU were classified taxonomically using RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) against the Greengenes database (McDonald *et al.*, 2012).

Confocal laser scanning microscopy (CLSM).

First, dairy matrices (WM and GWM) with microorganism were studied. For 1 mL of medium, milk fat was stained with 100 µL of non-polar Bodipy® 493/503 lipid probes (working solution concentration at 0.5% w/v in DMSO). 100 µL of amine reactive dye DyLight™ 550

NHS Ester 562/576 (working solution concentration at 0.5% w/v in DMSO) were added to stain proteins. 1 µL of the fluorescent SYTO® 61 nucleic acid stain 628/645 (commercial solution at 5 mM in DMSO) was used to stain microorganisms for 1 mL of microorganisms culture. Then 33 µL of each culture was added to the food matrix. For gel matrix, 20 µL of rennet (Naturen®, 520 mg/mL active chymosin Christian Hansen) were added to 1 mL of milk and placed at 37 °C for 40 min. After gelation, samples were transferred into wells of 96 wells µClear® cell culture microplate (Greiner Bio-One).

In a second time, the viability of microorganism during acid stress was determined with a Filmtracer™ LIVE/DEAD® Biofilm Viability Kit including two fluorescent markers. SYTO® 9 nucleic acid stain 482/500 (commercial solution at 3.34 mM in DMSO) labels all bacteria in a population (those with intact membranes and those with damaged membranes). In contrast Propidium iodide nucleic acid stain 490/635 (commercial solution at 20 mM in DMSO) penetrates only bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present (the mechanism is a combination of displacement of SYTO® 9 by Propidium Iodide and quenching of SYTO® 9 emissions by fluorescence resonance energy transfer; Stocks SM in Cytometry A, 2004).

The LIVE/DEAD staining was realized on the synthetic medium only. For this double staining, 0.5 µL of each stain were added directly in the 250 µL of sample in the wells of the microplate. HCl 0.05M was added to reach the concentration of 2.3 mM in the sample, corresponding to pH 2 in the Synthetic medium.

Samples were incubated in the dark at room temperature for at least 10 minutes for staining, and then mounted on the motorized stage of an inverted confocal microscope (Leica TCS SP8 AOBS, Leica Microsystems) at the MIMA2 platform (http://www.jouy.inra.fr/mima2/). A water immersion objective lens of 63X/1.2 N.A., (working distance 300 µm) was used. 2D sections were acquired at 600 Hz scan speed with bidirectional scan X direction mode, with numerical zoom 1, with 512 x 512 xy image definition (pixel size 361 nm), with a 8-bit dynamic range per pixel, with pinhole at Airy 1 (111.5 µm diameter) and with a z-step of 1 µm.

For analysis of matrix components, Bodipy® 493/503 (neutral lipids) was excited at 488 nm using an argon laser (output power at 30%, AOTF at 3%) and the emitted "green" fluorescence was recorded from 493 to 600 nm with a PMT detector with a 600 V gain. Simultaneously SYTO® 61 (microorganism) was excited with HeNe 633 nm laser (AOTF at 5%) and the emitted "red" fluorescence was recorded from 638 to 800 nm with a PMT detector with a 600 V gain.

Sequentially, DyLight™ 550 (proteins) was excited with 561 nm laser diode (AOTF at 0.6%) and the emitted "yellow" fluorescence was recorded from 570 to 615 nm with a PMT detector with a 625 V gain and Offset -1.

For the LIVE/DEAD fluorescence settings, Syto9 (live cells) was excited at 488 nm using an argon laser (output power at 30%, AOTF at 1.5%) and the emitted "green" fluorescence was recorded from 500 to 545 nm with a PMT detector with a 625 V gain. Simultaneously Propidium iodide (dead cells) was excited with the same laser and the emitted "red" fluorescence was recorded from 630 to 725 nm with a PMT detector with a 700 V gain. Image analysis was carried out using ImageJ software V1.49 (NIH, Bethesda, USA). The microscopic experiments were performed independently from DIDGI®'s one.

Calculation of matrix buffer capacity

The amount of HCl used to reach pH2 in the stomach phase of DIDGI system was determined through the SToRM software for each tested matrix. The resulting pH-titration curves where used to calculate the buffer capacity through equation (Eq.2) (Salaün *et al.*, 2005).

$$\frac{dB}{dpH} = \frac{Volume\ of\ acid\ (mL) x\ Normality\ of\ the\ acid}{Changes\ in\ pH\ x\ volume\ of\ sample} \quad \text{..... .....Eq. 2}$$

Dilution of sample with the volume of acid used was not taken in consideration, for not been considered to be significant in the resulting buffering curves (Upreti *et al.*, 2006).

On line viscosity measurement

Rheological characteristic of food matrix was measured on-line during gastric digestion with the same rheometer used for the gel characterization. These experiments were conduct simultaneously with DIDGI®'s ones. To better approach to DIDGI®'s digestion in term of matrix volume and pH adjustment, we developed a specific system to allow torque measurement during digestion and adapted it to the commercial rheometer. The system was composed by a glass vessel (high 165 mm, diameter 80 mm) surrounded with a heating mat to control temperature. A homemade stirrer was used to homogenize the matrix during digestion and to measure the evolution of the torque. The stirrer consisted of four stainless steel agitation blades (high 20 mm, long 35 mm) connected to the rheometer. The turbine blades were fully immerse in 200 mL of the matrix in the glass vessel. A pH, redox and temperature probe (Electrode InPro 480i/SG/120, reference 52003581, Mettler Toledo, France) was connected to the Storm software to measure and control the conditions during the experiment.

As describe for DIDGI®'s experiments, 200 mL of S, SM, WM, GSM or GWM were introduced in the glass vessel of the rheometer. Rennet gels were cut in cubic pieces with edges of 1.5 cm just before the introduction. The stirrer and the pH probe were placed in the matrix and the measurement of the torque began. Pepsin and HCl were incorporated in the same conditions than for the digestion (Table 2.5). The rotating speed of the stirrer was the same than for the digestion experiment (60 rpm).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to analysis, samples kept at – 20 °C were defrosted and homogenized during 15 s. at 15500 rpm using an Ultraturrax (T25, Janke & Kunkel,). Samples were stabilized at 25 °C for 20 min before analysis.

For the SDS-PAGE analysis, protein contents was calculated using the spectrometric method reported by Lüthi-Peng and Puhan (1999) with slight modifications. Briefly, 100 µL of sample were diluted in 1900 µL of 6 M guanidine-HCl in 0.1 M Na acetate buffer, pH 5.0. The absorbance at 280-320 nm was measured with a UV-Visible spectrophotometer (Evolution 201, ThermoFisher Scientific, France) and used to calculate the protein content of the samples. The

calibration curve was performed with the same dairy proteins content in the skimmed milk powder.

SDS-PAGE was performed using the 4-12% polyacrylamide NuPAGE SDS-PAGE precast gels (ThermoFisher Scientific, France). Samples were diluted (with running buffer) to reach a final concentration of 10 µg of proteins to be loaded in each lane. Gels were fixed in 700 mL of 20X NuPAGE MES SDS running buffer. Running was at 60 mA/ gel and 120 V for 2 h. After washing 3 x 5 min with deionized water, gels were stained 1h with Colloidal Blue Staining Kit (Simply Blue Safe Stain, ThermoFisher Scientific, France). A low molecular weight marker (SeeBluePlus 2, ThermoFisher Scientific, France) was used to identify the proteins in the gel. At least 2 gels were run of each food matrix. Image analysis of SDS-PAGE gels was carried out using PDQuest 2-D analysis software, version 6.2.1.

Free fatty acid by GC

For free fatty acid (FFA) analysis, digestion samples of SM, WM and GWM were obtained just at the digestion starting (T0), after 40 min and 70 min in the stomach (E40 and E70, respectively), and after 60 min, 120 min, and 240 min in the intestine (I60, I120, and I240, respectively). The SM sample was used as witness.

For FFA extraction, 50 µL of digestion sample was added with 100 µL of internal standard solution (0.49 µg tridecanoic acid/mL in $CHCL_3$) and dehydrated with Na_2SO_4 anhydrous. Next, it was extracted with 1 mL of diethyl ether/heptane (1:1 v/v) and shaken vigorously for 3 min. The organic supernatant was separated and the extraction was repeated and both extracts were combined.

Solid phase extraction (SPE) extraction of FFA was performed as described by Voigt, Chevalier, Qian, and Kelly (2010). Next, FFA extracts solvent was evaporated to dryness with a speed-vac system (Thermo) and dissolved with 100 µL of $CHCl_3$, put in a tight closed micro-vial and analyzed by GC-MS.

FFA extracts were analyzed by gas chromatography (Agilent Technologies 6890, Palo Alto, Ca.) coupled with a mass selective detector (MS 5,973 N, Agilent Technologies, France) and a CTC autosampler (CombiPAL, Agilent Technologies, France).

FFA were separated with a DB-FFAP column (30 m x 250 µm x 0.25 µm, Agilent Technologies) using Helium as carrier gas at 1.2 mL/min. oven temperature was programed as follows: after 1 min at 40 °C, temperature was increased at 10 °C/ min to 240 °C and hold 30 min. Injector temperature was set at 250 °C. Under these conditions, 1 µL sample was injected in the splitless mode. The mass spectra were recorded at 70 mV in the total ion mode (SCAN) with an m/z 33-550.

Quantification was performed with the internal standard method. FFA reference compounds were provided by Sigma-Aldrich (St. Louis, MO, USA) with a minimum purity of 98%. Standard calibration curves were obtained with successive dilution to obtain concentration range between 0.35 µg/mL and 935.0 µg/mL. Calibration curves were considered satisfactory if they showed a high correlation ($r^2 \geq 0.980$).

Statistics analysis

The statistical analysis was performed using Statistica software V12 (Statsoft Inc., Tulsa, OK, USA). Results are expressed as the mean ± standard deviation of the three biological independent experiments and were compared using a Student's test. Differences were considered statistically significant when $p < 0.01$. The comparison of the survival rates of the strains was performed in each compartment independently of the other. For each strain, at each time of sampling, Student's test was used to study the effect of the medium. For each strain in one medium, Student's test was used to study the effect of the time. For FFA, analysis of variance (ANOVA) and the Fisher's least square differences (LSD) were applied, they were considered statistically different when $p < 0.05$.

Results and Discussion

Survival of the microorganism to digestion

In vitro experiments

The effect of *in vitro* digestive stress on the survival of *HA*, *BA* and *ST* was tested in the dynamic digester DIDGI® and the protective effects of different dairy matrices (S, SM, WM, GSM, GWM) were compared (Figure 2.12 and 2.13). The statistic results on the effect of time or of the media are also reported in the figures. The strains exhibited different behavior in each digestive compartment.

The gastric phase

HA was the most resistant microorganism during all the digestion process. In stomach it remained constant, 7.08 ± 0.07 log CFU/mL, during the first 60 min (Figure 2.12A). When pH reaches 2 (at 70 min) the viability of microorganisms began to decrease to reach an average loss of 2.6 log CFU/mL at the end of gastric phase.

Slight differences between matrices were observed at the end of this phase (100 min). The viability decreased in all the matrices to less 5 log CFU/mL except in SM media, for which the level was 5.56 log CFU/mL.

Meanwhile, *BA* was more sensitive to gastric stress, than *HA* as shown in figure 2.13A. The viability level of *BA* decreased slightly, 0.38 log CFU/mL during the first 30 min in the gastric phase. When pH reached to 2.7, viability decreases on average 1.25 log CFU/mL. Slight differences between matrices were observed during the first 30 min of gastric digestion. WM (6.98 ± log CFU/mL) was slightly higher than the other matrices (6.35 ± 0.12 log CFU/mL) and SM was the lowest one (6.09 ± 0.32 log CFU/mL). At 50 min WM and GSM media exhibited higher viability (5.9 ± 0.1 log CFU/mL) when compared to S, SM and GWM (5.4 ± 0.13 log CFU/mL). However, differences were not statistically significant ($p < 0.01$). At 60 min *BA* was only found in SM at a level of 3.92 ± 0.97 log CFU/mL. Still, during the first 50 min, high level of microorganisms, between 7.9 and 8.6 log CFU, were capable to reach the duodenal phase.

ST was highly sensitive to the gastric phase. It remained viable (4.39 ± 1.7 log CFU/mL) during 30 min in the stomach, until pH reaches 4.6 (data not shown). Within the next 10 minutes, the concentration decreases very quickly and dropped under the detection limit and it was no longer found in any of the subsequent compartments within any of the tested matrices.

The duodenal phase

HA showed strong resistance to duodenal stress and the viability remained constant 80 min between 6.5 to 7.2 log CFU/mL (Figure 2.12B). Then, the viability lost an average of 1.03 log CFU/mL at the end of the duodenal phase. Slight differences in viability were observed between matrices at 120 min. S and WM media exhibited higher viability (6.37 log CFU/mL) when compared to SM, GWM and GSM (5.57 log CFU/mL). However differences were less than 1 log CFU/mL.

Meanwhile, *BA* was also resistant to duodenal stress (Figure 2.13B), although the decrease in viability was sharper that the one observed for *HA*. Viability of *BA* decreases constantly, losing on average 1.73 log CFU/mL in all matrices over the duodenal exposure. The drop was faster for SM and GWM matrices which exhibited the lowest levels at 120 min. However this could be due to a lower quantity of microorganism coming from the stomach compartment. In this compartment of the digester, *ST* could not be detected.

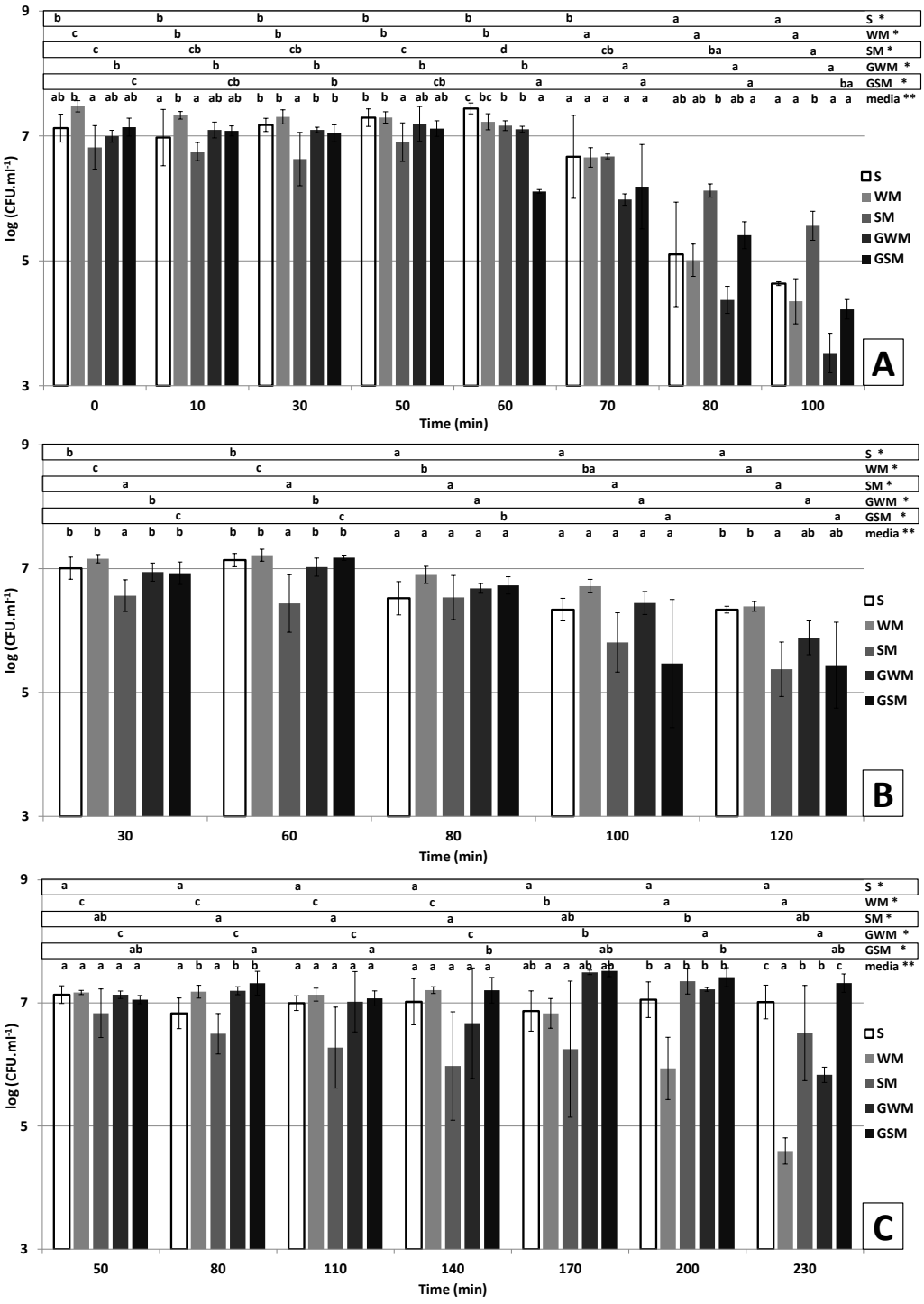


Figure 2.12: Survival of *HA* in five matrices (S, WM, SM, GWM, and GSM) *during in vitro* A) gastric, B) duodenal and C) intestinal compartments. * Result of statistical analysis of time effect by matrix**. Result of statistical analysis of media effect at the same time of digestion.

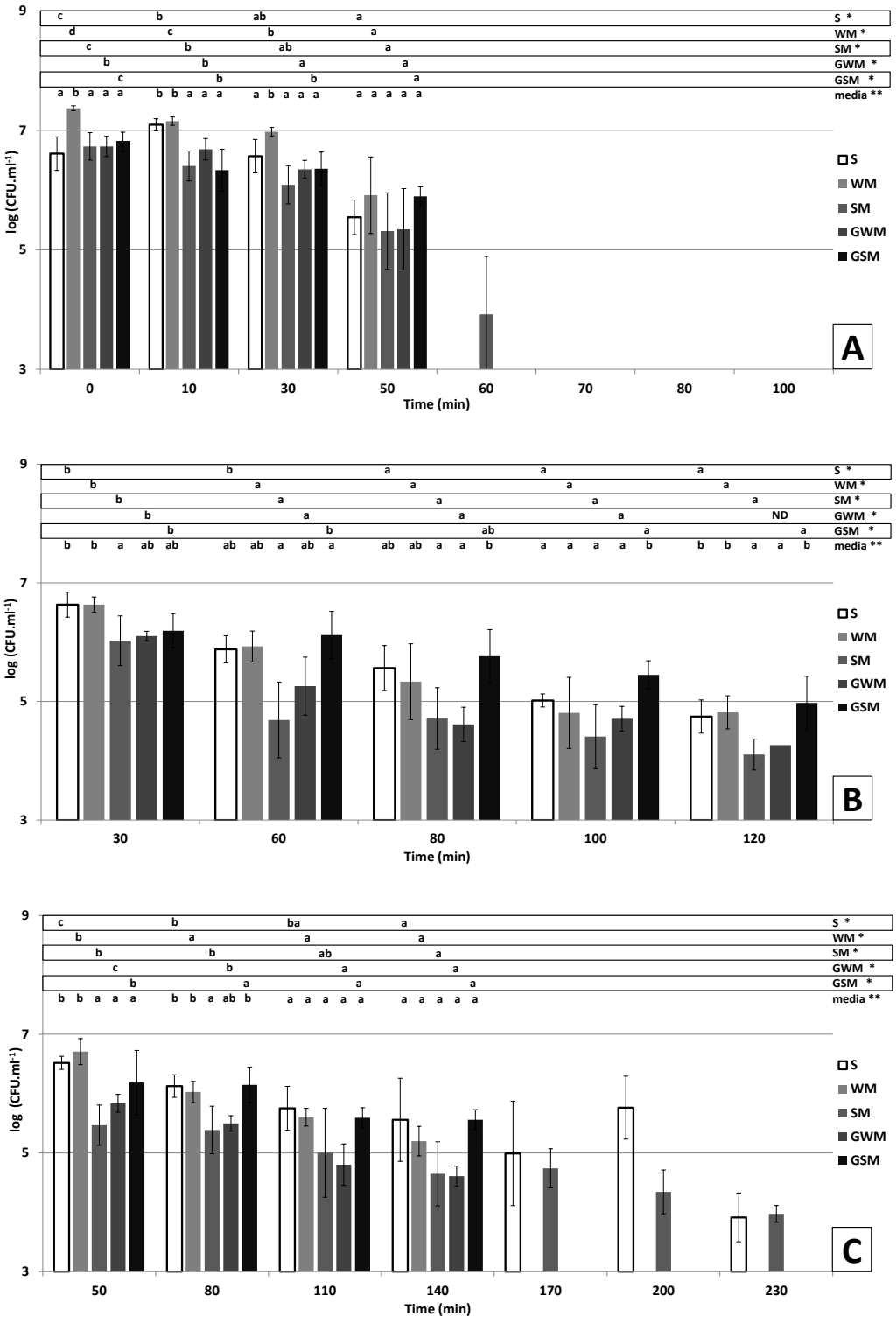


Figure 2.13. Survival of *BA* in five matrices (S, WM, SM, GWM, GSM) during *in vitro* A) gastric, B) duodenal and C) intestinal compartments. * Result of statistical analysis of time effect by matrix**. Result of statistical analysis of media effect at the same time of digestion.

The intestinal phase

HA also showed strong resistance to the stress in the intestinal phase (Figure 2.12C). All the matrices displayed similar initial level of viability in the small intestine (7.06 ± 0.05 log CFU/mL). Levels in S, WM, GSM and GWM remained constant over 140 min of intestinal digestion. *HA* level decreased 0.86 log CFU/mLin SM matrix in the same period but the difference was not statistically significant. However, significant differences ($p < 0.01$) were observed between matrices at 170 min. S, SM and GSM viability level remained relatively constant. GSM was the matrix with the highest viability of *HA,* 7.32 ± 0.15 log CFU/mL, for GSM 7.02 ± 0.27 log CFU/mL for S and 6.51 ± 0.77 log CFU for SM. The counts of *HA* in WM fell gradually after 170 min until lose 2.6 log CFU/mL by the end of the experiment. The same behavior was observed in GWM. From 200 min to the end of the intestinal phase (230 min) the level of *HA* had a significant drop of viability (1.3 log CFU/mL) compared with the initial value (Figure 2.12C).

On the other hand, *BA* was clearly more sensitive than *HA* to the intestinal stress (Figure 2.13C). *BA* reached the intestine in an initial concentration between 6.71 log CFU/mL (WM matrix) and 5.47 log CFU/mL (SM matrix). All the matrices displayed a similar behavior until 140 min. The viability of *BA* decreased slightly with an average loss of 1.03 log CFU/mL. The slight differences observed during this time were further attributed to differences in the initial count in this compartment and differences were not significants. After 140 min *BA* only survived in S and SM while the viability in WM, GSM and GWM descended below the detection limit of the plating method. *ST* could not be detected in this compartment of the DIDGI®.

The biggest loss of viability, occurred during the gastric phase, probing that low pH represents the most intense stress faced by the microorganism during all the digestive process. *HA* was resistant to the digestion stress up to pH 2 whereas *ST* and *BA* were sensitive to the gastric stress, and disappeared in the gastric compartment when the pH reached 3. However, due to the dynamic characteristics of the DIDGI®, the stress in all compartments was gradually applied, allowing to some microorganisms sensitive to the low pH to be transferred from the stomach to the duodenal and intestinal compartments before the gastric stress became more aggressive (it was the case for *BA*). Our first hypothesis assumed that the inclusion of

microorganisms in a food matrix might improve its ability to survive to the gastric stress. However our results did not reveal a protective effect of the dairy matrices tested. In contrast, in some cases the composition of the food matrix could be considered as negative environment for the microorganism as observed in the intestine with *HA* included in the matrices with fat content. These results makes raises questions about the effect of fat and the fatty acids liberated by the food matrix in the intestinal phase on the viability of *HA*.

Effect of the pH on the microorganism survey

The structure of WM and GWM were visualized by CLSM just after mixing with the strains (pHi = 6.5). Milk proteins were colored in yellow, fat spherical globules of different sizes in green and the microorganisms in bright red color (Figure 2.14). When the milk was coagulated by rennet, the proteins formed a porous network (pale yellow color) surrounded by lactoserum (unstained components) and containing fat spherical globules of different sizes (bright green color) (Figure 2.14B). The distribution of the microorganisms was not different between the two matrices. They were located in the aqueous phase without closeness marked for proteins or fat.

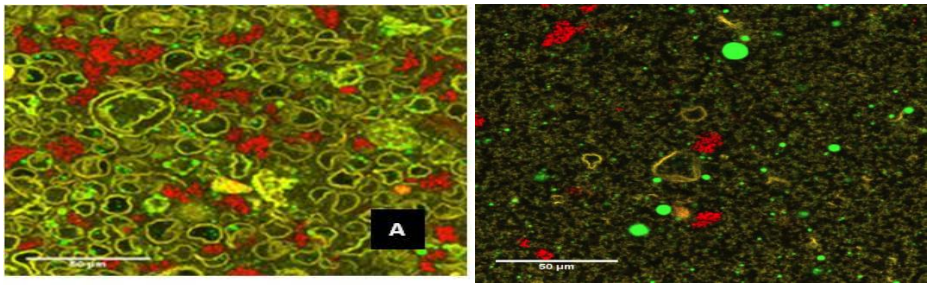


Figure 2.14. Confocal Laser Scanning Microscopy images showing the protein (yellow), the fat (green) and the microorganisms (red) in A) whole milk and B) in rennet gel of whole milk.

To support the effect of pH on the viability of the three strains, further investigations were conducted using LIVE/DEAD cell viability kit (Figure 2.15). The two different nucleic acid stains allowed distinguishing live bacteria with intact plasma membranes (green color) from dead bacteria with compromised membranes (red color). After incubation 20 min at pH 2, the micrographs confirmed the difference of viability between *HA*, *BA* and *ST*. In *HA* culture (Figure 2.15A), very few red cells were observed indicating that the cells had intact membrane. In *BA* and *ST* culture (Figure 2.15B and C respectively), the two type of fluorescence were visible. After

incubation, the proportions of damaged cells were respectively 2, 11 and 40% for *HA*, *BA* and *ST*. (ImageJ, statistical tools).

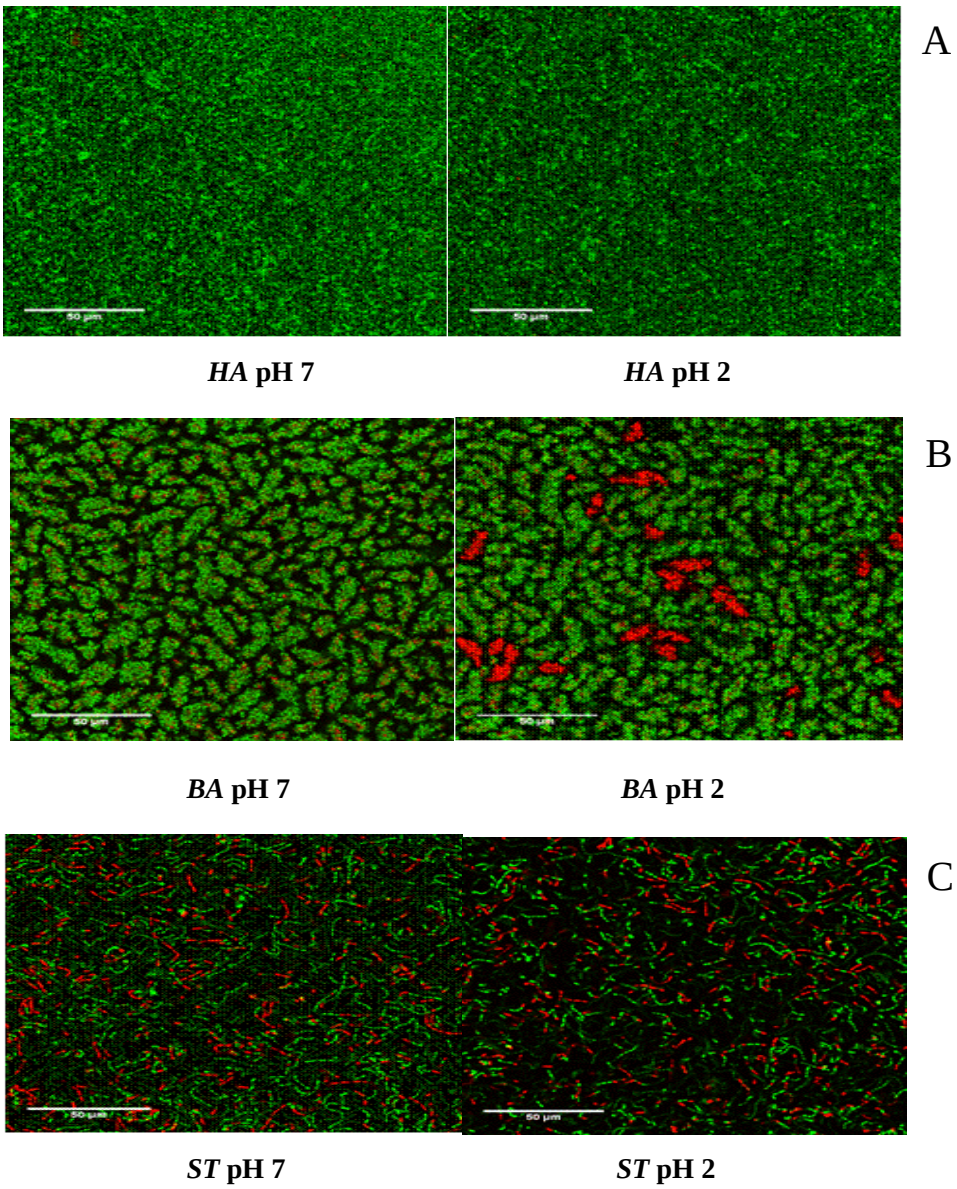


Figure 2.15. Confocal Laser Scanning Microscopy images showing live (green) and dead (red) cells of A) *HA*, B) *BA* and C) *ST* under the action of pH 2 after 20 min of incubation.

***In vivo* experiments**

A total of 3,103,358 16S rRNA (V3-V4 regions) sequencing reads were generated from the 72 mouse fecal samples. An average of 36,577 sequences per sample were obtained after

quality filtering and chimera removal. They were clustered into 244 bacterial OTUs with an abundance > 200 sequences in the entire dataset. It was not possible to detect *BA* and *HA*. *Streptococcus salivarius* was detected at low levels in almost all samples but this species is composed of two subspecies which are not distinguishable based on their 16S rRNA gene sequence, namely the cheese starter S. *salivarius subsp. thermophilus* and the commensal *S. salivarius subsp. salivarius*. The bacterial community structure did not vary neither according to the day of sampling nor the food matrix used for feeding (Figure 2.16), which indicated that the treatment (feeding mice with dairy matrix with or without microorganisms) did not modify the overall composition of the mouse microbiota.

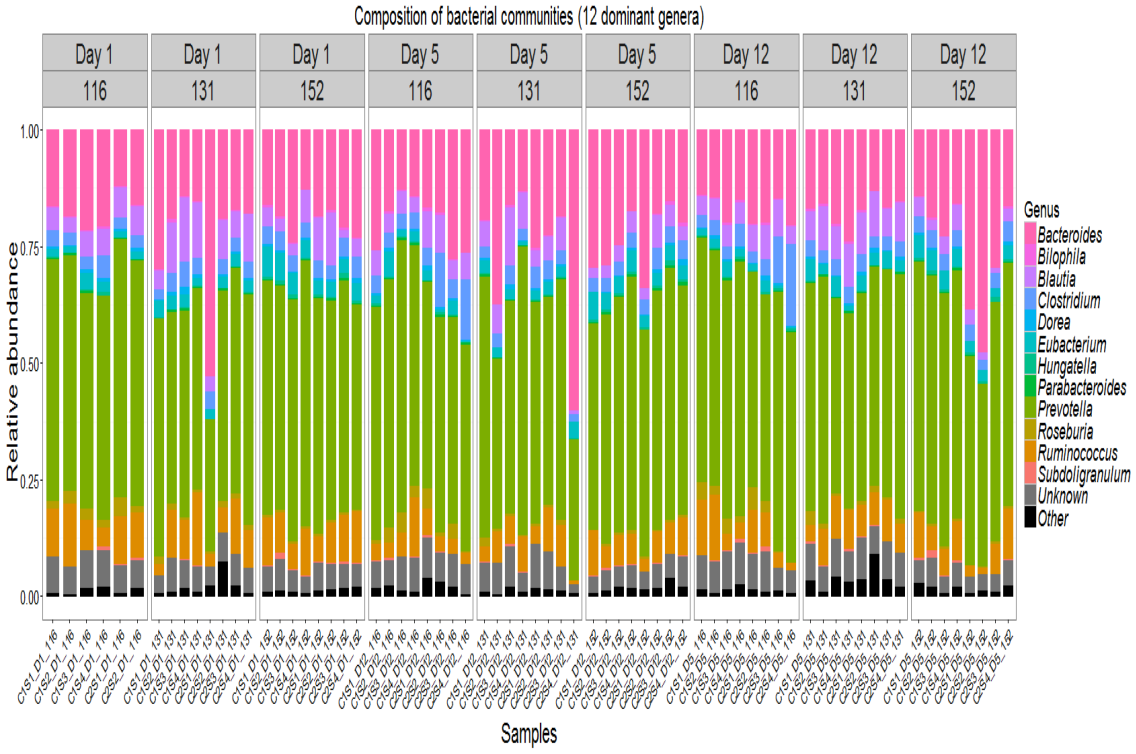


Figure 2.16. Composition of the bacterial communities from fecal samples of individual mouse according to the day of sampling (d1, d5, d12) and the tested group (116: standard feeding; 131: whole milk, WM; 152: whole milk + microorganisms).

Chemical and physical characterization of matrices during the digestion

Rheological properties of milks and gels during digestion

Figure 2.17 represents the average and standard error of the torque for the milk and rennet gel. We did not observed significant differences between matrices with our without fat, the average signal was calculated for the milk with or without fat and for the gel with or without fat.

To highlight the role of the digestion on the evolution of the matrix, effect of cutting and stirring on the rennet gel was tested replacing pepsine and HCl by water. Results, identified as Mechanical action are represented on figure 2.17. In these conditions without enzyme and acidification, initial torque of gel was 0.6 mN.m, descended slowly to 0.4 mN.m at 60 min and remained stable until the end. When enzyme and acid were added, the torque profile of gel during digestion changed. In the first minute torque of rennet gel decreased swiftly from the initial 0.6 to 0.5 mN.m. Then it continued decreasing linearly during 50 min until 0.15 nM.m. Between 55 – 70 minutes the signal increased slightly to 0.18 mN.m. After this period, torque went back down slowly and remained stable until the end of the experiment.

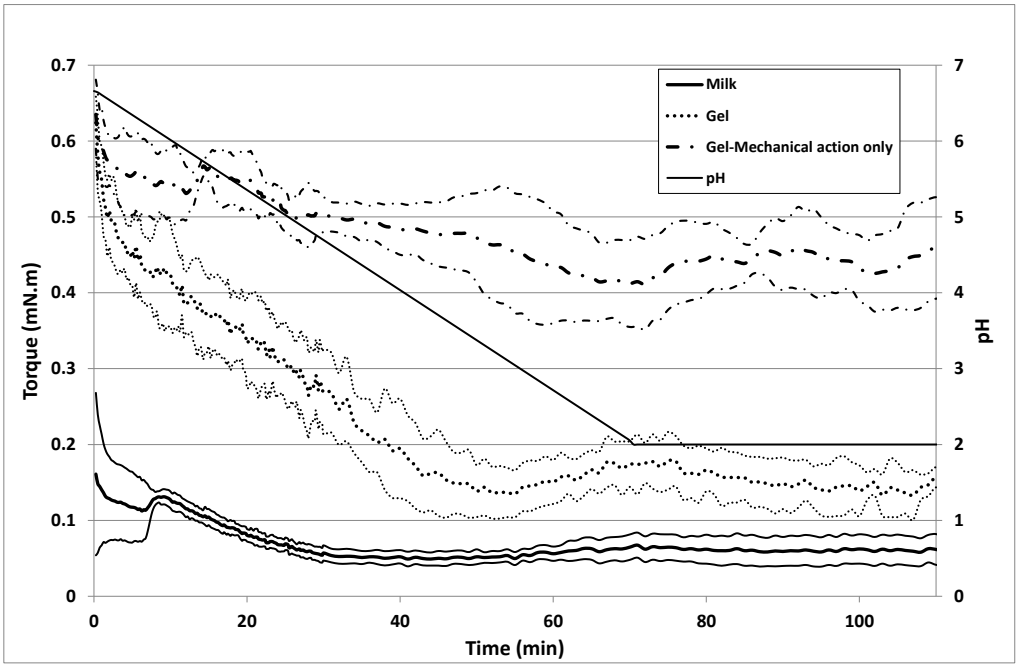


Figure 2.17. Evolution of the torque during digestion for milk and rennet gel matrices.

Moreover, significant differences were observed between milk and rennet gel. For milk matrix, torque decreased from 0.15 to 0.05 mN.m after 40 min and remained stable except a weak increase of 0.02 mM.m at 55 min corresponding to pH 3. This behavior might be due to the effect of stirring, that didn't allow the formation of a homogeneous acid curd. However we observed flocculation in the reactor.

Matrices buffering capacity

The buffer capacity (dB/dpH) of the 5 matrices S, SM, WM, GSM and GWM in the gastric was determined mathematically from the pH titration curves measured in the DIDGI® system. For the synthetic medium only 2.7 ± 0.4 mmol of HCl 0.5 m were sufficient to reach pH 2 (Figure 2.18).

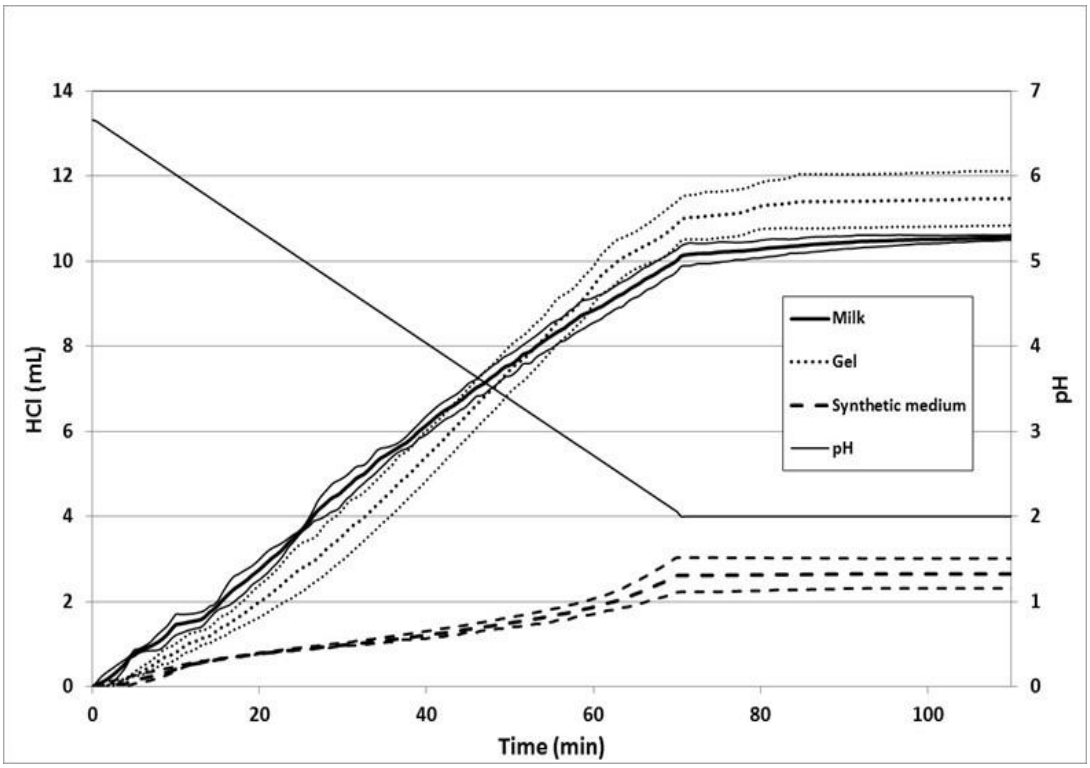


Figure 2.18. The change over time in HCl 0.5 M added during gastric phase in DIDGI® experiments, for each matrix.

For dairy matrices we observed not difference between SM and WM and between GSM and GWM. Thus fat addition was not significant for acidification. The average amount of HCl 0.5 M necessary to reach pH 2 was statically different; 10.6 ± 0.02 mM for milks and 11.5 ± 0.6 mM for gel. Thus, the quantity of HCl used to reach stomach pH in the DIDGI® system increased proportionally to the complexity of the matrix, imitating the *in vivo* conditions were the acid secretion in the stomach is highly related to the nature of the bolus (Kong & Singh, 2008).

Buffering capacity was calculated deriving the pH titration curve with the equation of Salaün *et al.,* (2005) (Figure 2.19). Therefore milk in figure 2.19 represents the average of SM and WM trials and gel the average of GSM and GWM trials, and their respective standard deviations. When the synthetic medium was acidified from pH 6.5 to 2, dB/dpH remained stable from pH 6.5 to 3 at a lower value than 0.005. Then, it increased to reach 0.0125 at pH 2. dB/dpH of milk and gel matrices increased between pH 6.5 and 5.2. For milk, the maximum of dB/dpH (0.017) was reached. Then, it decreased to 0.015 at pH 5 and remained stable. The values in gel continued to rise until 0.024 at pH 2.75 and then decreased to 0.015 at pH 2.

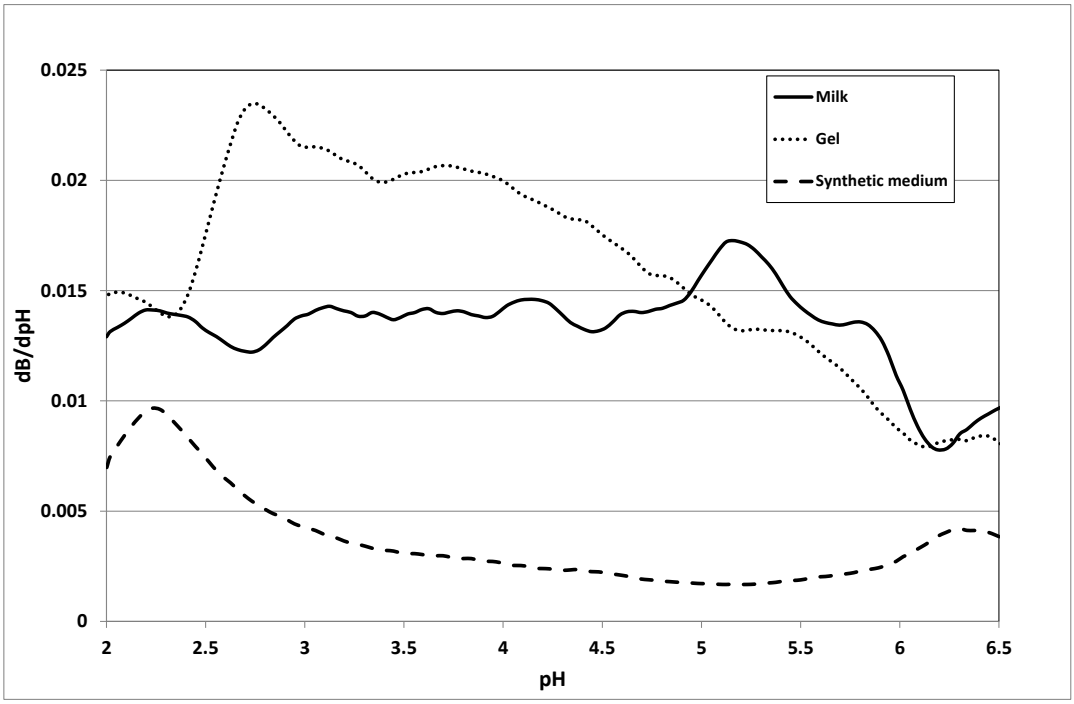


Figure 2.19. Buffering curves for milk and rennet gel matrices during gastric phase in DIDGI® experiments.

Protein profiles during *in vitro* digestion

Figure 2.20 shows SDS-PAGE profiles of WM and GWM matrices during gastric phase. Matrices with or without fat presented very similar trends therefore only results with fat were shown. The kinetics of hydrolysis differed between caseins, and whey protein (β-lactoglobulin and α-lactalbumin) but no or very weak significant differences were observed between the matrices. Caseins degradation in the stomach started immediately and the percentage of intact casein after 100 min was 70% (Figure 2.21A). At 10 min, a small matrix effect was observed where liquid matrices had lower with a lower percentage of intact casein. In spite of variation of percentages of whey proteins (Figure 2.21B), it could be assumed that β-lactoglobulin and α-lactalbumin were not degraded over the gastric phase and were resistant to pepsin hydrolysis.

All bands corresponding to caseins and whey proteins were not visualized for all the matrices in the intestine samples (data not shown).

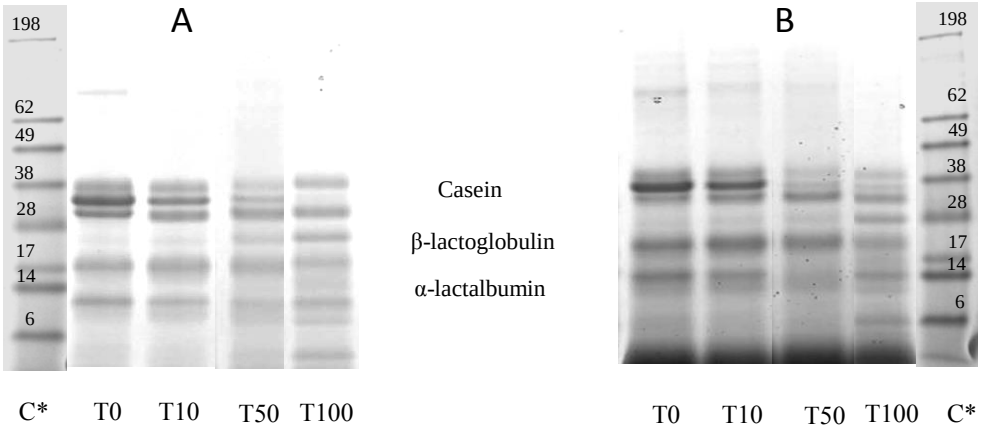
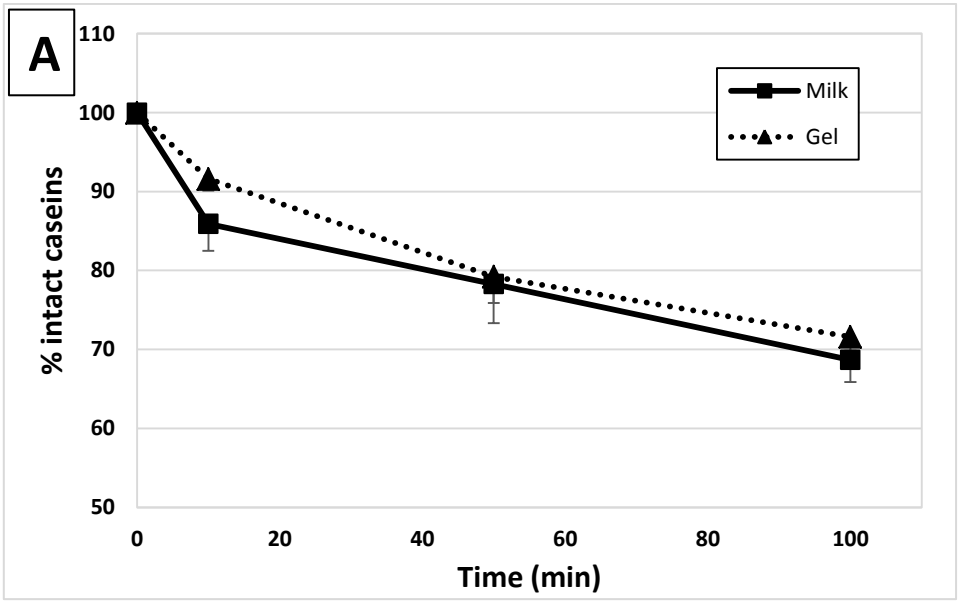


Figure 2.20: SDS page analysis of the evolution of proteolysis during gastric digestion of A) whole milk and B) rennet gel of whole milk. C*: Kda protein standard



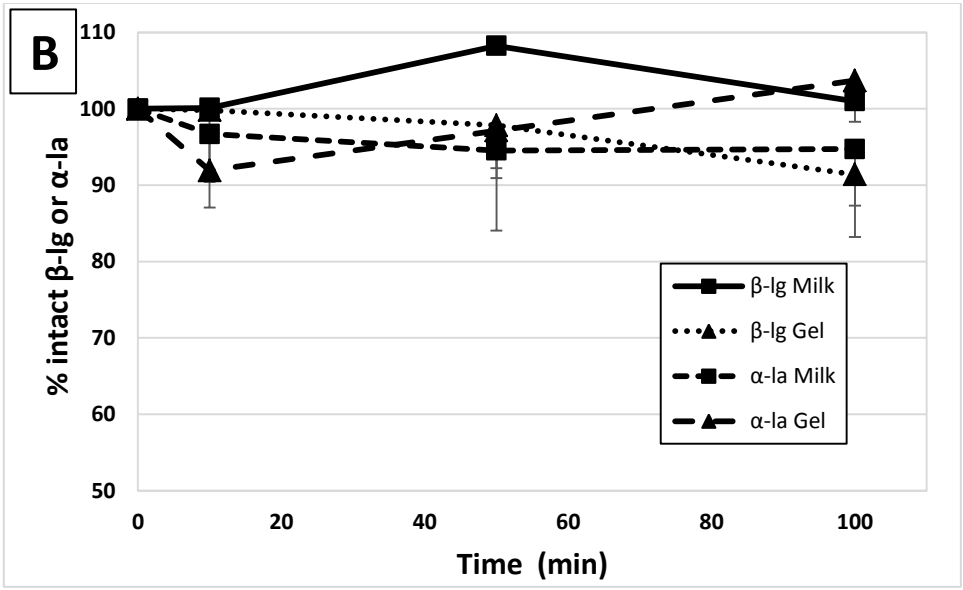


Figure 2.21: Kinetics of proteolysis of A) casein, B) β-lactoglobulin and αlactalbumin during gastric digestion of SM, GSM, WM and GWM.

Free fatty acid profiles during *in vitro* digestion

Figure 2.22 shows the free fatty acid (FFA) released during digestion process of WM and GWM matrices. The FFA profiles of both matrices had as the most abundant acids the C16:0, C18:1 n9, and C18. Short chain FFA showed low concentrations throughout digestion.

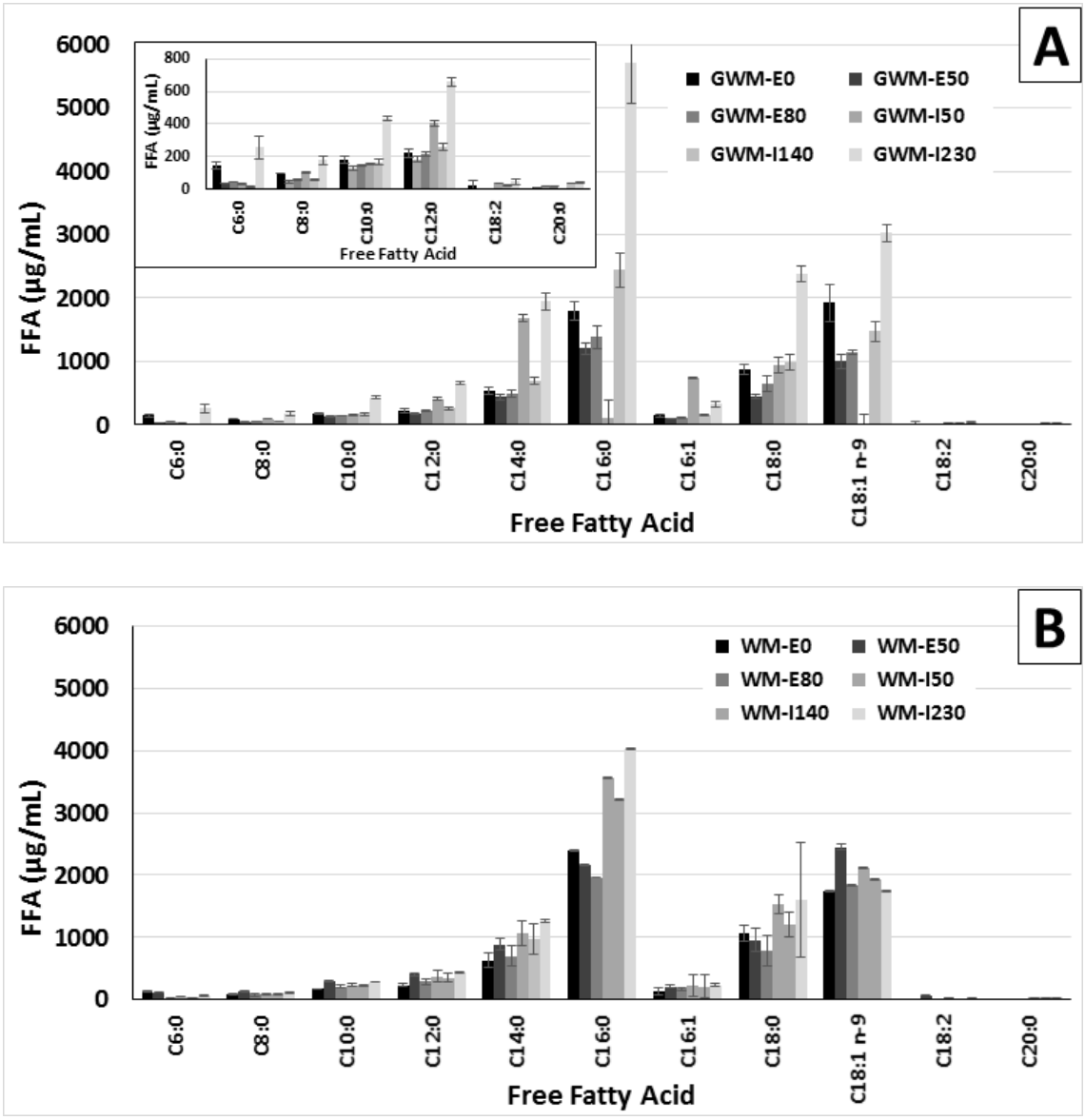


Figure 2.22. Free Fatty Acids released during digestion of A) Rennet gel of whole milk (GWM) and B) whole milk (WM).

For both WM and GWM all the analyzed FFA showed significant differences ($p<0.05$) according with the digestion time except C18:2 in WM. On the other hand, significant differences ($p < 0.05$) were observed between matrices all the FFA analyzed except for C6 and C20:0. Globally, the release of FFA was higher in WM samples than in GWM except at the end of

digestion (I230) where values were higher in GWM samples. Both matrices showed a lower FFA release during the stomach digestion than in the intestinal phase.

In the stomach, the lipolysis rate was significantly higher for WM samples than GWM samples most of the analyzed FFA showed higher concentration in WM. For WM, FFA concentration was remained steady throughout gastric digestion, while for GWM, FFA concentration was higher at the beginning of digestion than remained steady between E30 and E60.

On the other hand, in the intestine the lipolysis was higher than in the stomach for both matrices. For WM (Figure 2.22B) the behavior depended on the chain length, short chain FFA (C6-C12) remained steady between I50 and I140 and increased at the end of intestine digestion; medium chain saturated FFA (C14-C18) and C16:1 fluctuate throughout intestine digestion; while C20 increased steady. For GWM (Figure 2.22A), most of the evaluated FFA increased steady throughout digestion reaching higher values than in WM except for C16:1 and C20 that remained steady and increased only at the end of intestine digestion.

Discussion

One of the main objectives of this work was to evaluate the survival of cheese microorganisms in conditions of stomach and duodenal stress, expressed mainly by low pH and presence of bile, respectively. To further analyze the ability of a dairy matrix to enhance the survival rate and the degradation of the matrix during digestion.

Thus, we can consider that the *in vitro* digestive model, DIDGI®, used in our work, was adequate to test the digestion of dairy matrices, with different compositions and structures, using the same operation parameters, which allowed us to make a realistic comparison between matrices. It was already used to study the viability of the microorganisms (Adouard *et al.*, 2016) and the digestion of milk proteins (Ménard *et al.*, 2014).

Microorganism survival

When a microorganism goes through digestion, it suffers two main stresses: low pH in the gastric phase and the bile salts in the duodenal phase. The effect of enzymes will be no further discussed as according to Sumeri *et al.,* (2012) it is not significant in the survival of microorganisms.

Although the response of these strains to digestive stress has not been fully characterized, its development during cheese ripening gives us a trace to explain their behavior through digestion.

ST is part of the starter bacteria used at the beginning of cheese-making to transform lactose to lactate, descending pH in the matrix (Irlinger *et al.*, 2012). However, in our results we observed that *ST TIL257* was highly sensitive to low pH. When pH decreased beyond 4, after 30 min of gastric digestion, its viability descend below our detection limit. However, low levels of *Streptococcus* were detected through 16S rRNA gene sequence, in almost all samples from *in vivo* analyses.

Different works have focused on analyzing the ability of *ST* to resist in vitro and in vivo stress because it is wide use on dairy industry and of its potential health probiotic properties (Uriot *et al.*, 2016). Our *in vitro* results are in accordance with previous works of Adouard *et al.,* (2015) who observed that *ST* didn't survive to neither gastric and duodenal *in vitro* digestion; and partially in agreement with those of Marteau, Minekus, Havenaar, and Huis In´t Veld (1997) where authors observed that *ST* survived only briefly in the gastric phase during *in vitro* digestion of yogurt. However García-Hernández, Moreno, Chuan, and Hernández (2012) observed that *ST* was slightly resistant during gastric *in vitro* digestion of yogurt but highly resistant to duodenal digestion. Differences with our results in the duodenal phase might be due to the used of pancreatic juice without bile addition by García-Hernández *et al.,* (2012).

On the other hand, previous *in vivo* are results contradictory among researchers, while some authors have found an important number of viable cells of *ST* after *in vivo* digestions of cheese and yogurt (Firmesse *et al.*, 2008; García-Hernández *et al.*, 2012; Lay *et al.*, 2004; Mater

et al., 2005), others weren't able to find it in human feces after daily yogurt consumption (del Campo *et al.*, 2005; Elli *et al.*, 2006).

Interestingly Uriot *et al.,* (2016) tested the viability and mechanisms of stress response of 30 different strains of *ST* using an *in vitro* dynamic system. They found important differences in survival between strains, where sensitive to acid stress was attributed to deficiency in urease and small Heat Shock Proteins (sHSP).

BA was moderately sensitive to gastric stress. It was stable until pH reached 4, after this point its viability decreased dramatically, until below our detection limit after 50-60 min (pH 3-2.5). Gastric sensitivity of this microorganism was not surprising if we think in its growth characteristics while part of cheese microbiota. *BA* is part of the cheese ripening culture, that develops after deacidification of the medium by yeast metabolism, growing only when cheese pH reaches 6–7 (Irlinger *et al.*, 2012).

To our knowledge, the mechanisms of response to acid stress by *BA* have not been previously described. The only study related to this response is the one done by Halgasova *et al.*, (2002) on *Brevibacterium flavum* a related strain to *B. auranticum.* These authors probed the key role of *sigB* on growth and viability of *B.* f*lavum* as a general stress response protein and controls transcription of various stress related proteins in gram positive bacteria. Likewise Mounier *et al.*, (2007), analyzed the growth characteristics of *B. auranticum* 1-16-58 isolated from smear cheese and found that urease activity was not detected in this microorganisms which might give us an approach of why this microorganism is acid sensitive.

On the other hand, despite the sensitivity of *BA* to the gastric stress, enough viable cells reached the duodenal phase, between 7.9 and 8.6 log CFU, where they were rather resistant to bile stress. This behavior can be related with its ability to survive in cheese surface even when the matrix is salted in many occasions during cheese manufacture (Irlinger *et al.*, 2012; Mounier *et al.*, 2007). Genoma of *B. auranticum* 9174 exhibits an important number of osmoprotectant transporters (Monnet *et al.*, 2014) which might provide it a further cross-adaptation to other stress (Begley *et al.*, 2005). Additionally, the fact that this microorganisms was previously exposed to

acid stress in the gastric digestion could also provide it with a genetic adaptation (possible also expressed by sigB) that protects the microorganisms to further bile salts stress.

Still the mechanism of response of *BA* either to salt or to bile has not yet been completely elucidated and we can only hypothesizer about it. Our results about the survival to gastric and duodenal stress exhibited by this strain are in accordance to the previous works of Adouard *et al.,* (2015) and Adouard *et al.,* (2016), where authors studied the viability of several cheese microorganism during gastric and duodenal stress, in both ,batch and dynamic *in vitro* digestion, testing three different strains of *B. auranticum* concluding that strain 9174 was sensitive to gastric stress but resistant to duodenal conditions.

Finally, *HA* was highly resistant to digestive stress. During gastric phase its viability remained stable up to pH 2 (60 min) and then decreased to reach 4.46 log CFU/mL at 100 min. *HA* is a gram-negative bacteria part of the *Enterobacteriaceae* family. It is genetically and phenotypically related to *E. coli* (Janda & Abbott, 2006), a bacteria capable to proliferate in the gastrointestinal tract. Moreover, studies have proposed the role of clinical strains of *H. alvei* in gastric diseases (Bobko *et al.*, 2013; Vivas *et al.*, 2008) and therefore, its ability to survive gastric digestion.

Viability of *HA* during digestive stress could be attributed to the presences of an outer membrane, characteristic of all the gram-negative bacteria that provide them with an extra protection to all kind of environmental stresses. Additionally *HA* is an ornithine and urease positive bacteria (Janda & Abbott, 2006), both enzyme increases the pH through decarboxylation and production of ammoniac respectively. However both enzymes could be later inactivated by the action of the enzymes present in digestion, thus the real mechanism of response of *HA* could be more related to genetic adaptation.

When passing to the duodenal phase, *HA* was also highly tolerant to bile, with no significant loss of viability. There is no references describing the mechanism of response of *HA* trough bile stress, by its approach with *E. coli* we could attribute its high survival rate to the effect of efflux pumps or a cross-adaptation to the previous mechanism related to the acidity

stress (Begley *et al.*, 2005). The observed results are in agreement with the previous works of Adouard *et al.,* (2015) and Adouard *et al.,* (2016) who tested the viability of different strains of *H. alvei* from dairy origin, in pure cultures, rennet gel and cheese during gastric and duodenal stresses. Authors concluded that *H. alvei GB01* was a strain capable to survive digestive stress despite the matrix used.

The ability of *H. alvei* to resist both gastric and duodenal stress results interesting and a further description of its mechanism of response is necessary. Moreover, all the response mechanism previously proposed for the tested strain rests as hypothesis that to be validated will need a further genetic study. Indeed there is a dramatic lack of data on how does the gram-positive bacteria adapt to stressful environments and we can only conclude that response of a microorganism is not only dependent of the species but furthermore to the specific strain as suggested by Pitino *et al.,* (2010).

Thus, as observed, the three tested strains had different response to *in vitro* digestion. This selection let us to evaluate objectively the effect of the food matrix in the survival rates.All strains had in common a significant decrease in viability during their passage through the stomach, confirming the hypothesis that low pH represents the most important stress suffered by microorganism during digestion Which was later supported by the images obtained by confocal microscopy, where we visually observed the increased mortality of the three microorganisms when the pH dropped from 6.5 to 2; that was more dramatically for *ST* and *BA* than *HA*: Therefore reinforcing the previously observed higher resistance of *HA* to gastric stress.

Meanwhile our in vivo analysis failed to demonstrate the survival of *BA, HA* and *ST* after digestion. The traces of *Streptococcus* found in feces were further attributed to a similar strain part of the GI tract (*S. salivarius subsp. salivarius*) since related bacteria can mask the strain.-specific signal as previously suggested by Bogovic-Matijasic *et al.,* (2015). In their work authors observed an increased in the sequences assigned to *ST* after *in vivo* digestion of both, fermented (control) and probiotic fermented milk, concluding that the presence of *ST* was due to a methodological interference.

Our results are in agreement with those of del Campo *et al.*, (2005) who observed that *ST* part of fresh yogurt, was not capable to survive through *in vivo* digestion; and in disagreement with those of Lay *et al.*, (2004) who found that when part of Camembert cheese *ST* was capable to survive *in vivo* digestion but not when it was part of fermented milk; suggesting than a solid matrix was more effective than a liquid one. However in our results, we did not observe any increase in viability when gel was used instead of milk. In this same work Lay *et al.*, (2004) concluded that *H. alvei* part of Camembert microbiota was not able to survive during *in vivo* digestion. This last observation was in disagreement with our conclusion about *HA* being fairly resistant to *in vitro* digestion (regardless the matrix). Difference with our results could be further due to Lay *et al.*, (2004) method interferences, since authors used nalidixic acid to avoid contamination, but this drug is also toxic for *HA* and other gram negative (McBee & Schauer, 2006).

Effects of matrices on the survival

Some authors affirm that survival of microorganism can be improved by the protective action of carrier foods (Faye *et al.*, 2012; Gardiner *et al.*, 1998; Pitino *et al.*, 2012). The theories that explain the protective effect of the food matrix refers to a) the effect of the microorganism´s preadaptation to the matrix b) interactions between microorganisms and dairy components or microstructure itself and c) the buffer capacity of the matrix during the stomach phase.

It is important to emphasize that in this study, microorganisms were growth in pure culture and were further included into the matrix instead of growing directly in the food matrix, thus preadaptation effect was not evaluated in this work.

Effect of dairy components and structure

Thus in our original hypothesis we expected that the modifications of composition and structure of matrices would enhance the viability of the tested strains, especially in the stomach phase because of the buffering effect of matrix (Upreti *et al.*, 2006); and that this effect would be increased according with the complexity of the matrix, as proposed by Gardiner *et al.*, (1998).

Though, our results in the stomach and duodenal compartments didn't show any significant effect on the viability of *ST*, *BA* and *HA* related to the composition (presence of fat) or structure (milk or rennet gel) of the matrix used except for *HA*.

Previous works have focused on the protective effect of the food matrices on the viability of different microorganisms during digestion; however results are contradictory, while some of them conclude that the viability can be enhanced when the microorganisms are included in a dairy matrix (Lay *et al.*, 2004; Saxelin *et al.*, 2010) others found opposite results (Adouard *et al.*, 2015). Our results can be partially comparable with those of Adouard *et al.*, (2016) where authors observed that *HA* was more resistant (during gastric phase) when grown in synthetic medium than in cheese.

Furthermore our results are contrast with previous results of Ranadheera *et al.*, (2012) which observed that probiotics survival to *in vitro* digestion was better in ice cream than in yogurt. They suggested that the higher content of fat in ice cream (10%) compared to yogurt (5%) could provide better protection to acid and bile stress. However the effect of the food matrix observed in previous references seems to be more related to the preadaptation suffered by the microorganism while growing in this medium, than to the food matrix itself.

Meanwhile, during the intestinal phase we observed slight differences between matrices. For *BA*, SM had better results than other dairy matrices, but similar to the observed for the synthetic medium (S), thus the use of a dairy matrix did not increase the survival through digestion. This is in disagreement with previous observations of Adouard *et al.*, (2016), where authors observed that survival of *BA* to in vitro digestion was higher when contained in a rennet gel than in synthetic medium.

On the other hand, for *HA*, fat in the WM and GWM matrices had a negative effect on survival during the intestinal phase. Our results are interesting, since it has been assumed that matrices with higher fat content provided better protection to acid and bile stress (Ranadheera *et al.*, 2012).

It is important to remember that in the intestinal phase only diluent (MDR) is added, so the mortality exhibited in this compartment might be due to residual activities of enzymes from gastric and/or duodenal phase. Since the main effect observed was in the matrices with fat, we attribute this effect to the residual lipase that hydrolyze the triglycerides in the matrix releasing free fatty acids that act as antimicrobial. The antimicrobial properties of fatty acids released by gastric lipases has been previously described by Sun *et al.,* (2002) concluding that both gram positive and gram negative bacteria can be inhibited by the hydrolysis of milk fat in the digestive tract.

Therefore, the food matrix could also decrease the survival rate through negative interactions between microorganisms and food components, as observed in this study when fatty acids were released. Thus it results important to study the degradation of food matrix during digestion to better understand the resulting interactions between microorganisms and food components or microstructure.

The FFA profiles observed throughout digestion were consistent with the most reported proportions of these fatty acids in milk fat (DePeters, German, Taylor, Essex, & Perez-Monti, 2001; Parodi, 1982) or as result of lipolysis during milk or milk fat digestion (Devle *et al.*, 2014).

The lower release of FFA observed during the stomach lipolysis could be attributed to the *Rhizopus oryzae* lipase used in this work as well as the lack of pregastric lipase in the artificial saliva used. This gastric lipase shows the highest activity at pH 8 and 37 ºC, so its activity decreased as the pH during gastric digestion decrease. Some residual activity could be present at low activity despite of the of the change rate in this dynamic system suggesting a continuous release throughout gastric digestions. Nevertheless, we observed higher values in stomach lipolysis for both WM and GWM than those reported by Devle *et al.,* (2014) for milk digestion using human gastrointestinal juice.

On the other hand, the release of FFA in the intestine for both WM and GWM were similar to the results reported before by Devle *et al.,* (2014) for milk digestion with human duodenal juices.

Even though the antimicrobial activity attributed to C6 and C8 against gram-negative bacteria (Turgeon & Rioux, 2011), no significant correlations were found between the individual FFA and HA survival at the intestine. The concentrations obtained in this study were inferior to the minimum inhibitory concentration reported by Sun *et al.,* (2002) for those FFA. We cannot explain the decrease of HA survival only with the FFA concentration, there must be other factors involved that worth to be evaluated in future studies such as specific peptides-FFA interactions.

In a different approach, in our results with confocal microscopy, we observed that survival of *BA, HA* and *ST* at different pH (pHi and 2) was not affected by changes in the microstructure between liquid and gel matrix. This is in disagreement with previous works of Hannon *et al.,* (2006) who observed that survival of *L. lactis* during *in vitro* experiments was higher in a more homogeneous cheese matrix.

Thus, according to our results, fat and microstructure of the tested matrices did not enhance the viability of *ST*, *BA* and *HA* during digestive stress. Therefore the protective effect of dairy matrices might be related to other factors where the effect of the buffer capacity has always been the most concerned (Adouard *et al.*, 2016; Conway *et al.*, 1987; Charteris *et al.*, 1998; Gardiner *et al.*, 1998).

The digestion of milk proteins has been was widely studied. Many factors affect the kinetics of digestion, such as the origin of milk (Almass *et al.*, 2006; (Inglingstad *et al.*, 2010)), the type of proteins (caseins, whey protein), the structure of food matrix (Barbé *et al.*, 2014; Rinaldi *et al.*, 2014b), the physical treatments (Barbé *et al.*, 2014) but also the implemented method for the study (*in vitro/in vivo*). It should be noted that during *in vitro* study the gastric emptying and the duration of digestion have a fixed time, but during *in vivo* tests, this parameters are mostly dependent of the food matrix (Calbet & MacLean, 1997).

In our works, casein hydrolysis was continuous during the gastric phase while whey proteins remained intact. At the end of the gastric phase, about 70% of caseins remained intact. This is in accordance with the result of Inglingstad *et al.,* (2010) and Rinaldi, Gauthier, Britten,

and Turgeon (2014a) while working with in vitro digestion of proteins from different animal origin and liquid and semi liquid dairy matrixes, respectively. In contrast, our results are much higher than those reported by Gallier *et al.,* (2012) during the *in vitro* digestion of bovine milk and Bourlieu *et al.,* (2015) during the *in vivo* digestion of a standardized milk emulsion. These differences can probably be explained by the milk origin and preparation; as well as the type, amount and activity of the enzymes used in the experiments (Almass *et al.,* 2010). On the other hand, the resistance of native whey protein in unheated milk to pepsinolysis is well known. After 100 min of gastric digestion, β-lg was still detected in all the studied matrices. The impact of the fat in protein degradation (not highlighted in our study), has not been clearly established. For instance, Gallier *et al.,* (2012) reported that β-lg in whole (full fat) milk was resistant to pepsin digestion. Sarkar, Goh, Singh, and Singh (2009) reported that β-lg was resistant to pepsin in its native form but it was partially hydrolyzed when absorbed at the surface of oil droplet surface. However, Gallier *et al.,* (2012) explained that the milk used in their study was not homogenized as in our work therefore β-lg was not adsorbed at the surface of the milk fat globule.

Our results showed a slight difference of percentage (~5%) of intact casein between milk and gel after 10 min in the gastric phase that was attenuated after 50 min. During rennet gel digestion, flocculation was accompanied by an extensive syneresis indicating a strong contraction of the gel that could result in a more pronounced retention and limiting the accessibility to milk protein cleavages sites (Barbé *et al.,* 2014).

This structure effect of the food matrix structure was studied by Barbé *et al.,* (2014) who compared the kinetics of milk proteins degradation during *in vivo* digestion of acid and rennet gels. These authors observed differences in protein concentration in duodenum between acid and rennet gel, but these differences were completely smoothed in the jejunum.

Rheology

The increased of solids into a liquid matrix and the modification of structure change the viscosity of the food matrix. However in our results we didn't observe significant differences between matrices with or without fat, contrary to the observations of Devle *et al.,* (2012) who founded differences in viscosity at pH2 between skim milk and homogenized full fat milk . The

differences observed were attributed to an effect of softening by the large fat globule. Besides, we can think that our module of measure of torque is not enough sensitive to differentiate of low gap of viscosity.

Digestion of rennet gel can be compared to the cheese making process, as it involved cutting curd and stirring. Cutting gel creates more surface where syneresis can occur (Janhoj & Qvist, 2010) and stirring enhance syneresis by preventing sedimentation and rising pressure on the curd (Guinee & O´Callaghan, 2010).

The mechanical action of stirring in our experiment without gastric fluid lead to a loss of about 0.2mN.m in 2 phases, first an initial fast phase that lasted 5 minutes, then one slower phase that lasted 50 min. The effects of pepsin and HCl addition were substantial on the torque evolution of gel. For 50 minutes, the torque decreased 0.5 mN.m. The HCl addition conduct to low pH in the vessel, reducing the water present in the casein micelles and solubilizing calcium. The structure of the micelles changed, forming new bonds between caseins, reducing the size of the curd and increasing its permeability. Therefore increase the syneresis (Johnson & Law, 2010) This phenomena was relatively slow until pH 5.5, but when pH decreased, syneriesis increased and reached its maximum at pH 4.7-4.9 probably due to a reduction of overall charge as the isoelectric point of the casein is approached.

The decrease of torque below pH 4.7 also might be attributed to the action of pepsin, since the optimum pH zone is 2-4 (Schlamowitz & Peterson, 1959). Pepsin seems to have the same effect on the evolution of the torque than HCl, as the signal went down at the same rate. As previously described by Barbé *et al.*, (2014), rennet gel digestion was accompanied by an extensive syneresis indicating a strong contraction of the particles in the rennet gel decreasing the viscosity. The key role of the pepsin on the reduction of viscosity has been previously described by Prakash, Ma, and Bhandari (2014) which showed a reduction of the viscosity of infant formula in simulated gastric digestion.

The further increase of torque between 60-80 min might be attributed to the aggregation of the micelles. During these phase, the kinetics of aggregation become more important than the

hydrolysis by the pepsin and the size of the aggregates increased as the viscosity. The phenomenon is also visible in the digestion of the milk but with a lesser intensity

Effect of buffering capacity

Buffer capacity was measured from the titration curves observed in the stomach phase. Synthetic medium containing only NaCl and a low concentration of peptones showed a weak dB/dpH below 0.005 between pH 6.5 and 3. However an important increase in value (~ 0.01) was exerted at pH below 2.5. Still, the buffer capacity of S was wide below the dairy matrices.

We observed no significant differences between matrices with fat (WM and GWM) and matrices without fat (SM and GSM) (data not shown). Fat does not provide a direct contribution to the buffer capacity; its contribution is mostly related to the curd structure (Salaün *et al.*, 2005). The element contributing to the dB/dpH of milk are mainly salts (phosphate), organic acids and caseins.

Our dB/dpH curves can be compared with previous works on dairy buffer capacity as the reported by Salaün *et al.,* (2005) and Upreti *et al.,* (2006) who found two maximal dB/dpH, at about pH 5–5.5 due to the solubilization of colloidal calcium phosphate (Lucey, Hauth, Gorry, & Fox, 1993) and a strong increase below pH 3 due to acidic amino acids present in caseins. In rennet-curdled skim milk, Whittier (1929) described a maximal dB/dpH at pH 4.7 and the difference with milk was ascribed to the production of para casein by the rennet and to the coagulum structure. Lucey *et al.,* (1993) found a buffering peak at pH 4.2 during the titration of rennet whey.

At the beginning, dB/dpH values are slightly higher for liquid than for gel, this might be due to than in a liquid matrix, the ionizable groups, that give the buffering effect, are more accessible than in gel matrices. However after pH 5, values for liquid decreased slightly below 0.015 and remained steady the rest of the test, meanwhile for gel dB/dpH continued increasing. Higher dB/dpH values and therefore higher buffer capacity in gel than in liquid could be due to a structural effect, where the curd traps the elements that give the buffer capacity, longer than a liquid matrix (Salaün *et al.*, 2005). Furthermore it is well known that buffer capacity is depend of

the type of dairy product (Al-Dabbas *et al.*, 2011; Salaün *et al.*, 2005; Upreti *et al.*, 2006) since foods with the same components have not necessarily the same structural organization or same digestion kinetic (Rinaldi *et al.*, 2014a; Turgeon & Rioux, 2011).

In this regard, two main factors need to be considered, the action of the pepsin during the gastric phase and the dynamic evolution of digestion. Hydrolysis of milk proteins and the peptide release during the *in vitro* gastric digestion can modify the buffer capacity; through increasing the number of available ionizable groups (i.e. acidic amino acids) that before were inaccessible (Salaün *et al.*, 2005). Furthermore, proteolysis during digestion is different according to the type of dairy matrix (Barbé *et al.*, 2014). The dynamic functioning of the *in vitro* digester with a continuous transfer between the various compartments also modifies the composition of the matrix and consequently the buffer capacity.

Still, despite the higher buffer effect of dairy matrices, compared with synthetic medium, in our results we didn't observe a protective effect on microorganism survival through digestion. Our results are in disagreement with the previous works of Conway *et al*., (1987) and Charteris *et al.*, (1998) who in different works with probiotics suggested that milk proteins improved the gastric transit tolerance of microorganisms, because of its tampon effect. Our results are in accordance with the reported by Adouard *et al.*, (2016) who observed that survival of *Corynebacterium casei* and S*taphylococcus equorum* during *in vitro* digestion was similar when contained in cheese or synthetic medium.

Conclusions

In this work we studied the effect of dairy matrices different in composition (with and without fat) and structure (liquid and gel) on microorganism survival through digestion. The tested microorganisms had different survival rates during digestion; *S. thermophilus* was highly sensitive to gastric stress, and was not found in the duodenal compartment; *B. auranticum* was moderately sensitive to gastric stress but resistant to duodenal stress and *H. alvei* was highly resistant to both stresses. However, despite their evident buffer capacity (higher in gel than in liquid) the selected matrices did not exert a protective effect on microorganism´s survival during

in vitro and *in vivo* digestion. Moreover, the mortality of *H. alvei* increased during intestinal digestion but no relation was established with the production of free fatty acids.

Therefore our results suggest that protective effect of the dairy matrices during digestion is not dependent of buffer capacity or interactions between microorganisms and dairy matrices (components and microstructure). Suggesting that factors that provide protection during digestion could be strain-dependent or associated to changes in the digestive parameters. During *in vitro* assays, as the reported in this work, digestion parameters, as gastric emptying and duration of digestion, are set the same for all the tested matrices; however during *in vivo* digestion, these parameters change according to the type of food matrix.

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References

Adouard, N., Foligné, B., Dewulf, J., Bouix, M., Picque, D., & Bonnarme, P. (2015). In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese. *International Journal of Food MIcrobiology, 197*, 98-107. doi:10.1016/j.ijfoodmicro.2014.12.011

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Al-Dabbas, M. M., Al-Ismail, K., & Al-Abdullah, B. M. (2011). Effect of Chemical Composition on the Buffering Capacity of Selected Dairy Products. *Jordan Journal of Agricultural Sciences, 7*, 690-700.

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., . . . Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63, Part B*, 147-156. doi:10.1016/j.foodres.2014.02.015

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29*, 625-651. doi:10.1016/j.femsre.2004.09.003

Bobko, E., Tyras, M., & Jachymek, W. (2013). Reprint of ‘‘New complete structure of *Hafnia alvei* clinical isolate strain PCM 2670 semi-rough lipopolysaccharide". *Carbohydrate Research, 378*, 71-78. doi:10.1016/j.carres.2013.06.021

Bogovic-Matijasic, B., Obermajer, T., Lipoglavsek, L., Sernel, T., Locatelli, I., Kos, M., . . . Rogelj, R. (2015). Effects of synbiotic fermented milk containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. lactis BB-12 on the fecal microbiota of adults with irritable bowel syndrome: A randomized double-blind, placebo-controlled trial. *Journal of dairy science, 99*, 1-14. doi:10.3168/jds.2015-10743

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., . . . Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature methods, 10*(1), 57-59.

Bourlieu, C., Ménard, 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. doi:10.1016/j.foodchem.2015.03.001

Calbet, J., & MacLean, D. (1997). Role of caloric content on gastric emptying in humans. *The Journal of physiology, 498*(Pt 2), 553.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., . . . Gordon, J. I. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods, 7*(5), 335-336.

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of Lactic Acid Bacteria in the Human Stomach and Adhesion to Intestinal Cells. *Journal of dairy science, 70*(1), 1-12. doi:10.3168/jds.S0022-0302(87)79974-3

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology, 84*, 759-768. doi:10.1046/j.1365-2672.1998.00407.x

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Fischbach, M. A. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563. doi:10.1038/nature12820

del Campo, R., Bravo, D., Cantón, R., Ruiz-Garbajosa, P., García-Albiach, R., MOntesi-Libois, A., . . . Baquero, F. (2005). Scarce Evidence of Yogurt Lactic Acid Bacteria in Human Feces after Daily Yogurt Consumption by Healthy Volunteers. *Applied and Enviromental Microbiology, 71*(1), 547-549. doi:10.1128/AEM.71.1.547–549.2005

DePeters, E. J., German, J. B., Taylor, S. J., Essex, S. T., & Perez-Monti, H. (2001). Fatty Acid and Triglyceride Composition of Milk Fat from Lactating Holstein Cows in Response to Supplemental Canola Oil. *Journal of dairy science, 84*(4), 929-936. doi:10.3168/jds.S0022-0302(01)74550-X

Devle, H., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. E., Ekeberg, D., & Schüller, R. B. (2012). Rheological characterization of milk during digestion with human gastric and duodenal enzymes. *Annual Transactions of the Nordic Rheology Society, 20*.

Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G., & Ekeberg, D. (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of

bovine milk. *International Dairy Journal, 36*(1), 6-13. doi:10.1016/j.idairyj.2013.11.008

Do Espirito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2011). Influence of food matrices on probiotic viability - A review focusing on the fruity bases. *Trends in Food Science and Technology, 22*, 377-385. doi:10.1016/j.tifs.2011.04.008

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

Elli, M., Callegari, M. L., Ferrari, S., Bessi, E., Cattivelli, D., Soldi, S., . . . Antoine, J. (2006). Survival of Yogurt Bacteria in the Human Gut. *Applied and Enviromental Microbiology, 72*(7), 5113-5117. doi:10.1128/AEM.02950-05

Faye, T., Tamburello, A., Vegarud, G. E., & Skeie, S. (2012). Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human astric and duodenum juice. *Journal of dairy science, 95*, 558-566. doi:10.3168/jds.2011-4705

Firmesse, O., Alvaro, E., Mogenet, A., Bresson, J., Lemee, R., Le Ruyet, P., . . . Rigottier-Gois, L. (2008). Fate and effects of Camembert cheese micro-organisms in the human colonic microbiota of healthy volunteers after regular Camembert consumption. *International Journal of Food MIcrobiology, 125*, 176-181. doi:10.1016/j.ijfoodmicro.2008.03.044

García-Hernández, J., Moreno, Y., Chuan, C., & Hernández, M. (2012). In Vivo Study of the Survival of *Lactobacillus delbruecki* subsp. *bulgaricus* CECT 4005T and *Streptococcus thermophilus* CECT 801 by DVC-FISH after Consumption of Fermented Milk. *Journal of Food Science, 77*(10), M593- M597. doi:10.1111/j.1750-3841.2012.02918.x

Gardiner, G., Ross, R. P., Collins, J. K., Fitzgerald, G., & Stanton, C. (1998). Development of a Probiotic Cheddar Cheese Containing Human-Derived *Lactobacillus paracasei* Strains. *Applied and Enviromental Microbiology, 64*(6), 2192-2199.

Godon, J. J., Zumstein, E., Dabert, P., Habouzit, F., & Moletta, R. (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Applied and Environmental Microbiology, 63*(7), 2802-2813.

Guillemin, H., Perret, B., Picque, D., Menard, O., & Cattenoz, T. (2010). Logiciel StoRM-stomach and duodenum regulation and monitoring. *IDDNFR001230009000RP201000031235, 290*.

Guinee, T. P., & O´Callaghan, D. J. (2010). The formation of Cheese Curd. In B. A. Law & A. Y.

Tamime (Eds.), Techology of Cheesemaking (pp. 260-314). United Kingdom Wiley-Blackwell.

Halgasova, N., Bukovska, G., Ugorcakova, J., Timko, J., & Kormanec, J. (2002). The *Brevibacterium £avum* sigma factor SigB has a role in the environmental stress response. *FEMS Microbiology Letters, 216*, 77-84. doi:10.1111/j.1574-6968.2002.tb11418.x

Hannon, J. A., Lopez, C., Madec, M. N., & Lortal, S. (2006). Altering Renneting pH Changes Microstructure, Cell Distribution, and Lysis of *Lactococcus lactis* AM2 in Cheese Made from Ultrafiltered Milk. *Journal of dairy science, 89*(3), 812-823.

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., . . . Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Sci. Technol., 90*(5), 549-563.

Irlinger, F., Ah Yuen In Yung, S., Sarthou, A., Delbès-Paus, C., Montel, M., Coton, M., . . . Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food MIcrobiology, 153*, 332-338. doi:10.1016/j.ijfoodmicro.2011.11.022

Janda, J. M., & Abbott, S. L. (2006). The Genus Hafnia: from Soup to Nuts. *Clinical Microbiology Reviews, 19*(1), 12-18. doi:10.1128/CMR.19.1.12–28.2006

Janhoj, T., & Qvist, K. B. (2010). The formation of Cheese Curd. In B. A. Law & A. Y. Tamime (Eds.), Techology of Cheesemaking (pp. 130-156). United Kingdom Wiley-Blackwell.

Johnson, M., & Law, B. A. (2010). The Origins, Development and Basic Operations of Cheesemaking Technology. In B. A. Law & A. Y. Tamime (Eds.), Technology of CheesemakingSociety of Dairy Technology book series (2 ed., Vol. 1, pp. 68-96). United Kingdom Blackwell Publishing Ltd. Retrieved from https://books.google.com.mx/books?id=Turm77IMxnUC&pg=RA1-PT21&lpg=RA1-PT21&dq=ph+of+the+cheese+syneresis&source=bl&ots=r6DlG5fvj1&sig=fdISx_4-RYLtgsla-5D9OJD216c&hl=fr&sa=X&ved=0ahUKEwjyndST8YrMAhUKuYMKHdTADnYQ6AEIOzAF#v=onepage&q=ph%20of%20the%20cheese%20syneresis&f=false.

Kong, F., & Singh, R. P. (2008). Disintegration of Solid Foods in Human Stomach. *Journal of*

Food Science, 73(5), R67-R80. doi:10.1111/j.1750-3841.2008.00766.x

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C. (2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D. P., O'Donohue, M., & Hernandez-Raquet, G. (2015). Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. *Bioresource Technology, 196*, 241-249. doi:10.1016/j.biortech.2015.07.084

Lucey, J., Hauth, B., Gorry, C., & Fox, P. (1993). The acid-base buffering properties of milk. *Milchwissenschaft, 48*(5), 268-272.

Lüthi-Peng, Q., & Puhan, Z. (1999). Determination of protein and casein in milk by fourth derivative UV spectrophotometry. *Analytica Chimica Acta, 393*(1-3), 227-234. doi:10.1016/S0003-2670(98)00823-X

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963. doi:10.1093/bioinformatics/btr507

Marteau, P., Minekus, M., Havenaar, R., & Huis In´t Veld, J. H. J. (1997). Survival of Lactic Acid Bacteria in a Dynamic Model of the Stomach and Small Intestine: Validation and the Effects of Bile. *Journal of dairy science, 80*, 1031-1037. doi:10.3168/jds.S0022-0302(97)76027-2

Mater, D. D. G., Bretigny, L., Firmesse, O., Flores, M., Mogenet, A., Bresson, J., & Corthier, G. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiology Letters, 250*, 185-187. doi:10.1016/j.femsle.2005.07.006

McBee, M. E., & Schauer, D. B. (2006). The Genus Hafnia. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes: Volume 6: Proteobacteria: Gamma Subclass* (pp. 215-218). New York, NY: Springer New York.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., . . . Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J, 6*(3), 610-618.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039-1045. doi:10.1016/j.foodchem.2013.09.036

Monnet, C., Landaud, S., Bonnarme, P., & Swennen, D. (2014). Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters, 263*(1), 1-9. doi:10.1093/femsle/fnu025

Mounier, J., Rea, M. C., O´Connor, P. M., Fitzgerald, G. F., & Cogan, T. M. (2007). Characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Staphylococcus* spp. Isolated from Surface-Ripened Cheese. *Applied and Enviromental Microbiology, 73*(23), 7723-7739. doi:10.1128/AEM.01260-07

Parodi, P. W. (1982). Positional distribution of fatty acids in triglycerides from milk of several species of mammals. *Lipids, 17*(6), 437-442. doi:10.1007/BF02535223

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63. doi:10.1016/j.fm.2012.02.013

Pitino, I., Randazzo, C. L., Mandalari, G., Lo Curto, A., Faulks, R. M., Le Marc, Y., . . . Wickham, M. S. J. (2010). Survival of *Lactobacillus rhamnosus* strains in the upper gastrointestinal tract. *Food Microbiology, 27*, 1121-1127. doi:10.1016/j.fm.2010.07.019

Prakash, S., Ma, Q., & Bhandari, B. (2014). Rheological behaviour of selected commercially available baby formulas in simulated human digestive system. *Food Research International, 64*, 889-895. doi:10.1016/j.foodres.2014.08.028

Ranadheera, C. S., Evans, C. A., Adams, C. A., & Baines, S. K. (2012). In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International, 49*, 619-625. doi:10.1016/j.foodres.2012.09.007

Rinaldi, L., Gauthier, S. F., Britten, M., & Turgeon, S. L. (2014a). In vitro gastrointestinal digestion of liquid and semi-liquid dairy matrixes. *LWT - Food Science and Technology, 57*(1), 99-105. doi:10.1016/j.lwt.2014.01.026

Rinaldi, L., Rioux, L., Britten, M., & Turgeon, S. L. (2014b). In vitro bioaccessibility of peptides and amino acids from yogurt made with starch, pectin, or β-glucan. *International Dairy*

Journal, 1-7. doi:10.1016/j.idairyj.2014.09.005

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal, 15*, 95-109. doi:10.1016/j.idairyj.2004.06.007

Sanders, M. E., & Marco, M. L. (2009). Food Formats for Effective Delivery of Probiotics. *The Annual Reviews of Food Science and Technology, 1*, 65-85. doi:10.1146/annurev.food.080708.100743

Sarkar, A., Goh, K. K. T., Singh, R. P., & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model. *Food Hydrocolloids, 23*(6), 1563-1569. doi:10.1016/j.foodhyd.2008.10.014

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., . . . Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food MIcrobiology, 144*, 293-300. doi:10.1016/j.ijfoodmicro.2010.10.009

Schlamowitz, M., & Peterson, L. U. (1959). Studies on the Optimun pH for the Action of Pepsin on "Native" and Denatured Bovine Serum Albumin and Bovine Hemoglobin. *The Journal of Biological Chemistry, 234*(12), 3137-3145.

Sumeri, I., Adamberg, S., Uusna, R., Sarand, I., & Paalme, T. (2012). Survival of cheese bacteria in a gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41. doi:10.1016/j.idairyj.2011.12.016

Sun, C. Q., O´Connor, C. J., & Roberton, A. M. (2002). The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chemico-Biological Interactions, 140*, 185-198.

Turgeon, S. L., & Rioux, L. (2011). Food matrix impact on macronutrients nutritional properties *Food Hydrocolloids, 25*, 1915-1924. doi:10.1016/j.foodhyd.2011.02.026

Upreti, P., Bühlmann, P., & Metzger, L. E. (2006). Influence of Calcium and Phosphorus, Lactose, and Salt-to-Moisture Ratio on Cheddar Cheese Quality: pH Buffering Properties of Cheese. *Journal of dairy science, 89*, 938-950.

Uriot, O., Galia, W., Awussi, A. A., Perrin, C., Denis, S., Chalancon, S., . . . Roussel, Y. (2016). Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. *Food Microbiology, 53*, 18-29. doi:10.1016/j.fm.2015.05.007

Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., & Acosta, F. (2008). Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. *Veterinary microbiology, 129*, 150-155. doi:10.1016/j.vetmic.2007.11.007

Voigt, D. D., Chevalier, F., Qian, M. C., & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavour compounds in mature blue-veined cheese. *Innovative Food Science & Emerging Technologies, 11*(1), 68-77. doi:10.1016/j.ifset.2009.10.009

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261-5267. doi:10.1128/aem.00062-07

Whittier, E. (1929). Buffer intensities of milk and milk constituents I. The buffer action of casein in milk. *Journal of Biological Chemistry, 83*(1), 79-88.

GENERAL DISCUSSION

3. General discussion

Cheese is one of the most varied aliments, appreciated and consumed all over the world that has been recently highlighted because of its impact on human health (Adouard *et al.*, 2016; Lopez-Exposito & Recio, 2008; Silva *et al.*, 2012). The association of milk-microorganism (lactic bacteria, ripening microorganisms) that develops the sensorial characteristics of this dairy, also results in the production of bioactive peptides, that can potentially affect the course of some diseases (Choi *et al.*, 2012; De Simone *et al.*, 2009). Even the presence of these dairy microorganisms itself has attracted concerning about its effects on health when consumed, especially because some of them resist digestion and interact with intestinal microbiota (Adouard *et al.*, 2016; Lay *et al.*, 2004). This resistance to digestion has been associated to the effect of the dairy matrix containing these microorganisms (Do Espirito Santo *et al.*, 2011; Lay *et al.*, 2004; Saxelin *et al.*, 2010). In this context, this thesis was realized to respond to two particular objectives, first to determine the presence of bioactive peptides with activities as antioxidants and ACE inhibitors in Mexican cheeses; and second, to observe the effect of composition and structure of dairy matrices in survival of dairy microorganism through digestion.

The first part of the work was devoted to the measurement of bioactive peptides in Mexican cheeses. The presence of peptides with antioxidant and ACE inhibitory activities has been previously reported in a wide variety of European cheeses however very little is known about its Mexican homologues. For this purpose two Mexican artisan cheeses different in origin and characteristics, Cotija cheese and Fresh goat cheese were chosen based on their importance in Mexican culture and because of the interest of the artisan cheese makers to obtain a Denomination of Origin and a Collective Trade mark respectively. However, despite the interest of the producers, little work has been done on its full characterization, and until this work, no previous effort has been done on the observation of its functional properties.

Cotija cheese was made with cow raw milk fermented only with the autochthonous flora and ripened for six months in conditions similar to the artisan. Proteolysis during ripening was monitored, and changes in its antioxidant and ACE inhibitory activity were observed as ripening proceeded.

On the other hand, Fresh goat cheese, was made with goat milk from different seasons of the year, and fermented only with autochthonous flora. Cheeses were made with raw (NP) and pasteurized milk (P) to test the effect of heat treatment on the antioxidant and ACE inhibitory activity.

Cheeses were analyzed separately, and nitrogenous content of each cheese was fractionated to separate the peptides according to its size, in percentage of soluble nitrogen at pH 4.6 (ASN), 12 % TCA (NPN) and 70 % ethanol soluble (EtOH-SN). Nitrogenous content of all fractions was obtained and expressed in relation to the total nitrogen content (Chapter 2.1 and 2.2).

The ASN and NPN fractions were screened for the presence of antioxidant activity by measuring its ability to inhibit free radical DPPH. Meanwhile EtOH-SN and the 70 % ethanol insoluble precipitate, EtOH-NSN, were tested for ACE inhibitory activity through RP-HPLC method of Wang *et al.,* (2013) based on the production of hippuric acid (HA) from hippuryl-histidyl-leucine (HHL) catalyzed by the ACE. When a peptide with ACE inhibitory activity was present, the production of HA decreased according to the % of inhibition.

Meanwhile, the peptide profile of each nitrogenous fraction was further analyzed by RP-HPLC to determine the type of peptides present in each fraction and correlate them with the biological activity. The peptides in the chromatogram were divided according to their hydrophobicity following the criteria of Gonzalez de Llano *et al.,* (1995). Results are shown and discussed among Chapter 2.1 and 2.2. Therefore in this section we are only going to focus in its correlation with the biological activity.

The first part of the work was focused on Cotija cheese. Based on DPPH screening of the ASN and NON fractions, Cotija cheese released bioactive peptides with activity as antioxidants, from very early stages or ripening. Values observed during the first week were 67.3 % for NPN and 19.22 % for ASN. Antioxidant activty in both fractions increased during ripening. In NPN, the activity increased moderatly from 67.9 ± 1.2 % observed during the first month to 75.7 ± 9.70

% in six months cheese. Meanwhile, in ASN antioxidant activity was significant lower ($p<0.05$), compared to NPN, at early stages of ripening. During the first month activity in ASN was 17.6 ± 2.0 %, and remained relativale stable until the fourt moth(19.9 ± 10.1 %). However after this period ASN´s antioxidant activity increase substantially to 66.4 ± 19.1 %, reaching similar values to the observed for NPN in one month Cotija cheese.

The highest antioxidant activity observed for Cotija cheese was 75.66 ± 9.70 % in NPN and 66.4 ± 19.1 % in ASN in six months cheese. This values were lower to the observed by Silva *et al.,* (2012) in water soluble extracts of Coalho cheese (77.9 to 91.1 %) but similar to the reported by Gupta *et al.,* (2009) in water soluble extracts of 4 months old Cheddar (~ 70%).

Antioxidant activity was significant higher ($p<0.05$) in NPN than in ASN. This could be due to the size of peptides contained in each fraction. While ASN contained proteins, large peptides, small peptides and amino acids (McSweeney & Fox, 1997; Panizzolo *et al.*, 2011), NPN contained small peptides and amino acids soluble at 12% of TCA (McSweeney & Fox, 1997; Panizzolo *et al.*, 2011). Thus, our results suggest that smaller peptides, as the content in NPN, exerted higher antioxidant activity that bigger peptides in ASN.

The increase of antioxidant activity in ASN fraction of Cotija cheese observed at the end of ripening could be explained by action of bacterial proteinases and peptidases that degrade large peptides (product of caseins) releasing smaller peptides with higher antioxidant activity. Previous studies have reported the existent relationship between antioxidant activity and small peptides (Gómez-Ruiz *et al.*, 2008; Pihlanto, 2006; Pritchard *et al.*, 2010).

Meanwhile, the observed increase in antioxidant activity in both ASN and NPN fractions throug ripening of Cotija cheese, agrees with previous results of Perna *et al.,* (2015) who observed that antioxidant activity in water soluble extracts of Caciocavallo cheeses increased through 5 months of ripening. In contrast, Bottesini *et al.,* (2013) observed that antioxidant activity of water soluble extracts of Parmigiano Reggiano, remained stable through 41 months of ripening. Additionally, our results can be partially compared with those of Gupta *et al.,* (2009)

who observed that antioxidant activity of Cheddar cheese increased throug ripening, but only until certain level, because extensive proteolysis reduced the antioxidant activity.

The increasing trend in antioxidant activity in ASN and NPN was consistent with the increase in nitrogenous content in both fractions (ASN/NT and NPN/NT) (Chapter 2.1.3) Therefore antioxidant activity seems to be related to the formation of soluble peptides as previously reported by Gupta *et al.,* (2009) and Barác *et al.,* (2016), in Cheddar and white brined cheeses respectivley, where authors observed that the increase of antioxidant activity in water soluble extracts was related to the increase in nitrogenous content.

Additionally, it results noticeable that antioxidant activity and nitrogenous content in ASN and NPN were constant at early stages of ripening, but after four months a significant increase in both parameters was observed (Chapter 2.1). This could be related to the pattern of proteolysis in Cotija cheeses. During cheese proteolysis caseins are hydrolyzed by residual coagulant, plasmin and other indogenous proteolytic enzymes releasing large and intermediate peptides, that are moderatly bioactives, those peptides are later hydrolyzed by proteinases and peptidases from cheese microbiota (LAB, NSLAB, secondary flora, etc.) resulting in shorter peptides and amino acids with higher biological activities (McSweeney, 2004). Therefore, our results could suggest after four months or ripening, Cotija cheese goes through a major change in its proteolysis patern probably related to changes in its microbiota. However these results need to be further elucidated throug microbiological observations.

Meanwhile, ASN and NPN fractions were further studied through RP-HPLC looking to relate the peptide content with the biological activity. Obtained chromatograms were divided in hydrophilic (HI) and hydrophobic (HO) peptides based on its retention time (Chapter 2.1).

Antioxidant activity in ASN was highly correlated to its content in HI peptides (0.81). This type of peptides increased linearly as ripening proceeded (from initial 4011.2 ± 2954.1 till 33748.5 ± 7088.6), reaching its highest content at the end of ripening. At the same time as the highest antioxidant activity was observed for this fraction (66.4 ± 19.1 %).

Meanwhile antioxidant actitity in NPN was highly correlated with both HI and HO peptides (0.92 and 0.79 respectively (for 14 degree of freedom), however its correlation was slighty higher with the HI peptides.

In previous studies, antioxidant activity has been mainly attributed to hydrophobic peptides (Pihlanto, 2006), however in our results we found that this activity was mostly correlated with the HI peptide content, we could not fully explain this behavior, still, this could be related to properties of the amino acids in the peptide, that goes beyond the hydrophobic or hydrophilic nature of the peptides, for instance chelation properties, or the ability to produce glutathione (like cysteine). (Erdmann *et al.*, 2008).

On the other hand, the presence of bioactive peptides inhibitors of ACE in Cotija cheese was observed through screening its EtOH-SN and EtOH-NSN fractions. The ETOH-SN fraction was chosed for this screeming because it contained the smallest peptides, that according to Espejo-Carpio *et al.*, (2013) are the main holders of this activity. Meanwhile, EtOH-NSN fraction was also tested to observe if bigger peptides could also exert ACE inhibitory activity.

Cotija cheese exerted important ACE inhibitory activity since very early stages of ripening. Inhibitory activity in EtOH-SN during the first month was 82.2 ± 3.8 %. Activity in this fraction increased linearly during ripening, reaching 100% of ACE inhibiton after fourth months. A slight decrease of activity was observed during the fifth month (~3 %). Still, at the end of ripening ACE inhibitory activity was 100%.

Meanwhile, in EtOH-NSN fraction, inhibitory actvity during the first month was 49.9 ± 18.4%. It increased steply as ripening proceded, reaching its highest activity, 97.4 ± 3.3 %, during the fifht month, and remaining constant until the end of ripening (97.7 ± 0.4 %).

According to our results, Cotija cheese posses high content of bioactive peptides capables to inhibit ACE. Three months old Cotija cheese had almost 100 % of inhibition activity (~100% in EtOH-SN and > 90% in EtOH-NSN). This is of special importance if we consider that commercializable Cotija cheese most be at least three months old.

Values obtained for Cotija cheese were higher to the previously reported by Gómez-Ruiz *et al.,* (2008) in water soluble extracts of commercial Spanish varieties (56.6 to 76.1 %); and the observed by Ong *et al.,* (2007) in water soluble extracts of Cheddar cheese of different ages (<80 %). Also Ryhänen *et al.,* (2001) reported only ~ 50% ACE inhibitory activity in the fraction soluble at ethanol 80 % of three months old Festivo cheese.

In general, both EtOH-SN and EtOH-NSN exhibited high ACE inhibitory activtiy that increased as ripening proceeded. However activity in EtOH-SN fraction was significantly higher ($p < 0.05$) than in EtOH-NSN. This could be due to the size of peptides content in each fraction, since EtOH-SN fraction contained the smallest peptides that according to Espejo-Carpio *et al,.* (2013) and Hong *et al.,* (2008) are the most capable to interact with the active sites of ACE. Meanwhile, EtOH-NSN content bigger peptides that have more conformational difficulties sticking to ACE´s active sites.

This results are in agreement with previous works of Lignitto *et al.,* (2010), who observed that shorter peptides in the water soluble extracts of Asiago d´allevo cheese had higher contribution to the ACE inhibitory activity than bigger peptides.

However our results suggest that bigger peptides content in EtOH-NSN fraction could also exert important inhibitory activity over ACE. Furthermore, Cotija cheese ripenened between three and six months exerted almost 100 % of ACE inhibitory activity in both EtOH-SN and EtOH-NSN fractions.

Our observation of increasing ACE inhibitory activity during ripening of Cotija cheese, can be partially compared with previous works of Gupta *et al.,* (2013), Lignitto *et al,.* (2010) and Pritchard *et al.,* (2010) where authors observet that ACE inhibitory activity in water extracts of Asiago d´allevo and Cheddar cheeses increased as ripening advanced, but only until some extent to later decrease, suggesting that peptides inhibitors of ACE (and probably all type of bioactive peptides) depend on a balance between their formation and further catabolism. However, ACE inhibitory activity in EtOH-SN and EtOH-NSN fractions of Cotija cheese increased through all

ripening time without any signs of further catabolism. Disagreement with our results might be due to differences in the ripening time. While Cotija was ripenend for 6 months , Asiago d´allevo and Cheddar cheeses were ripened for 18 months and 9 months respectively.

As previously done for antioxidant activity, further RP-HPLC analysis was carried on EtOH-SN and EtOH-NSN fractions to observe if the peptide content was correlated with the ACE inhibitory activity (see Chapter 2.1).

In EtOH-NSN fraction ACE inhibitory activity was highly correlated with HO peptides (0.60).In contrast, activity in EtOH-SN was not significantly correlated either with HI or HO peptides,0.23 and 0.28 respectively (for 2 degree of freedom). ACE inhibitory activity has been always related to the hydrophobicity of the peptides (Espejo-Carpio *et al.,* 2013; Meisel, 2004), which explains the significant correlation between HO peptides and activity in EtOH-NSN. However, the lack of correlation with EtOH-SN fraction couldn't be explained, and it could be only attributed to other characteristics of the peptides present like the electrostatic charge of the amino acids or the peptide conformation (Meisel, 2004). However further studies need to be carried out to understand the precise relationship between the type of peptides in a determined nitrogenous fractions and its biological activity.

On the other hand, it is well known that proteolysis is more extensive during ripening, still, this biochemical process can also occur during the production of fresh cheeses (Paul & Van Hekken, 2011; Silva *et al.,* 2012; Torres-Llanez *et al.,* 2011). However until now, the majority of the studies about cheese bioactive peptides have focused on maturated cheeses. Therefore the second part of this work was focused on Fresh goat cheese, and its content on bioactive peptides with activities as antioxidants and ACE inhibitors and furthermore to observe the effect of pasteurization on these bioactivities.

As done for Cotija cheese ASN and NPN were selected, based on its peptide content, to screen its capacity to scavenging free radicals by the method of DPPH:

Fresh goat cheeses made with raw (NP) and pasteurized milk (P) exhibited important antioxidant activity in both NPN and ASN fractions. Values observed for NP cheeses were 22.3 ± 2.1 % and 61.6 ± 1.9% for ASN and NPN respectively. Meanwhile in P cheeses values obtained were 21.88 ± 5.35 % and 64.22 ± 0.04 % for ASN and NPN.

Our results suggest that there were no significant differences ($p>0.05$) in antioxidant activity between NP and P cheeses. In contrast antioxidant activity was significantly higher ($p<0.05$) in NPN than in ASN fraction in all cheeses.

Antioxidant activity (in NPN fraction) in Fresh goat cheese, 62.9 % was lower that the reported by Silva *et al.,* (2012) in water soluble extract of Coalho cheese (77.9 to 91.1 %) but higher that the reported by Meira *et al.,* (2012) in Feta-type cheese (32.7 ± 1.8 %). Additionally, when compared to Cotija cheese, antioxidant activity in Fresh goat cheese was similar to the observed in fresh (one week) cheese (67.3 % for NPN and 19.2 % for ASN).

Lack of differences in antioxidant activity between NP and P cheeses is in disagreement with previous reports of Silva *et al.,* (2006) where authors observed that ovine and caprine cheese-like systems made with raw milk exerted higher antioxidant activity than those made with sterilized milk. However authors worked with sterilized (not pasteurized) milk and their cheeses were 45 days old, meanwhile our cheese was fresh. McSweeney *et al.,* (1993) observed that differences in proteolysis between raw and pasteurized cheeses appear and increase as ripening progressed, therefore, despite our Fresh goat cheeses showed no differences between each other, but those differences could appear in older cheeses.

Meanwhile, higher activity in NPN than in ASN was in agreement with the previously observed in Cotija cheese, where differences in antioxidant activity were attributed to the size of the peptides present in each fraction since according to Pihlanto (2006) the antioxidant peptides derived from milk are small peptides mainly composed of 5 to 11 aminoacides.

The peptide profile of ASN and NPN fractions were correlated to the antioxidant activity. Activity in ASN was positive correlated to the HI peptides (0.85). Meanwhile in NPN,

antioxidant activity was highly and negatively correlated to HI and HO peptides (-0.94 and -0.98 respectively). These results are partially in agreement with the previously observed for Cotija cheese where activity in ASN was positive correlated with HI peptides and in NPN was correlated with both type of peptides. However, further studies need to be carried out to understand the precise relationship between the type of peptides in a determined nitrogenous fractions and its biological activity.

Furthermore, it has been established that peptides with antioxidant activity are mainly released by secondary proteolysis during cheese ripening, Gupta *et al.,* (2009) as also observed for Cotija cheese. However, our results suggest that the release of these peptides can also occurs during cheese making where according to McSweeney (2004) casein breakdown is mainly due to the coagulant retained in the curd, endogenous enzymes or starter microorganisms (added or autochthonous). For our Fresh goat cheeses, the role of microorganisms was not significant as observed by the lack of differences between NP and P cheeses. Meanwhile previous studies have probed the existence of antioxidant peptides present naturally in goat milk (Bezerra *et al.*, 2013; El-Salam & El-Shibiny, 2012). Therefore we can suggest that bioactive peptides with antioxidant activity in our Fresh goat cheeses were naturally present in goat milk and released by coagulant or endogenous milk enzymes.

On the other hand, EtOH-SN and EtOH-NSN fractions were screened to observe if Fresh goat cheese contained bioactive peptides inhibitors of ACE. Although many studies have been carried on to measure the ACE inhibitory activity of peptides in cheeses with different ripening degrees, only few studies have focused on the evaluation of this activity in fresh cheeses (Paul & Van Hekken, 2011; Torres-Llanez *et al.*, 2011).

Both NP and P cheeses exhibited high ACE inhibitory activity, attributed do its content in bioactive peptides. Values for NP cheeses were 89.4 ± 14.4 % and 94.7 ± 3.0 % for EtOH-SN and EtOH-NSN fractions respectively. Meanwhile in P cheeses values obtained were 99.8 ± 0.2 % and 85.2 ± 11.2 % for EtOH-SN and EtOH-NSN fractions. Despite the slight differences observed ANOVA showed no statistical difference ($p > 0.05$) between cheeses or fractions.

ACE inhibitory activity in EtOH-SN and EtOH-NSN fractions of Fresh goat cheese, (ranging between 73.0 % and 100 %) was higher to the reported by Meira *et al.,* (2012) in water soluble extracts of Feta cheese (46.5 %) and similar to previous reports of Torres-Llanez *et al.,* (2011) and Silva *et al.,* (2012) in water soluble extracts of Mexican Fresco (95.3 % and 99.8 %) and Coalho cheeses (75.9 % and 91.1 %) respectively.

It is interesting to notice that, inhibitory activity in Fresh goat cheese was higher than the reported by Ong *et al*., (2007)in water soluble extracts of Cheddar cheese at different ripening ages (< 80%). When compared to Cotija cheese, ACE inhibitory activity in Fresh goat cheese was similar to the observed in six months Cotija cheese but higher to the observed in young Cotija.

The release of ACE- inhibitory peptides has always been considered as dependent of the proteolysis degree during cheese ripening (Gupta *et al.,* 2009; Ong *et al.,* 2007; Pritchard *et al.,* 2010). Meanwhile, the studies done on fresh cheeses fabricated with the natural flora, attribute the release of ACEI peptides to the enzymatic action of starter cultures (Torres-Llanez *et al.,* 2011). Nevertheless our Fresh goat cheese was unripened and fabricated without starter culture, therefore we assume that these peptides were either be already present in the milk used for cheese making and released by endogenous enzymes (Bezerra *et al.,* 2013), or by coagulant during casein breakdown (Silva *et al.,* 2006).

Our primary results suggest that there was no difference in ACE inhibitory activity between NP and P cheeses. This is disagreement with the previously observed by Silva *et al.,* (2006) in caprine cheese-like systems, where authors observed that cheeses made with raw milk (44. 9 and 79.4 %) had higher inhibition of ACE than those made with sterilized milk (16.6 and 33. 7 %). In contrast, Paul and Van Hekken (2011) observed that ACE inhibitory activity in Queso Fresco cheeses, made without starter cultures, was similar in cheeses made with raw and pasteurized milk (87 to 93 %). Authors attributed this lack of differences to further spoilage bacteria in both type of cheeses providing the same enzymes in raw and pasteurized cheeses, to hydrolyze caseins and release ACE inhibitory compounds.

On the other hand lack of different between EtOH-SN and EtOH-NSN fractions is in disagreement with the previously observed for Cotija cheese. This suggests that size of peptides is not the only factor that determines ACE inhibitory activity. The type of peptides content in each fraction has been previously shown to be related with the biological activity. ACE inhibitory activity in EtOH-SN was positive correlated with the HO peptides (0.57), meanwhile activity in EtOH-NSN was highly and negative correlated with HI peptides (-0.96).

It is well known that pasteurization of milk prior to cheese making can affects the proteolysis, its effect over the proteolysis and the type of peptides produced in cheese has been previously reported (Albenzio *et al.*, 2001; Kırmacı *et al.*, 2014). However its impact on Fresh goat cheese was not evident at first sight. Though, further PCA analysis showed that pasteurization increased the production of HO peptides (Chapter 2.2) that were highly correlated with both antioxidant and ACE inhibitory activity. This variation of HO peptides affects the ACEI activity in the EtOH-SN fraction differentiating between NP and P cheeses. Meanwhile, biological activity in NP cheeses seems to be more related to the HI peptides of the ASN fraction. These observations are in accordance with previous observation on Cotija cheese, where a high correlation was observed between HO peptides and ACE inhibitory activity; meanwhile HI peptides influenced the antioxidant activity. Therefore despite initial results, the effect of pasteurization on biological activity of Fresh goat cheeses cannot be discharged.

In brief, according to our results, Cotija and Fresh goat cheeses contained peptides with activities as antioxidant and ACE inhibitors, that can be released during cheese manufacture, by coagulant or other endogenous proteolytic enzymes (in Fresh goat cheese), and increase during ripening (in Cotija) probably because of the proteinases and peptidases from cheese microorganisms (LAB, NSLAB, secondary flora, etc.) (McSweeney, 2004).

These microorganisms have been recently highlighted because after consumption in cheese, some of them are capable to resist to low pH and bile salts during digestion and reach the colon where they can continue to release bioactive peptides, or interact with the intestinal microbiota producing some other health effects (Adouard *et al.*, 2015; Ibrahim *et al.*, 2010; Lay *et al.*, 2004). Beyond the mechanism of response to stress own of each microorganism (Begley *et*

al., 2005; De Angelis & Gobbetti, 2004; Mills *et al.*, 2011), it has been hypothesized that the food matrix could enhance their resistance through interaction between microorganisms and food components (Do Espirito Santo *et al.*, 2011; Lay *et al.*, 2004; Saxelin *et al.*, 2010). However, study of cheese as a food matrix could be extremely complex, difference between varieties, are reflected in differences in digestion patterns (macrostructure) (Lamothe, Corbeil, Turgeon, & Britten, 2012) and preadaptation of the microorganisms to the matrix produce cross adaptation to further digestive stress (Begley *et al.*, 2005; Pitino *et al.*, 2012).

Thus this part of the work was devoted to the study of the effect of dairy matrices in the survival of dairy microorganisms through digestion. However, since the microorganisms that form the microbiota of Cotija and Fresh goat cheese have not been yet characterized and their study would be the topic of whole new work, we decided to select dairy microorganisms that have been already identified by is role in yogurt and cheese production (Irlinger *et al.*, 2012; McSweeney *et al.*, 1993; Spinnler & Gripon, 2004): *Streptococcus thermophilus* TIL 257 (*ST*), *Brevibacterium aurantiacum* ATCC9174 (*BA*) and *Hafnia alvei* GB01 (*HA*). These microorganisms have attracted interest for their potential effect on human health (Adouard *et al.*, 2015; Uriot *et al.*, 2016): Therefore all the information regarding its survival during digestive stress results interesting.

The first step was to characterize the survival of each microorganism, growth and contained in synthetic medium (S), through digestion; to later observe if their inclusion into a dairy matrix could enhance their survival.

The tested dairy matrices were made from the same skim milk powder but differentiated from each other by their composition, with and without fat; and their structure, liquid and gel (as in rennet cheese): Skim milk (SM), whole milk (WM), rennet gel of skim milk (GSM) and rennet gel of whole milk (GWM).

Digestion was carried on an *in vitro* dynamic digester (DIDGI) with three separated compartments that simulates the stomach, duodenum and small intestine (jejune and ileum), taking into consideration the real *in vivo* interactions between compartments and its kinetic and

sequential aspects. Parameters of digestion (pH, gastric emptying and intestinal transit) were the same for all the matrices tested. The *in vitro* results were compared with *in vivo* experiments with mice. And the degradation of the dairy matrices through *in vitro* digestion was analyzed by electrophoreses and GC-Mass.

Microorganisms grown in pure culture were included together into the dairy matrices just before digestion. Although the response of these strains to digestive stress has not been fully characterized, its development during cheese ripening gives us a trace to explain their behavior through digestion.

BA was moderately sensitive to gastric stress, it remained viable the first 50-60 min (6.3 ± 0.8 log CFU/mL) to then decreases below our detection limit. In duodenum BA was tolerant to the stress of the bile salts losing on average 1.7 log CFU/mL. In the intestine viability (5. 8 ± 0.5 log CFU/mL) decreased 1.07 log CFU/mL through 140 min regardless the matrix. Beyond 140 min, BA was only found in S and SM (4.2 ± 0.7 log CFU/mLrespectively).

Our results are in accordance to the previous works of Adouard *et al.,* (2015) and Adouard *et al.,* (2016), where authors studied the viability *B. auranticum* through batch and dynamic *in vitro* digestion founding that this strain was sensitive to gastric stress but resistant to duodenal conditions.

*BA´*s behavior was no surprising when considering that while part of cheese microbiota, it only grown after deacidification of the medium when cheese pH reaches 6–7 (Irlinger *et al.*, 2012). Thus its sensitivity to gastric stress was expected. To our knowledge, the mechanisms of response to acid stress by *BA* have not been previously described. The only study related to this response is the one done by Halgasova *et al.,* (2002) on *Brevibacterium flavum* a related strain to *B. auranticum,* where authors probed the key role of *sigB* on growth and viability of *B.* f*lavum* as a general stress response protein and controls transcription of various stress related proteins in Gram positive bacteria. Likewise Mounier *et al.,* (2007), analyzed the growth characteristics of *B.*

auranticum 1-16-58 isolated from smear cheese and found that urease activity was not detected in this microorganisms which does not help it to resist drops in pH.

On the other hand, tolerance of *BA* to bile salts could be related to its ability to survive to high salt concentrations during cheese manufacture (Irlinger *et al.*, 2012; Mounier *et al.*, 2007). In this field previous authors observed that salt tolerance of this microorganisms could be related to osmoprotectant transporters in its genoma (Monnet *et al.*, 2014) that could provide *BA* with a further cross- adaptation to other stress (Begley *et al.*, 2005). Furthermore, the previous exposition of BA to low pH could increase this bile resistance.

Meanwhile, *HA* was highly resistant to gastric stress with a decrease of only 2.78 log CFU/mL. It was the only strain capable of surviving throughout all the gastric phase. When passing to the duodenal phase *HA* was also highly tolerant to bile salts, losing only 1.08 log CFU/mL. In the intestine, viability of *HA* within matrices without fat (S, SM and GSM) remained stable thought all the phase (6. 52 ± 0.41 log CFU/mL). However viability within matrices with fat (WM and GWM) was significantly lower ($p < 0.05$) in the intestinal phase.

Our results are in agreement with those of Adouard *et al.*, (2015) and Adouard *et al.*, (2016) who observed that *H. alvei* in syntetic medium was capable of surviving throughout batch and dynamic *in vitro* digestion. Survival of HA to gastric stress could be explained by the genetic characteristics of this microorganism: it is a gram negative bacteria related to *E. coli* (Janda & Abbott, 2006), a bacteria capable of surviving digestive stress. Additionally, previous clinical studies have suggested the ability of some medical strains *H. alvei* to colonize GI tract (Bobko *et al.*, 2013; Vivas *et al.*, 2008). In this field, previous authors have observed that this microorganisms is an ornithine and urease positive bacteria (Janda & Abbott, 2006), with a mechanism or response highly effective against acidity stress.

Finally, *ST* was highly sensitive to digestive stress, it was only stable during the first 40 min of gastric digestion was not found beyond the first compartment. This behavior was partially surprising when considering that *ST* has been widely considered as a probiotic microorganism (Mater *et al.*, 2005; Uriot *et al.*, 2016). However, when looking at its role in the dairy industry, *ST*

is a strain normally used as a starter culture to reduce the pH of the milk, but to a limited extent (~ 5) therefore its intolerance to very low pH in the stomach phase is consistent. In this field Uriot *et al.,* (2016) observed that survival of different strains of *ST* to digestion was mainly dependent of the metabolism of urease and small Heat Shock Proteins (sHSP).

Thus the three microorganisms *BA, HA* and *ST* had different behaviors during digestion related to the mechanism of strain specific response. However all strains had in common a significant decrease in viability during their passage through the stomach. This confirmed the hypothesis that low pH represents the most important stress suffered by these microorganisms during digestion. This aspect was later supported by the images obtained by confocal microscopy, where we visually observed the increased mortality of the three microorganisms when the pH dropped from 4 to 2, and it was more dramatic for *ST* and *BA* than *HA*, (40, 11 and 2 % damaged cells, ImageJ, statistical tools) therefore reinforcing the previously observed higher resistance of *HA* to gastric stress. In these images of microscopy we also noticed the preference of these microorganisms to settle in the protein matrix, in agreement as previously reported by Pitino *et al.,* (2012) about the localization of *L. rhamnosus* into the protein-fat interface of a cheese matrix.

The effect of a food matrix to enhance microorganism survival through digestion has been widely hypothesized; and matrices of dairy origin have been the most studied case because of its complex composition and its structural variety (Castro *et al.*, 2014).

In our work, we analyzed the effect of structure and composition of dairy matrices different in composition (with and without fat) and structure (liquid and gel), in the survival of *BA*, *HA* and *ST*, through digestion.

For *BA*, the effect of dairy matrices was not significant. During the intestinal phase SM had better results than other dairy matrices, but similar to that observed for the synthetic medium (S), thus the use of a dairy matrix did not increase the survival of *BA* through digestion. Our results are in contrast with previous observations of Adouard *et al.,* (2016), where the authors observed that the survival of *B. aurantiacum* in *in vitro* digestion was higher when contained in a rennet gel than in a synthetic medium.

Meanwhile for *HA*, fat in the WM and GWM matrices had a negative effect on survival through the intestinal phase. Our results can be partially comparable with those of Adouard *et al.,* (2016) who observed that *H. alvei* grown in synthetic medium, survived better the gastric stress, than when in cheese; however, in further compartments, survival was the same for both matrices.

Our results are interesting, since previous works by Ranadheera *et al.,* (2012), have suggested that matrices with higher fat content provided better protection to acid and bile stress. Mortality of *HA* in fat containing matrices might be due to the effect of residual lipases from previous compartments that passed into the intestine and continued to be active, hydrolyzing triglycerides and releasing fatty acids with antimicrobial effects; as proposed by (Sun *et al.*,2002) who observed that gram positive and gram negative bacteria can be inhibited by the hydrolysis of milk fat in the GI tract. However in our work, the decrease in *HA´*s viability could not be correlated with the concentration of fatty acids released from milk fat during digestion.

Meanwhile for *ST* dairy matrices showed any type of effect on survival through digestion. *S. thermophilus* has been previously screened to observe its ability to survive digestive stress when part of a dairy matrix (del Campo *et al.*, 2005; Lay *et al.*, 2004; Mater *et al.*, 2005) however, results are inconsistent. Therefore, the comparison of our results with the literature was difficult. Mater *et al.,* (2005) observed that *S. thermophilus* part of fresh yogurt, was capable to survive through *in vivo* digestion. But, in a similar work, del Campo *et al.,* (2005) found no viability of *S. thermophilus in vivo* digestion of yogurt.

Meanwhile Lay *et al.,* (2004) found that *S. thermophilus* was capable to survive *in vivo* digestion when part of Camembert cheese were digested but not when it was part of fermented milk; suggesting than a solid matrix was more protective than a liquid one. In this same work, authors concluded that *H. alvei* part of Camembert microbiota was not able to survive during *in vivo* digestion. This last observation was in disagreement with our conclusion about *H. alvei* being fairly resistant to *in vitro* digestion (regardless the matrix). Difference with our results could be further due to interferences in the method of Lay *et al.,* (2004) who used nalidixic acid

to avoid contamination, but this drug is also toxic for *H. alvei* and other gram negative bacteria (McBee, 2006).

In a different approach, in our results with confocal microscopy, we observed that survival of *BA, HA* and *ST* was not affected by changes in the microstructure between liquid and gel matrix. This is in disagreement with previous works of Hannon *et al.,* (2006) who observed that survival of *L. lactis* during *in vitro* experiments was higher in a more homogeneous cheese matrix.

Finally our *in vivo* analysis failed to show survival of *BA; HA* and *ST* after their digestion in a dairy matrix. The traces of *Streptococcus* found in feces were further attributed to a similar strain part of the GI tract as previously suggested by Bogovic-Matijasic *et al.,* (2015). Differences between our *in vitro* and *in vivo* results could be because the amount of ingested microorganisms (10^6) was not enough to implant in the colon.

Thus, according to our results, the dairy matrices tested WM, SM, GSM and GWM didn't show any clear protective effect on *BA, HA* and *ST* survival through digestive stress, despite its high buffer capacity (higher in gel than in milk). Our results are in disagreement with the previous works of (Conway *et al.*, 1987; Charteris *et al.*, 1998, {Conway, 1987 #96) who in different works with probiotics suggested that milk proteins improved the gastric transit tolerance of microorganisms, because of milk buffer capacity. Still, our results are in accordance with work of Adouard *et al.,* (2016) who observed that survival of *Corynebacterium casei* and S*taphylococcus equorum* during *in vitro* digestion was similar when contained in cheese or synthetic medium. Thus the role of the composition and structure of dairy matrix in the microorganism´s survival through digestion remains unclear.

References

Adouard, N., Foligné, B., Dewulf, J., Bouix, M., Picque, D., & Bonnarme, P. (2015). In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese. *International Journal of Food MIcrobiology, 197*, 98-107. doi:10.1016/j.ijfoodmicro.2014.12.011

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Albenzio, M., Corbo, M. R., Rehman , S. U., Fox, P. F., De Angelis, M., & A. Corsetti , A. S., M. Gobbetti. (2001). Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food MIcrobiology, 67*. doi:10.1016/S0168-1605(00)00533-X

Barác, M., Smiljanic, M., Zilic, S., Pesic, M., Stanojevic, S., Vasic, M., & Vucic, T. (2016). Protein profiles and total antioxidant capacity of water soluble and insoluble protein fractions of white cow cheese at different stage of ripening. *Mljekarstavo, 66*(3), 187-197. doi:10.15567/mljekarstvo.2016.0303

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29*, 625-651. doi:10.1016/j.femsre.2004.09.003

Bezerra, V. S., Campos, J. F., da Silva, R. A., Porto, T. S., de Lima-Filho, J. S., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. *Food Science and Technology, 33*(3), 513-520. doi:10.1590/S0101-20612013005000074

Bobko, E., Tyras, M., & Jachymek, W. (2013). Reprint of ‘‘New complete structure of *Hafnia alvei* clinical isolate strain PCM 2670 semi-rough lipopolysaccharide". *Carbohydrate Research, 378*, 71-78. doi:10.1016/j.carres.2013.06.021

Bogovic-Matijasic, B., Obermajer, T., Lipoglavsek, L., Sernel, T., Locatelli, I., Kos, M., . . . Rogelj, R. (2015). Effects of synbiotic fermented milk containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. lactis BB-12 on the fecal microbiota of adults with irritable bowel syndrome: A randomized double-blind, placebo-controlled

trial. *Journal of dairy science, 99*, 1-14. doi:10.3168/jds.2015-10743

Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., . . . Sforza, S. (2013). Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. *Food Science and Nutrition, 68*(8), 963-968. doi:10.3109/09637486.2013.821696

Castro, J. M., Tornadijo, M. E., Fresno, J. M., & Sandoval, H. (2014). Biocheese: A Food Probiotic Carrier. *BioMed Research International, 2015*. doi:10.1155/2015/723056

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of Lactic Acid Bacteria in the Human Stomach and Adhesion to Intestinal Cells. *Journal of dairy science, 70*(1), 1-12. doi:10.3168/jds.S0022-0302(87)79974-3

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology, 84*, 759-768. doi:10.1046/j.1365-2672.1998.00407.x

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12. doi:10.1111/j.1471-0307.2011.00725.x

De Angelis, M., & Gobbetti, M. (2004). Environmental stress responses in Lactobacillus: A review. *Proteomics, 4*, 106-122. doi:10.1002/pmic.200300497

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., & Vanacore, D. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258. doi:10.1002/psc.1093.

del Campo, R., Bravo, D., Cantón, R., Ruiz-Garbajosa, P., García-Albiach, R., MOntesi-Libois, A., . . . Baquero, F. (2005). Scarce Evidence of Yogurt Lactic Acid Bacteria in Human Feces after Daily Yogurt Consumption by Healthy Volunteers. *Applied and Enviromental Microbiology, 71*(1), 547-549. doi:10.1128/AEM.71.1.547–549.2005

Do Espirito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2011). Influence of food matrices on probiotic viability - A review focusing on the fruity bases. *Trends in Food Science and Technology, 22*, 377-385. doi:10.1016/j.tifs.2011.04.008

El-Salam, M. H. A., & El-Shibiny, S. (2012). Bioactive Peptides of Buffalo, Camel, Goat, Sheep, Mare, and Yak Milks and Milk Products. *Food Reviews International, 29*(1), 1-23. doi:10.1080/87559129.2012.692137

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654. doi:10.1016/j.jnutbio.2007.11.010

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183. doi:10.1016/j.idairyj.2013.04.002

Gómez-Ruiz, J. Á., López-Expósito, I., Pihlanto, A., Ramos, M., & Recio, I. (2008). Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC–MS/MS. *European Food Research and Technology, 227*(4), 1061-1067. doi:10.1007/s00217-008-0820-3

Gonzalez de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of Proteolysis in Liquid Chromatography Artisanal Cheeses: High Performance of Peptides. *Journal of dairy science, 78*, 1018-1024. doi:10.3168/jds.S0022-0302(95)76717-0

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Halgasova, N., Bukovska, G., Ugorcakova, J., Timko, J., & Kormanec, J. (2002). The *Brevibacterium £avum* sigma factor SigB has a role in the environmental stress response. *FEMS Microbiology Letters, 216*, 77-84. doi:10.1111/j.1574-6968.2002.tb11418.x

Hannon, J. A., Lopez, C., Madec, M. N., & Lortal, S. (2006). Altering Renneting pH Changes Microstructure, Cell Distribution, and Lysis of *Lactococcus lactis* AM2 in Cheese Made from Ultrafiltered Milk. *Journal of dairy science, 89*(3), 812-823.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*, 1062-1071. doi:10.1016/j.peptides.2008.02.005

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2010). Probiotics and immunosenescence: cheese as a carrier. *FEMS Immunology and Medical Microbiology, 59*, 53-59. doi:10.1111/j.1574-695X.2010.00658.x

Irlinger, F., Ah Yuen In Yung, S., Sarthou, A., Delbès-Paus, C., Montel, M., Coton, M., . . . Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food MIcrobiology, 153*, 332-338. doi:10.1016/j.ijfoodmicro.2011.11.022

Janda, J. M., & Abbott, S. L. (2006). The Genus Hafnia: from Soup to Nuts. *Clinical Microbiology Reviews, 19*(1), 12-18. doi:10.1128/CMR.19.1.12–28.2006

Kırmacı, H. A., Hayaloglu, A. A., Özer, H. B., Atasoy, A. F., & Türkoglud, H. (2014). Evolution of proteolysis in Urfa cheese made from ewe's milkby wild type starter culture systems. *Small Ruminant Research, 119*, 120-129. doi:10.1016/j.smallrumres.2014.01.006

Lamothe, S., Corbeil, M., Turgeon, S. L., & Britten, M. (2012). Influence of cheese matrix on lipid digestion in a simulated gastro-intestinal environment. *Food and Function, 3*, 724-731. doi:10.1039/c2fo10256k

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C. (2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Lignitto, L., Cavatorta, V., Balzan, S., G., G., Galaverna, G., Novelli, E., . . . Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal, 20*, 11-17. doi:10.1016/j.idairyj.2009.07.001

Lopez-Exposito, I., & Recio, I. (2008). Protective Effect of Milk Peptides: Antibacterial and Antitumor Properties. In Z. Bösze (Ed.), *Bioactive components of milk* (Vol. 606, pp. 271-293). New York: Springer.

Mater, D. D. G., Bretigny, L., Firmesse, O., Flores, M., Mogenet, A., Bresson, J., & Corthier, G. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiology Letters, 250*, 185-187. doi:10.1016/j.femsle.2005.07.006

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127-144. doi:10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H., & Fox, P. F. (1997). Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait, 77*, 41-76.

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N., & Cogan, T. M. (1993). Contribution of the Indigenous Microflora to the Maturation of Cheddar Cheese. *International Dairy Journal, 3*, 613-634. doi:10.1016/0958-6946(93)90104-8

Meira, S. M. M., Daroit, D. J., Helfer, V. E., Corrêa, A. P. F., Segalin, J., Carro, S., & Brandelli, A. (2012). Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and Uruguay. *Food Research International, 48*(1), 322-329. doi:10.1016/j.foodres.2012.05.009

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Mills, S., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2011). Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories, 10*(1), 1-15. doi:10.1186/1475-2859-10-S1-S19

Monnet, C., Landaud, S., Bonnarme, P., & Swennen, D. (2014). Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters, 263*(1), 1-9. doi:10.1093/femsle/fnu025

Mounier, J., Rea, M. C., O´Connor, P. M., Fitzgerald, G. F., & Cogan, T. M. (2007). Characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Staphylococcus* spp. Isolated from Surface-Ripened Cheese. *Applied and Enviromental Microbiology, 73*(23), 7723-7739. doi:10.1128/AEM.01260-07

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Panizzolo, L. A., Arujo, A. C., Taroco, L. V., Rodriguez, A., & Schopf, G. (2011). Evolución de la proteólisis durante la maduración de quesos Danbo elaborados con distintos cultivos iniciadores. *REVISTA DEL LABORATORIO TECNOLÓGICO DEL URUGUAY, 6*, 24-27.

Paul, M., & Van Hekken, D. L. (2011). Assessing antihypertensive activity in native and model Queso Fresco cheeses. *Journal of dairy science, 94*, 2280-2284. doi:10.3168/jds.2010-3852

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2015). Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening. *Journal of dairy science, 98*, 3690-3694. doi:10.3168/jds.2014-9097

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63. doi:10.1016/j.fm.2012.02.013

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548. doi:10.1016/j.foodres.2010.03.007

Ranadheera, C. S., Evans, C. A., Adams, C. A., & Baines, S. K. (2012). In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International, 49*, 619-625. doi:10.1016/j.foodres.2012.09.007

Ryhänen, E.-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal, 11*, 441-447. doi:10.1016/S0958-6946(01)00079-6

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., . . . Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food MIcrobiology, 144*, 293-300. doi:10.1016/j.ijfoodmicro.2010.10.009

Silva, R. A., Lima, M. S. F., Viana, J. B. M., Bezerra, V. S., Pimentel, V. S., Porto, A. L. F., . . . Lima-Filho, J. L. (2012). Can artisanal ‘‘Coalho’’ cheese from Northeastern Brazil be used as a functional food? *Food Chemistry, 135*, 1533-1538. doi:10.1016/j.foodchem.2012.06.058

Silva, S., Pihlanto, A., & Malcata, F. (2006). Bioactive Peptides in Ovine and Caprine Cheeselike Systems Prepared with Proteases from< i> Cynara cardunculus</i>. *Journal of dairy science, 89*(9), 3336-3344. doi:10.3168/jds.S0022-0302(06)72370-0

Spinnler, H. E., & Gripon, J. C. (2004). Surface Mould-ripened Cheeses. In P. F. Fox, P. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: Chemistry, Physics and Microbiology (1 ed., Vol. 2, pp. 157-174). Retrieved from http://www.sciencedirect.com/science/article/pii/S1874558X04800435.

doi:10.1016/S1874-558X(04)80043-5

Sun, C. Q., O´Connor, C. J., & Roberton, A. M. (2002). The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chemico-Biological Interactions, 140*, 185-198.

Torres-Llanez, M., González-Córdova, A., Hernandez-Mendoza, A., Garcia, H., & Vallejo-Cordoba, B. (2011). Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. *Journal of dairy science, 94*(8), 3794-3800. doi:10.3168/jds.2011-4237

Uriot, O., Galia, W., Awussi, A. A., Perrin, C., Denis, S., Chalancon, S., . . . Roussel, Y. (2016). Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. *Food Microbiology, 53*, 18-29. doi:10.1016/j.fm.2015.05.007

Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., & Acosta, F. (2008). Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. *Veterinary microbiology, 129*, 150-155. doi:10.1016/j.vetmic.2007.11.007

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910

CONCLUSIONS AND PERSPECTIVES

4. Conclusions and perspectives

This thesis is the result of a collaborative work between two international research teams, the Tecnológico de Monterrey and the AgroParisTech-INRA; that had as objective to determine the presence of bioactive peptides in Mexican cheeses; and to observe the effect of dairy matrices on microorganism´s survival during digestion.

Therefore, in this work, we put in evidence the presence of bioactive peptides with activities as antioxidant and ACE inhibitors in Cotija and Fresh goat cheeses. Our results proved that both cheeses had important antioxidant activity. This activity was higher in fractions rich in small peptides, and was correlated with the hydrophilic peptides.

Young Cotija cheese and Fresh goat cheese had similar antioxidant activity. However, antioxidant activity increased throughout ripening and six months Cotija cheese had higher antioxidant activity than Fresh goat cheese. This increase was consistent with the production of soluble peptides that remained active as antioxidants, during all ripening. Meanwhile, the pasteurization of goat milk did not cause any significant change on the antioxidant activity in Fresh goat cheeses.

So far only few cheeses varieties (Cheddar, Coalho Brazilian cheese, Caciocavallo and Parmigiano-Reggiano) have been screened for antioxidant acitiviy, and this is the first report about antioxidant peptides present naturally in Mexican cheeses.

On the other hand, peptide fractions of Cotija and Fresh goat cheese, also showed, *in vitro*, high inhibition of ACE, a mechanim highly related with the regulation of blood pressure. ACE inhibitory activity in Fresh goat cheese was slightly higher than in young Cotija cheese (two months), however values increased throughout ripening and Cotija cheese (three months old) from the market exerted similar values than Fresh goat cheese.

This finding was really interesting, since the release of peptides with ACE inhibitory activity has always been associated to the proteolysis during cheese ripening. However, our

observation in Fresh goat cheese, suggest that this peptides can also appear during cheese manufacture (released by coagulant or naturally present in milk) and can be even higher than the observed in some ripened cheeses.

The ACE inhibitory activity was highly correlated with the hydrophobic peptides in nitrogenous fractions of Cotija and Fresh goat cheese. Meanwhile, pasteurization of milk in Fresh goat cheeses, increased the amount of hydrophobic peptides, impacting on the ACE inhibitory activity and differentiating between pasteurized and non pasteurized Fresh goat cheeses.

In conclusion, Cotija and Fresh goat cheese had important amount of bioactive peptides with activities as antioxidants and potentially inhibitors of ACE, providing these cheeses with an important added value based on their biological properties. Both cheeses are an important part of the Mexican culture and are on their way to obtain a Denomination of Origin and Collective Trade Mark, respectively. Thus this work was an effort to increase the knowledge about them. Furthermore as far as we know, this is the first approach to the study of the biochemistry of Cotija cheese ripening.

However few questions remain on the air and further studies need to be carried out to understand the precise relationship between the type of peptides (hydrophobicity) in a determined nitrogenous fractions and its biological activity. Therefore we strongly recomend a separation of both types of peptides to test their activity separately to later identify the peptides with higher activity.

Also the impact of pasteurization on bioactive peptides should be further analyzed on ripened cheeses, since in Fresh goat cheese, its effect was not evident at first sight. Ripening time should also be deeper analyzed since antioxidant and ACE inhibitor peptides in Cotija cheese increased through six months, however this cheese can also be ripened for longer periods (Cotija añejo cheese) therefore it would also be interesting to observe the effect of a longer time of ripening on bioactive peptides in Cotija cheese, since previous authors have claimed that these peptides suffer further catabolism that decrease their biological activity.

Moreover, beyond the determination of bioactive peptides in Cotija and Fresh goat cheeses, it results necessary to measure the stability of these peptides during digestion, to make sure that they are not further hydrolysed into inactive peptides and amino acids, and they can reach intact the target sites where they must perform their bioactivity (bioaccesibility).

Furthermore, release of bioactive peptides in cheeses, has always been associated to the ezymatic action of the cheese microbiota. The presence of these bioactive peptides in Fresh goat cheeses made without starter cultures raises the question if these peptides were endogenous in milk or released by enzymatic action of rennet; therefore goat milk should be analyzed to clarify this interrogation.

In this regard, microbiota of Cotija cheese, has not been fully characterized, and as suggested by our results (changes in proteolysis rates, chapter 2.1) it seems to be very dynamic during ripening. Therefore, it results imperative to obtain an identification of the microorganisms present in Cotija cheese to correlate them, based on their enzymatic characteristics, with the release of bioactive peptides. Furthermore, it would be very interesting to evaluate the potential effect of the microorganisms present on Cotija cheese on human health, beyond metabolite production. Since previous studies have shown that some cheese microorganisms (isolated from European varieties) are capable to survive digestion and interact with the intestinal microbiota, producing effects on human health. With the further hypothesis that the presence of a dairy matrix (i. e. cheese) could improve their chances to survive digestion.

From this idea, the second part of this thesis, was devoted to the study of the effect of dairy matrices on microorganisms survival during digestion. Several mechanisms have been proposed to explain a claimed protective effect of food matrices during digestive stress, in this work we focused on the effect of the interaction between microorganisms and dairy components and/or microstructure; as well as on the buffer effect exerted by these matrices during gastric digestion.

Selected microorganisms, *Streptococcus thermophilus*, *Brevibacterium aurantiacum* and *Hafnia alvei* had different survival rates during *in vitro* digestion. *H. alvei* was highly resistant to

gastric and duodenal stress; *B. auranticum* was moderately resistant to gastric stress but highly resistant to duodenal stress. Meanwhile *S. thermophilus* was very sensitive to gastric stress and was not found in the duodenal compartment. However our *in vivo* analyses by 16S rRNA gene metabarcoding failed to confirm these observations because any of the tested microorganisms were detected in mouse feces.

In vitro results suggested that, the most important stress suffered by microorganisms during digestion is given in the gastric phase because of low pH, as was later confirmed by confocal laser scanning microscopy images (CLSM). This CLSM also pointed the preference of microorganisms for protein network despite the type of microstructure.

In the gastric compartment, the high buffer capacity of dairy matrices was dependent on the matrix microstructure (higher in gel than in milk) but was unaffected by fat addition.

However despite this high buffer capacity, the inclusion of the microorganisms into the dairy matrices did not enhance microorganism´s survival during digestion. Instead, fat addition increased the mortality of *H. alvei* during its pass through the intestinal phase.

To better understand the relationship between dairy matrix and microorganism´s survival, we followed the digestion of the matrix, through protein and fat degradation. Our results suggested that either fat addition or microstructure affected the protein degradation. Caseins were 30 % degraded during gastric phase, while whey proteins remained stable and the protein digestion was complete after the duodenum compartment.

On the other hand, fat degradation of both milk and gel, was lower in the stomach than in the intestine; interestingly it is in the later compartment that fat seemed to have a negative effect on survival. Meanwhile, microstructure had an effect on fat degradation that was higher in milk than in gel during the gastric phase, but the behavior is reversed during the intestinal phase where fatty acid release was higher in gel. However, despite the suggested fat effect on *H. alvei´*s survival our results were not conclusive.

In conclusion, our result suggests that the claimed protective effect of dairy matrices on microorganism´s survival during digestion might not be so dependent on the buffer capacity or the interactions between microorganisms and dairy matrices (components and microstructure). The most used hypothesis about buffer effect was not enough to enhance microorganism´s survival during digestion; and the interaction with dairy components (fat) could even decrease survival. Thus suggesting that the protective effect of the dairy matrices is mostly strain-dependent or related to changes in the digestive parameters associated to the matrix, two parameters that were not evaluated in this work.

About, the fat effect, we could not relate the concentration of the released fatty acids with microorganism´s survival, and further analysis need to be carried out on this field. We recommend a specific sensitivity test of *H. alvei* against the fatty acids released from milk fat. Furthermore, the study of possible interactions between the free fatty acids and the bioactive peptides, with antimicrobial properties, released from the dairy matrix may also give interesting results; about microorganisms survival but also on the peptide efficiency.

In this study we did not highlighted the release of this type of peptides during digestion, since protein degradation was only measured globally through caseins and whey proteins. However a more specific analysis through HPLC methods could results in more specific protein pattern degradation and a better understanding about microorganisms and dairy components interactions.

General references

Abadía-García, L., Cardador, A., Martín del Campo, S. T., Arvízu, S. M., Castaño-Tostado, E., Regalado-González, C., . . . Amaya-Llano, S. L. (2013). Influence of probiotic strains added to Cottage Cheese on potentially-antioxidant peptides generation, anti-Listerial activity, and survival of probiotic microorganisms in simulated astrointestinal conditions. *International Dairy Journal, 33*(2), 191–197. doi:10.1016/j.idairyj.2013.04.005

Adouard, N. (2015). *Les micro-organismes du microbiote fromager survivent-ils à la digestion et peuvent-ils avoir un effet immunomodulateur sur l'hôte ?* (Docteur), AgroParisTech, Thiverval-Grignon.

Adouard, N., Foligné, B., Dewulf, J., Bouix, M., Picque, D., & Bonnarme, P. (2015). In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese. *International Journal of Food MIcrobiology, 197*, 98-107. doi:10.1016/j.ijfoodmicro.2014.12.011

Adouard, N., Magne, L., Cattenoz, T., Guillemin, H., Foligné, B., Picque, D., & Bonnarme, P. (2016). Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract. *Food Microbiology, 53 part A*, 30–40. doi:10.1016/j.fm.2015.03.002

Agboola, S., Chen, S., & Zhao, J. (2004). Formation of bitter peptides during ripening of ovine milk cheese made with dierent coagulants. *Le Lait, 84*(6), 567-578. doi:10.1051/lait:2004032

Ahmed, A. S., El-Bassiony, T., Elmalt, L. M., & Ibrahim, H. R. (2015). Identification of potent antioxidant bioactive peptides from goat milk proteins. *Food Research International, 74*, 80-88. doi:10.1016/j.foodres.2015.04.032

Al-Dabbas, M. M., Al-Ismail, K., & Al-Abdullah, B. M. (2011). Effect of Chemical Composition on the Buffering Capacity of Selected Dairy Products. *Jordan Journal of Agricultural Sciences, 7*, 690-700.

Albenzio, M., Corbo, M. R., Rehman , S. U., Fox, P. F., De Angelis, M., & A. Corsetti , A. S., M. Gobbetti. (2001). Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food MIcrobiology, 67*. doi:10.1016/S0168-1605(00)00533-X

Aloğlu, H. Ş., & Öner, Z. (2011). Determination of antioxidant activity of bioactive peptide

fractions obtained from yogurt. *Journal of dairy science, 94*, 5305-5314. doi:10.3168/jds.2011-4285

Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences, 90*, 7915-7922.

Baliarda, A. (2003). *Evaluation de la reponse au stress chez les bacteries lactiques partenant aux genres Pediococcus et Tetragenococcus. Approches physiologiques et genetiques.* (Docteur), Universite Bordeaux, Bordeaux. Retrieved from http://grenet.drimm.u-bordeaux1.fr/pdf/2003/BALIARDA_AURELIE_2003.pdf (2747)

Barác, M., Smiljanic, M., Zilic, S., Pesic, M., Stanojevic, S., Vasic, M., & Vucic, T. (2016). Protein profiles and total antioxidant capacity of water soluble and insoluble protein fractions of white cow cheese at different stage of ripening. *Mljekarstavo, 66*(3), 187-197. doi:10.15567/mljekarstvo.2016.0303

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., . . . Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63, Part B*, 147-156. doi:10.1016/j.foodres.2014.02.015

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29*, 625-651. doi:10.1016/j.femsre.2004.09.003

Bertola, N. C., Califano, A. N., Bevilacqua, A. E., & Zaritzky, N. E. (2000). Effects of ripening conditions on the texture of Gouda cheese. *International Journal of Food Science and Technology, 35*, 207-214. doi:10.1046/j.1365-2621.2000.00347.x

Bezerra, V. S., Campos, J. F., da Silva, R. A., Porto, T. S., de Lima-Filho, J. S., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. *Food Science and Technology, 33*(3), 513-520. doi:10.1590/S0101-20612013005000074

Bobko, E., Tyras, M., & Jachymek, W. (2013). Reprint of "New complete structure of *Hafnia alvei* clinical isolate strain PCM 2670 semi-rough lipopolysaccharide". *Carbohydrate Research, 378*, 71-78. doi:10.1016/j.carres.2013.06.021

Bogovic-Matijasic, B., Obermajer, T., Lipoglavsek, L., Sernel, T., Locatelli, I., Kos, M., . . . Rogelj, R. (2015). Effects of synbiotic fermented milk containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. lactis BB-12 on the fecal microbiota of adults with irritable bowel syndrome: A randomized double-blind, placebo-controlled trial. *Journal of dairy science, 99*, 1-14. doi:10.3168/jds.2015-10743

Boke, H., Aslim, B., & Alp, G. (2010). The role of resistance to bile salts and acid tolerance of exopolysaccharides (EPSS) produced by yogurt starter bacteria. *Archives of biological sciences (Beograd), 62*(2), 323-328. doi:10.2298/abs1002323b

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., . . . Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature methods, 10*(1), 57-59.

Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., . . . Sforza, S. (2013). Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. *Food Science and Nutrition, 68*(8), 963-968. doi:10.3109/09637486.2013.821696

Bourlieu, C., Ménard, 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. doi:10.1016/j.foodchem.2015.03.001

Calbet, J., & MacLean, D. (1997). Role of caloric content on gastric emptying in humans. *The Journal of physiology, 498*(Pt 2), 553.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., . . . Gordon, J. I. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods, 7*(5), 335-336.

Castro, J. M., Tornadijo, M. E., Fresno, J. M., & Sandoval, H. (2014). Biocheese: A Food Probiotic Carrier. *BioMed Research International, 2015*. doi:10.1155/2015/723056

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of Lactic Acid Bacteria in the Human Stomach and Adhesion to Intestinal Cells. *Journal of dairy science, 70*(1), 1-12. doi:10.3168/jds.S0022-0302(87)79974-3

Cotter, P. D., & Hill, C. (2003). Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbioology and Molecular Biology Reviews, 67*(3), 429-453. doi:10.1128/MMBR.67.3.429–453.2003

Cushman, D., & Cheung, H. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical pharmacology, 20*(7), 1637-1648.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology, 84*, 759-768. doi:10.1046/j.1365-2672.1998.00407.x

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry, 46*, 49-53. doi:10.1021/jf970649w

Choi, H., Yang, C., Choi, K., & Bae, I. (2015). Characteristics of Gouda cheese supplemented with fruit liquors. *Journal of Animal Science and Technology, 57*(15). doi:10.5851/kosfa.2011.31.6.872

Choi, J., Sabikhi, L., Hassan, A., & Anand, S. (2012). Bioactive peptides in dairy products. *International Journal of Dairy Technology, 65*(1), 1-12. doi:10.1111/j.1471-0307.2011.00725.x

Chombo-Morales, P., Kirchmayr, M., Gschaedler, A., Lugo-Cervantes, E., & Villanueva-Rodríguez, S. (2016). Effects of controlling ripening conditions on the dynamics of the native microbial population of Mexican artisanal Cotija cheese assessed by PCR-DGGE. *LWT - Food Science and Technology, 65*, 1153-1161. doi:10.1016/j.lwt.2015.09.044

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Fischbach, M. A. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563. doi:10.1038/nature12820

De Angelis, M., & Gobbetti, M. (2004). Environmental stress responses in Lactobacillus: A review. *Proteomics, 4*, 106-122. doi:10.1002/pmic.200300497

De Simone, C., Picariello, G., Mamone, G., Stiuso, P., Dicitore, A., & Vanacore, D. (2009). Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey. *Journal of Peptide Science, 15*, 251-258. doi:10.1002/psc.1093.

del Campo, R., Bravo, D., Cantón, R., Ruiz-Garbajosa, P., García-Albiach, R., MOntesi-Libois, A., . . . Baquero, F. (2005). Scarce Evidence of Yogurt Lactic Acid Bacteria in Human Feces after Daily Yogurt Consumption by Healthy Volunteers. *Applied and Enviromental*

Microbiology, 71(1), 547-549. doi:10.1128/AEM.71.1.547–549.2005

DePeters, E. J., German, J. B., Taylor, S. J., Essex, S. T., & Perez-Monti, H. (2001). Fatty Acid and Triglyceride Composition of Milk Fat from Lactating Holstein Cows in Response to Supplemental Canola Oil. *Journal of dairy science, 84*(4), 929-936. doi:10.3168/jds.S0022-0302(01)74550-X

Devle, H., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. E., Ekeberg, D., & Schüller, R. B. (2012). Rheological characterization of milk during digestion with human gastric and duodenal enzymes. *Annual Transactions of the Nordic Rheology Society, 20*.

Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G., & Ekeberg, D. (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of bovine milk. *International Dairy Journal, 36*(1), 6-13. doi:10.1016/j.idairyj.2013.11.008

Do Espirito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2011). Influence of food matrices on probiotic viability - A review focusing on the fruity bases. *Trends in Food Science and Technology, 22*, 377-385. doi:10.1016/j.tifs.2011.04.008

Dunne, C., O´Mahony, L., Murphy, L., Thorton, G., Morrissey, D., O´Halloran, S., . . . Collins, J. K. (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *The American Journal of Clinical Nutrition, 73*(2), 386S-392S.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Rolet-Repecaud, O., Duboz, G., . . . Mackie, A. R. (2010). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition and Food Research, 54*, 1677-1689. doi:10.1002/mnfr.200900582

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461. doi:10.1093/bioinformatics/btq461

El-Salam, M. H. A., & El-Shibiny, S. (2012). Bioactive Peptides of Buffalo, Camel, Goat, Sheep, Mare, and Yak Milks and Milk Products. *Food Reviews International, 29*(1), 1-23. doi:10.1080/87559129.2012.692137

El Galiou, O., Zantar, S., Bakkali, M., & Laglaoui, A. (2013). Lipolysis and Proteolysis During the Ripening of Fresh Moroccan Goats' Milk Cheese. *World Journal of Dairy & Food Sciences, 8*, 201-206. doi:10.5829/idosi.wjdfs.2013.8.2.8140

Elli, M., Callegari, M. L., Ferrari, S., Bessi, E., Cattivelli, D., Soldi, S., . . . Antoine, J. (2006). Survival of Yogurt Bacteria in the Human Gut. *Applied and Enviromental Microbiology, 72*(7), 5113-5117. doi:10.1128/AEM.02950-05

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry, 19*(10), 643-654. doi:10.1016/j.jnutbio.2007.11.010

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183. doi:10.1016/j.idairyj.2013.04.002

Faye, T., Tamburello, A., Vegarud, G. E., & Skeie, S. (2012). Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human astric and duodenum juice. *Journal of dairy science, 95*, 558-566. doi:10.3168/jds.2011-4705

Firmesse, O., Alvaro, E., Mogenet, A., Bresson, J., Lemee, R., Le Ruyet, P., . . . Rigottier-Gois, L. (2008). Fate and effects of Camembert cheese micro-organisms in the human colonic microbiota of healthy volunteers after regular Camembert consumption. *International Journal of Food MIcrobiology, 125*, 176-181. doi:10.1016/j.ijfoodmicro.2008.03.044

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *The Journal of nutrition, 134*(4), 980S-988S.

Flores-Magallón, R., Oliva-Hernández, A. A., & Narváez-Zapata, J. A., . (2011). Characterization of Microbial Traits Involved with the Elaboration of the Cotija Cheese. *Food Science and Biotechnology, 20*(4), 997-1003. doi:10.1007/s10068-011-0137-z

Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Reviews International, 12*(4), 457-509. doi:10.1080/87559129609541091

Franco, I., Prieto, B., Urdiales, R., Fresno, J. M., & Carballo, J. (2001). Study of the biochemical changes during ripening of Ahumado de Áliva cheese: a Spanish traditional variety. *Food Chemistry, 74*, 463-469. doi:10.1016/S0308-8146(01)00164-9

García-Hernández, J., Moreno, Y., Chuan, C., & Hernández, M. (2012). In Vivo Study of the Survival of *Lactobacillus delbruecki* subsp. *bulgaricus* CECT 4005T and *Streptococcus thermophilus* CECT 801 by DVC-FISH after Consumption of Fermented Milk. *Journal of Food Science, 77*(10), M593- M597. doi:10.1111/j.1750-3841.2012.02918.x

Gardiner, G., Ross, R. P., Collins, J. K., Fitzgerald, G., & Stanton, C. (1998). Development of a Probiotic Cheddar Cheese Containing Human-Derived *Lactobacillus paracasei* Strains. *Applied and Enviromental Microbiology, 64*(6), 2192-2199.

Gobbetti, M. (2004). Extra-hard varieties. *Cheese: Chemistry, Physics and Microbiology, 2*, 51-70. doi:10.1016/S1874-558X(04)80039-3

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., & Di Cagno, R. (2002). Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Critical Reviews in Food Science and Nutrition, 42*(3), 223-239. doi:10.1080/10408690290825538

Godon, J. J., Zumstein, E., Dabert, P., Habouzit, F., & Moletta, R. (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Applied and Environmental Microbiology, 63*(7), 2802-2813.

Gómez-Ruiz, J. Á., López-Expósito, I., Pihlanto, A., Ramos, M., & Recio, I. (2008). Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC–MS/MS. *European Food Research and Technology, 227*(4), 1061-1067. doi:10.1007/s00217-008-0820-3

González-Córdova, A. F., Yescas, C., Ortiz-Estrada, A. M., De la Rosa-Alcaraz, M. A., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2016). Invited review: Artisanal Mexican cheeses. *Journal of dairy science, 99*, 1-13. doi:10.3168/jds.2015-10103

Gonzalez de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of Proteolysis in Liquid Chromatography Artisanal Cheeses: High Performance of Peptides. *Journal of dairy science, 78*, 1018-1024. doi:10.3168/jds.S0022-0302(95)76717-0

Gripon, J. C. (1997). Flavour and textrure in soft cheese. In B. A. Law (Ed.), *Microbiology and Biochemistry of Cheese and Fermented Milk* (Vol. 1, pp. 193-203). Berkshire, U. K.: Blackie Academic and Professional

Guerra-Martínez, J. A., Montejano, J. G., & Martín-del-Campo, S. T. (2012). Evaluation of proteolytic and physicochemical changes during storage of fresh Panela cheese from Queretaro, Mexico and its impact in texture. *CyTA - Journal of Food, 10*(4), 296-305. doi:10.1080/19476337.2011.653791

Guillemin, H., Perret, B., Picque, D., Menard, O., & Cattenoz, T. (2010). Logiciel StoRM-stomach and duodenum regulation and monitoring. *IDDNFR001230009000RP201000031235, 290*.

Guinee, T. P., & O´Callaghan, D. J. (2010). The formation of Cheese Curd. In B. A. Law & A. Y. Tamime (Eds.), Techology of Cheesemaking (pp. 260-314). United Kingdom Wiley-

Blackwell.

Gunn, J. S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes and Infection, 8*, 907-913. doi:10.1016/S1286-4579(00)00392-0

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International Journal of Dairy Technology, 62*(3). doi:10.1111/j.1471-0307.2009.00509.x

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2013). ACE-Inhibitory Activity of Cheddar Cheeses Made with Adjunct Cultures at Different Stages of Ripening. *Advances in Dairy Research, 1*(1). doi:10.4172/2329-888X.1000102

Hajirostamloo, B. (2010). Bioactive component in milk and dairy product. *World Acad Sci Eng Technol, 72*, 162-166.

Halgasova, N., Bukovska, G., Ugorcakova, J., Timko, J., & Kormanec, J. (2002). The *Brevibacterium £avum* sigma factor SigB has a role in the environmental stress response. *FEMS Microbiology Letters, 216*, 77-84. doi:10.1111/j.1574-6968.2002.tb11418.x

Hannon, J. A., Lopez, C., Madec, M. N., & Lortal, S. (2006). Altering Renneting pH Changes Microstructure, Cell Distribution, and Lysis of *Lactococcus lactis* AM2 in Cheese Made from Ultrafiltered Milk. *Journal of dairy science, 89*(3), 812-823.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current opinion in biotechnology, 18*(2), 163-169. doi:10.1016/j.copbio.2007.01.013

Hayaloglu, A. A., & Brechany, E. Y. (2007). Influence of milk pasteurization and scalding temperature on the volatile compounds of Malatya, a farmhouse Halloumi-type cheese. *Lait, 87*, 39-57. doi:10.1051/lait:2006025

Hayaloglu, A. A., Ozer, B. H., & Fox, P. F. (2008). Cheeses of Turkey: 2. Varieties ripened under brine. *Dairy Science and Technology, 88*, 225-244. doi:10.1051/dst:2007014

Hernández-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Angiotensin Converting Enzyme Inhibitory Activity in Commercial Fermented Products. Formation of Peptides under Simulated Gastrointestinal Digestion. *Journal of Agricultural and Food Chemistry, 52*(6), 1504-1510. doi:10.1021/jf034997b

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and*

Interface Science, 165(1), 23-35. doi:10.1016/j.cis.2010.11.001

Hernández-Morales, C., Hernández-Montes, A., Aguirre-Mandujano, E., & Villegas, A. (2010). Physicochemical, microbiological, textural and sensory characterisation of Mexican Añejo cheese. *International Journal of Dairy Technology, 63*(4), 552-560. doi:10.1111/j.1471-0307.2010.00615.x

Hernández Galán, L., Cardador Martínez, A., Picque, D., Spinnler, H. E., López del Castillo Lozano, M., & Martín del Campo Barba, S. T. (2016). Angiotensin converting enzyme inhibitors and antioxidant peptides release during ripening of Mexican Cotija hard cheese. *Journal of Food Research, 5*(3).

Hernández, V., Navarro, A., & Quirasco, M. (2009a). Antioxidant capacity in the Cotija "Región de Origen" Cheese, the case of an artisanal cheese. *Carnilac Industrial, 24*(1), 24-31.

Hernández, V., Quirasco, M., & Quintero, B. (2009b). Un acercamiento al mundo del queso Cotija Región de Origen MC: arte y tradición de México. *Culinaria, Revista Virtual Gastronómica 5,* 5-19.

Hnosko, J., Clark, S., & Van Hekken, D. (2009). Latin American Cheeses. In S. Clark, Costello, M., Drake, M., Bodyfelt, F. (Ed.), *The Sensory Evaluation of Dairy Products* (pp. 489-504). Lincoln, New England: Springler

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides, 29*, 1062-1071. doi:10.1016/j.peptides.2008.02.005

Hutkins, R. W., & Nannen, N. L. (1993). pH Homeostasis in Lactic Acid Bacteria. *Journal of dairy science, 76*(8), 2354-2365. doi:10.3168/jds.S0022-0302(93)77573-6

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., & Ouwehand, A. C. (2010). Probiotics and immunosenescence: cheese as a carrier. *FEMS Immunology and Medical Microbiology, 59*, 53-59. doi:10.1111/j.1574-695X.2010.00658.x

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., . . . Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Sci. Technol., 90*(5), 549-563.

Irlinger, F., Ah Yuen In Yung, S., Sarthou, A., Delbès-Paus, C., Montel, M., Coton, M., . . . Helinck, S. (2012). Ecological and aromatic impact of two Gram-negative bacteria

(*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food MIcrobiology, 153*, 332-338. doi:10.1016/j.ijfoodmicro.2011.11.022

Jäkälä, P., & Vapaatalo, H. (2010). Antihypertensive Peptides from Milk Proteins. *Pharmaceuticals, 3*(1), 251-272.

Janda, J. M., & Abbott, S. L. (2006). The Genus Hafnia: from Soup to Nuts. *Clinical Microbiology Reviews, 19*(1), 12-18. doi:10.1128/CMR.19.1.12–28.2006

Janhoj, T., & Qvist, K. B. (2010). The formation of Cheese Curd. In B. A. Law & A. Y. Tamime (Eds.), Techology of Cheesemaking (pp. 130-156). United Kingdom Wiley-Blackwell.

Johnson, M., & Law, B. A. (2010). The Origins, Development and Basic Operations of Cheesemaking Technology. In B. A. Law & A. Y. Tamime (Eds.), Technology of CheesemakingSociety of Dairy Technology book series (2 ed., Vol. 1, pp. 68-96). United Kingdom Blackwell Publishing Ltd. Retrieved from https://books.google.com.mx/books?id=Turm77IMxnUC&pg=RA1-PT21&lpg=RA1-PT21&dq=ph+of+the+cheese+syneresis&source=bl&ots=r6DlG5fvj1&sig=fdISx_4-RYLtgsla-5D9OJD216c&hl=fr&sa=X&ved=0ahUKEwjyndST8YrMAhUKuYMKHdTADnYQ6AEIOzAF#v=onepage&q=ph%20of%20the%20cheese%20syneresis&f=false.

Katsiari, M. C., Alichanidis, E., Voutsinas, L. P., & Roussis, I. G. (2000). Proteolysis in reduced sodium Feta cheese made by partial substitution of NaCl by KCl. *International Dairy Journal, 10*, 635 - 646. doi:10.1016/S0958-6946(00)00097-2

Kedare, S. B., & Singh, R. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of food science and technology, 48*(4), 412-422.

Kırmacı, H. A., Hayaloglu, A. A., Özer, H. B., Atasoy, A. F., & Türkoglud, H. (2014). Evolution of proteolysis in Urfa cheese made from ewe's milkby wild type starter culture systems. *Small Ruminant Research, 119*, 120-129. doi:10.1016/j.smallrumres.2014.01.006

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323. doi:10.2174/1381612033454883

Kong, F., & Singh, R. P. (2008). Disintegration of Solid Foods in Human Stomach. *Journal of Food Science, 73*(5), R67-R80. doi:10.1111/j.1750-3841.2008.00766.x

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods, 1*(2), 177-187. doi:10.1016/j.jff.2009.01.007

Korhonen, H., & Pihlanto, A. (2003). Food-derived Bioactive Peptides – Opportunities for Designing Future Foods. *Current Pharmaceutical Design, 9*, 1297-1308. doi:10.1016/j.idairyj.2005.10.012

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945-960.

Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., . . . Etherton, T. D. (2002). Bioactive Compounds in Foods: Their Role in the Prevention of Cardiovascular Disease and Cancer. *The American Journal of Medicine, 113*(9B), 71-88. doi:10.1016/S0002-9343(01)00995-0

Lacroix, C., Verret, P., & Paquin, P. (1996). Regional and Seasonal Variations of Nitrogen Fractions in Commingled Milk. *International Dairy Journal, 6*, 947-961. doi:10.1016/S0958-6946(96)00022-2

Lamothe, S., Corbeil, M., Turgeon, S. L., & Britten, M. (2012). Influence of cheese matrix on lipid digestion in a simulated gastro-intestinal environment. *Food and Function, 3*, 724-731. doi:10.1039/c2fo10256k

Lay, C., Sutren, M., Lepercq, P., Juste, C., Rigottier-Gois, L., Lhoste, E., . . . Andrieux, C. (2004). Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats. *British Journal of Nutrition, 92*, 429-438. doi:10.1079/BJN20041192

Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D. P., O'Donohue, M., & Hernandez-Raquet, G. (2015). Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium. *Bioresource Technology, 196*, 241-249. doi:10.1016/j.biortech.2015.07.084

Li, G. (2012). *Intestinal Probiotics: Interactions with Bile Salts and Reduction of Cholesterol*. Paper presented at the 2011 International Conference on Environmental Science and Engineering (ICESE 2011).

Lignitto, L., Cavatorta, V., Balzan, S., G., G., Galaverna, G., Novelli, E., . . . Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal, 20*, 11-17. doi:10.1016/j.idairyj.2009.07.001

Lopez-Exposito, I., & Recio, I. (2008). Protective Effect of Milk Peptides: Antibacterial and Antitumor Properties. In Z. Bösze (Ed.), *Bioactive components of milk* (Vol. 606, pp. 271-293). New York: Springer.

Lucey, J., Hauth, B., Gorry, C., & Fox, P. (1993). The acid-base buffering properties of milk. *Milchwissenschaft, 48*(5), 268-272.

Lucey, J. A., & Fox, P. F. (1993). Importance of Calcium and Phosphate in Cheese Manufacture: A Review. *Journal of dairy science, 76*(6), 1714-1724. doi:10.3168/jds.S0022-0302(93)77504-9

Lüthi-Peng, Q., & Puhan, Z. (1999). Determination of protein and casein in milk by fourth derivative UV spectrophotometry. *Analytica Chimica Acta, 393*(1-3), 227-234. doi:10.1016/S0003-2670(98)00823-X

Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics, 27*(21), 2957-2963. doi:10.1093/bioinformatics/btr507

Marteau, P., Minekus, M., Havenaar, R., & Huis In´t Veld, J. H. J. (1997). Survival of Lactic Acid Bacteria in a Dynamic Model of the Stomach and Small Intestine: Validation and the Effects of Bile. *Journal of dairy science, 80*, 1031-1037. doi:10.3168/jds.S0022-0302(97)76027-2

Martínez Augustin, O., & Martínez de Victoria, E. (2006). Proteins and peptides in enteral nutrition. *Nutrición Hospitalaria, 21*(2), 1-14.

Mater, D. D. G., Bretigny, L., Firmesse, O., Flores, M., Mogenet, A., Bresson, J., & Corthier, G. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiology Letters, 250*, 185-187. doi:10.1016/j.femsle.2005.07.006

Mayer, H. K., Rockenbauer, C., & Mlcak, H. (1998). Evaluation of proteolysis in Parmesan cheese using electrophoresis and HPLC. *Dairy Science and Technology, 78*(4), 425-438. doi:10.1051/lait:1998441

McBee, M. E., & Schauer, D. B. (2006). The Genus Hafnia. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes: Volume 6: Proteobacteria: Gamma Subclass* (pp. 215-218). New York, NY: Springer New York.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., . . .

Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J, 6*(3), 610-618.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127-144. doi:10.1111/j.1471-0307.2004.00147.x

McSweeney, P. L. H., & Fox, P. F. (1997). Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait, 77*, 41-76.

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N., & Cogan, T. M. (1993). Contribution of the Indigenous Microflora to the Maturation of Cheddar Cheese. *International Dairy Journal, 3*, 613-634. doi:10.1016/0958-6946(93)90104-8

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait, 80*, 293-324.

Meira, S. M. M., Daroit, D. J., Helfer, V. E., Corrêa, A. P. F., Segalin, J., Carro, S., & Brandelli, A. (2012). Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and Uruguay. *Food Research International, 48*(1), 322-329. doi:10.1016/j.foodres.2012.05.009

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *BioFactors, 21*, 55-61. doi:10.1002/biof.552210111

Meisel, H. (2005). Biochemical Properties of Peptides Encrypted in Bovine Milk Proteins. *Current medicinal chemistry, 12*, 1623-1629. doi:10.2174/0929867054546618

Meisel, H., & Günther, S. (1998). Food proteins as precursors of peptides modulating human cell activity. *Food / Nahrung, 42*, 175-176.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039-1045. doi:10.1016/j.foodchem.2013.09.036

Michalski, M. (2007). On the supposed influence of milk homogenization on the risk of CVD, diabetes and allergy. *British Journal of Nutrition, 97*, 598-610. doi:10.1017/S0007114507657900

Mills, S., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2011). Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories, 10*(1), 1-15. doi:10.1186/1475-2859-10-S1-S19

Mollet, B., & Rowland, I. (2002). Functional foods: at the frontier between food and pharma.

Current in Opinion and Biotechnology, 13, 483-486. doi:10.1016/S0958-1669(02)00375-0

Monnet, C., Landaud, S., Bonnarme, P., & Swennen, D. (2014). Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters, 263*(1), 1-9. doi:10.1093/femsle/fnu025

Mounier, J., Rea, M. C., O´Connor, P. M., Fitzgerald, G. F., & Cogan, T. M. (2007). Characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Staphylococcus* spp. Isolated from Surface-Ripened Cheese. *Applied and Enviromental Microbiology, 73*(23), 7723-7739. doi:10.1128/AEM.01260-07

Mullally, M. M., Mehra, R., & Fitzgerald, R. J. (1998). Thermal effects onthe conformation and susceptibility of beta-lactoglobulin to hydrolysis by gastric and pancreatic endoproteinases. *Irish Journal of Agricultural and Food Research, 37*(1), 51-60.

Ng-Kwai-Hang, K. F., Hayes, J. F., Moxley, J. E., & Monardes, H. G. (1984). Variability of Test-Day Milk Production and Composition and Relation of Somatic Cell Counts with Yield and Compositional Changes of Bovine Milk. *Journal of dairy science, 67*, 361-366. doi:10.3168/jds.S0022-0302(84)81309-0

Olvera-García, V., Cardador-Martínez, A., & Martín del Campo, S. T. (2015). Effect of Thermal Process and Filtration on the Antioxidant Activity and Physicochemical Parameters of Agave atrovirens Extracts. *Journal of Food Research, 4*, 155-167. doi:10.5539/jfr.v4n1p155

Ong, L., Henriksson, A., & Shah, N., P. (2007). Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic Lactobacillus casei sp. *Lait, 87*, 149-165. doi:10.1051/lait:2007004

Panizzolo, L. A., Arujo, A. C., Taroco, L. V., Rodriguez, A., & Schopf, G. (2011). Evolución de la proteólisis durante la maduración de quesos Danbo elaborados con distintos cultivos iniciadores. *REVISTA DEL LABORATORIO TECNOLÓGICO DEL URUGUAY, 6*, 24-27.

Parada, J., & Aguilera, J. M. (2007). Food Microstructure Affects the Bioavailability of Several Nutrients. *Journal of Food Science, 72*(2), 21-32. doi:10.1111/j.1750-3841.2007.00274.x

Parodi, P. W. (1982). Positional distribution of fatty acids in triglycerides from milk of several species of mammals. *Lipids, 17*(6), 437-442. doi:10.1007/BF02535223

Paul, M., & Van Hekken, D. L. (2011). Assessing antihypertensive activity in native and model

Queso Fresco cheeses. *Journal of dairy science, 94*, 2280-2284. doi:10.3168/jds.2010-3852

Pavia, M., Trujillo, A. J., Guamis, B., & Ferragut, V. (2000). Ripening control of salt-reduced Manchego-type cheeseobtained by brine vacuum-mpregnation. *Food Chemistry, 70*, 155-162. doi:10.1016/S0308-8146(99)00249-6

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2015). Short communication: Effect of genetic type on antioxidant activity of Caciocavallo cheese during ripening. *Journal of dairy science, 98*, 3690-3694. doi:10.3168/jds.2014-9097

Piddock, L. J. V. (2006). Multidrug-resistance efflux pumps, not just for resistance. *Nature Reviews Microbiology, 4*, 629-636. doi:10.1038/nrmicro1464

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*(11), 1306-1314. doi:10.1016/j.idairyj.2006.06.005

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 31*, 57-63. doi:10.1016/j.fm.2012.02.013

Pitino, I., Randazzo, C. L., Mandalari, G., Lo Curto, A., Faulks, R. M., Le Marc, Y., . . . Wickham, M. S. J. (2010). Survival of *Lactobacillus rhamnosus* strains in the upper gastrointestinal tract. *Food Microbiology, 27*, 1121-1127. doi:10.1016/j.fm.2010.07.019

Poméon, T. (2007). *El queso Cotija, México. Un producto con marca colectiva queso "Cotija Región de origen", en proceso de adquisición de una Denominación de Origen*. Retrieved from

Prakash, A., Rigelhof, F., & MIller, E. (2001). Antioxidant activity. *Medallion laboratories analytical progress, 19*(2), 1-4.

Prakash, S., Ma, Q., & Bhandari, B. (2014). Rheological behaviour of selected commercially available baby formulas in simulated human digestive system. *Food Research International, 64*, 889-895. doi:10.1016/j.foodres.2014.08.028

Pritchard, S. R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International, 43*, 1545-1548. doi:10.1016/j.foodres.2010.03.007

Ranadheera, C. S., Evans, C. A., Adams, C. A., & Baines, S. K. (2012). In vitro analysis of

gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Research International, 49*, 619-625. doi:10.1016/j.foodres.2012.09.007

Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K., & da Silva-Pinto, M. (2012). Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British Journal of Clinical Pharmacology, 75*(3), 588-602. doi:10.1111/j.1365-2125.2012.04425.x

Rinaldi, L., Gauthier, S. F., Britten, M., & Turgeon, S. L. (2014a). In vitro gastrointestinal digestion of liquid and semi-liquid dairy matrixes. *LWT - Food Science and Technology, 57*(1), 99-105. doi:10.1016/j.lwt.2014.01.026

Rinaldi, L., Rioux, L., Britten, M., & Turgeon, S. L. (2014b). In vitro bioaccessibility of peptides and amino acids from yogurt made with starch, pectin, or β-glucan. *International Dairy Journal*, 1-7. doi:10.1016/j.idairyj.2014.09.005

Ruas-Madiedo, P., Bada-Gancedo, J. C., Delgado, T., Gueimonde, M., & De los Reyes-Gavilán, C. G. (2003). Proteolysis in rennet-coagulated Spanish hard cheeses made from milk preserved by refrigeration and addition of carbon dioxide. *Journal of Dairy Research, 79*, 115-122. doi:10.1017/S0022029902005952

Ruiz, L., Margolles, A., & Sanchez, B. (2013). Bile resistence mechanisms in Lactobacillus and Bifidobacterium *Frontiers in Microbiology, 4*, 396. doi:10.3389/fmicb.2013.00396

Ryhänen, E.-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal, 11*, 441-447. doi:10.1016/S0958-6946(01)00079-6

Saarela, M., Mogensen, G., Fonden, R., Matto, J., & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology, 84*, 197-215. doi:10.1016/S0168-1656(00)00375-8

Sachs, G., Weeks, D. L., Wen, Y., Marcus, E. A., & Scott, D. R. (2005). Acid Acclimation by Helicobacter pylori. *American Physiological Society, 20*(6), 429-438. doi:10.1152/physiol.00032.2005

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese. *Journal of dairy science, 83*(7), 1434-1440. doi:10.3168/jds.S0022-0302(00)75013-2

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal, 15*, 95-109. doi:10.1016/j.idairyj.2004.06.007

Sánchez-Macías, D., Morales-delaNuez, A., Moreno-Indias, I., Hernández-Castellano, L. E., Mendoza-Grimón, V., Castro, N., & Argüello, A. (2011). Lipolysis and proteolysis profiles of fresh artisanal goat cheese made with raw milk with 3 different fat contents. *Journal of dairy science, 94*, 5786-5793. doi:10.3168/jds.2011-4423

Sanders, M. E., & Marco, M. L. (2009). Food Formats for Effective Delivery of Probiotics. *The Annual Reviews of Food Science and Technology, 1*, 65-85. doi:10.1146/annurev.food.080708.100743

Sangabriel Hernandez, H. (2011). *Red Regional de Microqueserías Artesanales con Leche de Cabra* Veracruz: Comite Nacional Sistema Producto Caprinos Retrieved from http://www.cnsp.caprinos.org.mx/documentos/conferencias2011/RedRegionalMicroqueserias Ver.pdf.

Sarkar, A., Goh, K. K. T., Singh, R. P., & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model. *Food Hydrocolloids, 23*(6), 1563-1569. doi:10.1016/j.foodhyd.2008.10.014

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., . . . Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food MIcrobiology, 144*, 293-300. doi:10.1016/j.ijfoodmicro.2010.10.009

Schlamowitz, M., & Peterson, L. U. (1959). Studies on the Optimun pH for the Action of Pepsin on "Native" and Denatured Bovine Serum Albumin and Bovine Hemoglobin. *The Journal of Biological Chemistry, 234*(12), 3137-3145.

Scholz, W. (1997). Elaboración de quesos de oveja y de cabra (pp. 145). Zaragoza, Spain: Acribia.

Silva, R. A., Lima, M. S. F., Viana, J. B. M., Bezerra, V. S., Pimentel, V. S., Porto, A. L. F., . . . Lima-Filho, J. L. (2012). Can artisanal ‘‘Coalho’’ cheese from Northeastern Brazil be used as a functional food? *Food Chemistry, 135*, 1533-1538. doi:10.1016/j.foodchem.2012.06.058

Silva, S., Pihlanto, A., & Malcata, F. (2006). Bioactive Peptides in Ovine and Caprine Cheeselike Systems Prepared with Proteases from< i> Cynara cardunculus</i>. *Journal of dairy*

science, 89(9), 3336-3344. doi:10.3168/jds.S0022-0302(06)72370-0

Spinnler, H. E., & Gripon, J. C. (2004). Surface Mould-ripened Cheeses. In P. F. Fox, P. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: Chemistry, Physics and Microbiology (1 ed., Vol. 2, pp. 157-174). Retrieved from http://www.sciencedirect.com/science/article/pii/S1874558X04800435. doi:10.1016/S1874-558X(04)80043-5

Sumeri, I., Adamberg, S., Uusna, R., Sarand, I., & Paalme, T. (2012). Survival of cheese bacteria in a gastrointestinal tract simulator. *International Dairy Journal, 25*, 36-41. doi:10.1016/j.idairyj.2011.12.016

Sun, C. Q., O´Connor, C. J., & Roberton, A. M. (2002). The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chemico-Biological Interactions, 140*, 185-198.

Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochemical and Biophysical Research Communications, 453*, 254-267. doi:10.1016/j.bbrc.2014.05.090

Theolier, J., Hammami, R., Fliss, I., & Jean, J. (2014). Antibacterial and antifungal activity of water-soluble extracts from Mozzarella, Gouda, wiss, and Cheddar commercial cheeses produced in Canada. *Dairy Science and Technology, 94*, 427-438. doi:10.1007/s13594-014-0170-9

Timón, M. L., Parra, V., Otte, J., Broncano, J. M., & Petrón, M. J. (2014). Identification of radical scavenging peptides (<3 kDa) from Burgos-type cheese. *LWT - Food Science and Technology, 57*(1), 359-365. doi:10.1016/j.lwt.2014.01.020

Torres-Llanez, M., González-Córdova, A., Hernandez-Mendoza, A., Garcia, H., & Vallejo-Cordoba, B. (2011). Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. *Journal of dairy science, 94*(8), 3794-3800. doi:10.3168/jds.2011-4237

Turgeon, S. L., & Rioux, L. (2011). Food matrix impact on macronutrients nutritional properties *Food Hydrocolloids, 25*, 1915-1924. doi:10.1016/j.foodhyd.2011.02.026

Upreti, P., Bühlmann, P., & Metzger, L. E. (2006). Influence of Calcium and Phosphorus, Lactose, and Salt-to-Moisture Ratio on Cheddar Cheese Quality: pH Buffering Properties of Cheese. *Journal of dairy science, 89*, 938-950.

Uriot, O., Galia, W., Awussi, A. A., Perrin, C., Denis, S., Chalancon, S., . . . Roussel, Y. (2016).

Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut. *Food Microbiology, 53*, 18-29. doi:10.1016/j.fm.2015.05.007

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 357–366. doi:10.1079/BJN20041189

Vivar-Quintana, A. M., Blanco López, M. A., Revilla, I., González-Martín, I., Hernández-Hierro, J. M., & González-Pérez, C. (2009). Seasonal Evolution of Hydrophilic and Hydrophobic Peptide Contents in Cheeses Made from Ewe's Goat's or Cow's Milk. *Czech Journal of Food Science, 27*(special issue), 5106-5108.

Vivas, J., Padilla, D., Real, F., Bravo, J., Grasso, V., & Acosta, F. (2008). Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. *Veterinary microbiology, 129*, 150-155. doi:10.1016/j.vetmic.2007.11.007

Voigt, D. D., Chevalier, F., Qian, M. C., & Kelly, A. L. (2010). Effect of high-pressure treatment on microbiology, proteolysis, lipolysis and levels of flavour compounds in mature blue-veined cheese. *Innovative Food Science & Emerging Technologies, 11*(1), 68-77. doi:10.1016/j.ifset.2009.10.009

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261-5267. doi:10.1128/aem.00062-07

Wang, W., Wang, N., Zhang, Y., Cai, Z., Chen, Q., & He, G. (2013). A Convenient RP-HPLC Method for Assay Bioactivities of Angiotensin I-Converting Enzyme Inhibitory Peptides. *ISRN Biotechnology, 2013*. doi:10.5402/2013/453910

Whittier, E. (1929). Buffer intensities of milk and milk constituents I. The buffer action of casein in milk. *Journal of Biological Chemistry, 83*(1), 79-88.

WHO, & FAO. (2003). Diet, Nutrition and The prevention of chronic diseases (pp. 160). Geneva: WHO

ÉCOLE DOCTORALE
Agriculture, alimentation, biologie, environnement, santé (ABIES)

Titre: Potentiel des écosystèmes microbiens fromagers pour la formation de peptides bioactifs, et effets dûs aux matrices laitières sur la survie des microorganismes d´intérêt fromager au cours de la digestion.

Mots clés : peptides bioactifs, fromage, microorganismes, digestion, matrice laitière

Résumé : Cette thèse eu deux objectifs: A) Déterminer la présence de peptides bioactifs avec des activités antioxydantes ou inhibitrices d´enzyme de conversion de l'angiotensine (ECA) dans les fromages mexicains. B) Observer l'effet de la composition et de la structure de matrices laitières dans la survie des microorganismes laitières pendant la digestion. Les fromages ont été analysés séparément: fromage Cotija, affiné 6 mois et fromage de chèvre frais (non affiné) produit à partir de lait cru et pasteurisé. L´activité antioxydante a été plus élevé dans les fractions contenant des petits peptides. Le fromage Cotija jeune a donné des valeurs similaires au fromage de chèvre frais mais l'activité augmente pendant l´affinage (en raison de la production de peptides solubles). La pasteurisation n'a pas impacté l'activité antioxydante dans les fromages de chèvre frais. Cette activité a été corrélée avec les peptides hydrophiles. Les deux fromages ont une activité inhibitrice de l´ECA qui a été plus élevée dans le fromage de chèvre que dans le fromage Cotija jeune. Cette activité était corrélée avec les peptides hydrophobes. Les fromages de chèvre frais, fait avec du lait pasteurisés, ont une concentration en peptides hydrophobes plus élevé. La libération de peptides bioactifs dans le fromage a toujours été considérée comme dépendante de la protéolyse par action des microorganismes. Certains de ces microorganismes sont capables de survivre à la digestion et l'hypothèse que la présence d'une matrice alimentaire pourrait améliorer leur résistance pendant la digestion a été testée. Nous avons mesuré la viabilité des microorganismes; *Streptococcus thermophilus*, *Brevibacterium aurantiacum* et *Hafnia alvei* pendant la digestion (*in vitro* et *in vivo)*. Les microorganismes ont été inclus dans des matrices laitières liquides et gélifiées avec et sans matière grasse: lait écrème (SM), lait entier (WM), gel présure de lait écrèmé (GSM) et gel présure de lait entier (GWM). La digestion *in vitro* a été réalisée sur un digesteur dynamique (DIDGI) qui simule l'estomac, le duodénum et l'intestin grêle. Les paramètres de la digestion ont été les mêmes pour toutes les matrices. La dégradation des matrices pendant digestion *in vitro* a été analysée par électrophorèse et GC-Mass. La microscopie confocale à balayage laser (CLSM) a été aussi réalisée pour observer la microstructure. Pendant la digestion *in vitro*, il a été montré que *S. thermophilus* était très sensible au stress gastrique, et n'a pas été trouvé dans le compartiment duodénale. *B. auranticum* était modérément sensible au stress gastrique, mais résistant au stress duodénale. *H. alvei* était très résistant aux deux stress, par contre aucun effet des matrices laitières n'a été observé. Les images de CLSM ont montré l'effet de l´acidité sur la survie des microorganismes. Mais les analyse *in vivo* n'ont pas réussi à confirmer les observations *in vitro* parce que les microorganismes testés ne sont pas détectés.

Title : Potential of cheese microorganisms ecosystems for the production of bioactive peptides, and effect of the dairy matrices in the survival of dairy microorganisms through digestion

Keywords : bioactive peptides, cheese, microorganisms, digestion, dairy matrix

Abstract : This thesis was realized to respond to two particular objectives a) to determine the presence of bioactive peptides with activities as antioxidants and inhibitors of the angiotensin converting enzyme (ACE) in Mexican cheeses; and b), to observe the effect of composition and structure of dairy matrices in survival of dairy microorganism through digestion.
Two Mexican cheeses were analyzed separately: Cotija cheeses ripened 6 months; and Fresh goat cheese (unripened) produced from raw and pasteurized milk. Studied cheeses had important antioxidant activity that was higher in fractions with smaller peptides. Young Cotija cheese had similar values than Fresh goat cheese. But activity increased throughout ripening (because of the production of soluble peptides). Milk pasteurization (Fresh goat cheeses) did not affect the antioxidant activity. This activity was correlated with the hydrophilic peptides. Also, both cheeses possess ACE inhibitor activity that was higher in Fresh goat cheese than in young Cotija cheese. This inhibitor activity was more correlated with the hydrophobic peptides. Pasteurization of Fresh goat cheeses increased the production of hydrophobic peptides. The release of bioactive peptides in cheeses has always been considered as dependent of the proteolysis caused by cheese microbiota. Some of these microorganisms are capable to survive digestion. It has been hypothesized that the presence of a food matrix could enhance their resistance through digestion. We measured the viability of three dairy microorganisms; *Streptococcus thermophilus*, *Brevibacterium aurantiacum* and *Hafnia alvei* during (*in vitro* and *in vivo*) digestion. Microorganisms were included in liquid and gel dairy matrices with and without fat: Skim milk (SM), whole milk (WM), rennet gel of skim milk (GSM) and rennet gel of whole milk (GWM). *In vitro* digestion was carried on with a dynamic digester (DIDGI) that simulated the stomach, duodenum and small intestine. Parameters of digestion were the same for all the matrices tested. The degradation of the dairy matrices through *in vitro* digestion was analyzed by electrophoreses and GC-Mass. Confocal laser scanning microscopy (CLSM) was performed to observe matrix microstructure.During *in vitro* digestion, the microorganisms had different survival rates; *S. thermophilus* was highly sensitive to gastric stress, and was not found in the duodenal compartment. *B. auranticum* was moderately sensitive to gastric stress but resistant to duodenal stress. *H. alvei* was highly resistant to both stresses. Despite its buffer capacity, we did not observe any effect of the dairy matrices on microorganisms survival. CLSM images, probed the effect of low pH on microorganisms survival. However our *in vivo* analyses failed to confirm *in vitro* observations and tested microorganisms were not detected.

Université Paris-Saclay-Ecole Doctoral ABIES (AgroParisTech)
UMR 782 – Génie et Microbiologie des Procédés Alimentaires
Bâtiment CBAI, Avenue Lucien Brétignières
78850 THIVERVAL-GRIGNON