Suivi de la survie de *Geotrichum candidum* pendant la digestion *in vitro* du fromage de type Camembert

Mémoire

Rihab Farhat

Maîtrise en sciences et technologie des aliments
Maître ès sciences (M. Sc.)

Québec, Canada

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Sous la direction de :

Sylvie Turgeon, directrice de recherche
Steve Labrie, codirecteur de recherche

Résumé

Au contraire du Camembert traditionnel, la pré-acidification du Camembert stabilisé est limitée par l'inoculation des bactéries lactiques thermophiles à une température inférieure à celle de leur croissance optimale (35-39 °C). *Geotrichum candidum* est une levure essentielle pour l'affinage du fromage Camembert grâce à ses activités biochimiques. En outre, quelques études ont rapporté que cette levure a été détectée dans les fèces humaines suite à la digestion du Camembert. Cette présence pourrait être due soit à la résistance intrinsèque des souches de *G. candidum* ou en lien avec les propriétés protectrices de la matrice fromagère. L'objectif de notre étude était d'examiner l'effet protecteur procuré à la souche *G. candidum* LMA-1028, par les propriétés de la matrice du fromage Camembert pendant la digestion statique *in vitro.* Afin d'y parvenir, deux matrices liquides (i.e. lait 3,25 % matières grasses et un milieu de culture) ainsi que deux matrices fromagères (i.e. Camembert traditionnel et Camembert stabilisé) ont été analysées. La survie de *G. candidum* et la désintégration de matrices étudiées ont été évaluées à différents temps de digestion aux étapes buccale, gastrique et duodénale. La désintégration du lait et du milieu de culture était plus élevée que celle des matrices fromagères en raison de leur structure liquide. La désintégration du Camembert stabilisé est plus importante que celle du Camembert traditionnel, ceci pourrait être attribué entre autres à une composition en lipides plus élevée. Globalement, la teneur en matière grasse des matrices laitières contrôle la progression de la désintégration. Lors de la digestion *in vitro*, la survie de *G. candidum* a été évaluée. Les résultats sur la viabilité de *G. candidum* LMA-1028 ont montré que cette souche est hautement résistante. La composition, la structure et les propriétés physicochimiques des matrices laitières n'ont pas amélioré la viabilité de *G. candidum* LMA-1028 pendant le transit gastro-intestinal.

Abstract

Compared to traditional Camembert-type cheese, stabilized Camembert's pre-acidification is limited using thermophilic lactic acid bacteria that are inoculated and used under their optimal growth temperature (35-39 °C). *Geotrichum candidum* is an essential ripening yeast of Camembert cheese due to its biochemical activities. Incidentally, it has been detected in human feces after Camembert consumption. However, this observation could be due either to the intrinsic *G. candidum* resistance to the gastrointestinal condition or to the protective properties of the Camembert cheese matrix**.** This study examines the putative protective effect of the cheese matrix on *G. candidum* LMA-1028 viability during static *in vitro* digestion. For this purpose, two liquid matrices (i.e. culture medium and pasteurized whole milk (3.25 %fat)) and two Camembert-type cheese variety (i.e. traditional and stabilized) were analyzed. *G. candidum* LMA-1028 survival under digestive stress was investigated at five digestion times (oral: 2 min, gastric: 60 and 120 min and duodenal: 60 and 120 min), while matrix disintegration was evaluated at three times (oral: 2 min, gastric: 120 min and duodenal: 120 min)**.** Milk and culture medium matrices displayed higher disintegration than cheese matrices due to their liquid nature. The lowest measured disintegration of traditional Camembert compared to stabilized cheese matrix could be attributed to the higher fat content. Overall, dairy matrices disintegration was significantly modulated by the matrix fat content. The structure of the casein networks of milk and Camembert cheeses appears to modulate the accessibility of digestive juice into these matrices during gastric digestion. The difference in the original structure of both Camembert cheese matrices led to different rates of gastric disintegration and resulted in different rates of fat release. When comparing viability counts, *G. candidum* LMA-1028 showed a high intrinsic resistance to simulated gastrointestinal stresses. Camembert cheese matrices as well as milk didn't bring additional protection to the studied strain LMA-1028.

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Abbreviation list

CFU	Colony Forming Unit
CM	Culture medium
FAA	Free Amino Acids
FFA	Free Fatty Acids
FRQNT	Fonds de recherche du Québec - Nature et les Technologies
FRAP	Fluorescence Recovery After Photobleaching
HDM	Human Duodenal Model
LAB	Lactic Acid Bacteria
NPN	Non Protein Nitrogen
PDO	Protected Designation of Origin
TN	Total Nitrogen
TPA	Texture Profile Analysis
YEG	Yeast Extract Glucose

Remerciements

Je remercie tout particulièrement ma directrice de maitrise, Dre Sylvie Turgeon, pour son assistance et ses conseils si précieux, et surtout de m'avoir aidé à développer mon esprit critique.

Par ailleurs, j'adresse mes remerciements les plus sincères à mon codirecteur, Dr Steve Labrie, pour sa bienveillance et son excellent mentorat de toute l'équipe.

Je remercie principalement Dr Yves Pouliot pour son soutien, sa confiance et surtout de m'avoir recommandé pour intégrer deux équipes de recherche au sein desquelles j'ai pu beaucoup apprendre.

J'adresse aussi mes remerciements, aux membres de deux équipes de recherche pour leur collaboration et au personnel de l'Université Laval: Catherine Viel, Diane Gagnon, Dre Laurie-Eve Rioux et Dre Marie-Hélène Lessard pour l'aide et les conseils qu'elles m'ont offerts au laboratoire.

Le bouquet de mes remerciements, je le présente affectueusement à mes parents, mes frères, mon oncle et Fourat pour leur soutien si efficace tout le long de mon cheminement.

Je souhaite également remercier tous mes amis et plus particulièrement Adriana Paredés pour son écoute, ses conseils et soutien moral tout le long de ma maitrise, ainsi Awa, Olfa, Zeineb, Dorra, Nesrine Fatma et Mariem. Merci pour votre temps, votre aide, votre bonne humeur et pour les agréables moments passés ensemble.

Avant-propos

Les travaux de ce mémoire font partie d'un projet de recherche financé par le Fonds de recherche du Québec – Nature et technologies (FRQNT). L'objectif général de ce mémoire était l'évaluation de l'effet protecteur des matrices fromagères sur la survie de *Geotrichum candidum* pendant la digestion *in vitro* du fromage Camembert.

Le mémoire comprend deux chapitres. Le premier chapitre correspond à une *Revue de la littérature* abordant les propriétés physico-chimiques du fromage Camembert, une description de la levure *G. candidum* et ses activités enzymatiques (lipolytique et protéolytique), la caractérisation du stress gastro-intestinal (i.e. stress acide et biliaire), la description des modèles de digestion *in vitro* utilisés en recherche et la cinétique de la désintégration des matrices laitières suite à la digestion orale, gastrique et duodénale.

Le deuxième chapitre, intitulé « Survival of *Geotrichum candidum* from Camembert-type cheese during simulated gastro-intestinal transit », est un article qui sera soumis à une revue scientifique à déterminer. J'ai contribué à ce travail en réalisant toutes les expérimentations au laboratoire et en rédigeant entièrement l'article. Sylvie Turgeon, Steve Labrie, Marie-Hélène Lessard et Laurie-Eve Rioux ont apporté leur soutien scientifique dans les décisions expérimentales et lors de la rédaction et la correction de l'article. Sylvie Turgeon et Steve Labrie ont également obtenu le financement pour la réalisation de la recherche.

Introduction

The worldwide production of cheese increased with an average annual growth rate of 4.0% over the last 30 years (Fox *et al.*, 2017). This increase is due to their nutritive quality, their appealing flavors, textural properties and the large possibility to use them in a meal (Fox *et al.*, 2017). In Canada, cheese consumption reached 13.38 kg per capita per annum in 2016 (Canadian Dairy Commission, 2016). Among all cheese categories, the highest increase of consumption belongs to Cheddar and specialty cheese types (Canadian Dairy Commission, 2016). For instance, soft cheese is the third largest cheese production with 77,194 Kg in 2016 (Canadian Dairy Commission, 2017).

From a structural point of view, cheese matrix is a protein-based gel consisting in a cross-linked casein-calcium phosphate network; physically entrapping fat globules. The final structure of the gel is a function of pH, calcium concentration and milk processing history. The variation of cheese-making steps (e.g., coagulation, maturation, whey draining, salting, pressing, and ripening) provides a large variety of cheese products. For instance, a mixed coagulation by microbial acidification and rennet action on casein gel gives a Camembert-type cheese curd. Depending on the nature of the selected starter, two categories of Camembert cheese are produced; namely traditional- and stabilized-Camembert cheeses. For the traditional Camembert cheese, acidification requires the use of mesophilic bacteria (25 °C), whereas for the stabilized curd, thermophilic species are used below their optimal growth temperature (34-39 °C) to limit acidification (Lawrence *et al.*, 1987). Camembert cheese stabilization results in different physicochemical properties, such as pH and water activity, which in turn influences the composition of Camembert cheese microflora (Arteau *et al.*, 2010; Lessard *et al.*, 2012; Spinnler and Gripon, 2004). Specifically, *Penicillium camemberti* predominates on the rind of stabilized cheeses whereas *Geotrichum candidum* seems more abundant on traditional curd (Arteau *et al.*, 2010).

G. candidum is an aerobic acid-tolerant and salt-sensitive yeast. It is a key microorganism in the development of the organoleptic properties of Camembert-type cheese. It governs the lipolytic and proteolytic activities during the ripening of Camembert cheese matrix (Boutrou *et al.*, 2006b; Dugat-Bony *et al.*, 2015; Leclercq-Perlat *et al.*, 2004a; Lessard *et al.*, 2014). *G. candidum* simultaneously assimilates the lactate produced by lactic acid bacteria and produces ammonia which contributes to the alkalinization of the curd. This subsequently promotes the proteolytic activity of the surface microflora during the first 21 days of the cheese ripening (Boutrou *et al.*, 2006b). As with proteolysis, lipolysis occurs extensively within mould-ripened cheeses matrices; about 5-20% of the triglycerides are hydrolyzed depending on the ripening period (Fox *et al.*, 2004).

The metabolic activities (i.e. proteolysis and lipolysis) of the cheese microflora, in addition to technological processing, modulate the particular microstructural and physicochemical properties of the cheese matrix. These properties have an important influence on nutrient bioaccessibility during the gastrointestinal transit (Turgeon and

Rioux, 2011). Few studies focused on the disintegration and the nutrients release of different cheese matrices such as aged, young, mild and light Cheddar, Mozzarella and stabilized Camembert-type cheeses (Ayala-Bribiesca *et al.*, 2016; Fang *et al.*, 2016; Lamothe *et al.*, 2012). Overall, high-fat commercial cheese varieties (Camembert and Cheddar) showed higher disintegration rates compared to low-fat Mozzarella (Fang *et al.*, 2016). A high calcium content limited the lipolysis extent, decreased the Cheddar disintegration, and also limited and delayed the fatty acids bioavailability (Ayala-Bribiesca *et al.*, 2016). Specifically, the gastric disintegration of cheeses is modulated by the composition and the textural profile of the matrix (i.e., elasticity, hardness, and cohesiveness) (Lamothe *et al.*, 2012). These studies showed that the bioaccessibility of the cheese nutrients was proportional to the disintegration kinetics of the cheese matrix during the gastrointestinal transit (Lamothe *et al.*, 2012; Rinaldi *et al.*, 2014). Noteworthy, liquid and semi-liquid dairy matrices such as milk and yogurt reached full ″disintegration″ more rapidly, i.e. after about 120 min of gastric digestion (Rinaldi *et al.*, 2014), while the majority of the analyzed cheese matrices showed an almost complete disintegration after 300 min of gastrointestinal digestion.

The slow disintegration of cheese matrix made this product a favorable delivery system of probiotic microorganisms (Ouwehand *et al.*, 2010; Stanton *et al.*, 1998). Mainly, the dense matrix (i.e., high protein and fat contents), the high pH in some varieties and the buffering capacity have been reported to bring protection against gastrointestinal stresses (Champagne *et al.*, 2011; Plessas *et al.*, 2012). These properties also promoted the development of a diversified microflora within cheese during ripening which provides an average of 10^8–10^9 CFU microorganisms per g of ready-to-eat cheese (Beresford *et al.*, 2001). The diversity of cheese ecosystems raises the question whether this microflora has a beneficial effect on human microbiota when ingested (Montel *et al.*, 2014). Lay *et al.* (2004), showed that the microflora of traditional Camembert cheese enhanced the metabolic activity of the human-associated rat's microbiota during consumption. Subsequently, the same team confirmed the beneficial effect of Camembert-type cheese consumption on the microbiota of a human subject group. *G. candidum* showed a significant resistance to gastrointestinal stresses (Firmesse *et al.*, 2008). Adouard *et al.* (2015b) , compared the viability of microbial mixture, including *G. candidum* ATCC 204307 when inoculated into liquid culture medium, rennet gel, and smear-ripened cheese, using a dynamic *in vitro* digestion model. They observed that during the digestion of smear-ripened cheese, the strains of the yeast species *D. hansenii, K. lactis* and *G. candidum* displayed high resistance to digestive stresses.

A high viability of *G. candidum* strains is observed through digestive stress, after the consumption of Camembert type cheese (Firmesse *et al.*, 2008). Given this, it may be hypothesized that the resistance of *G. candidum* to these harsh conditions is due to the protective effect of the structural and physicochemical properties of cheese matrix. Our study aims to investigate the viability of *G. candidum* LMA-1028*,* during the gastrointestinal transit using an *in vitro* static

model. The protective effect of pasteurized whole milk (3.25 % fat content) and of two Camembert-type cheese matrices with different protein and fat content was studied.

Chapter 1 : Literature review

I. Surface mold-ripened cheese: Camembert

Numerous soft-cheese varieties are produced such as Pont-l'Évêque, Munster, and Italian Crescentia. Camembert and Brie-type cheeses are the best known and their popularity is increasing especially in Australia, USA, and Canada (Tamime and Law, 2001). Those varieties are made from either raw or pasteurized milk and do not have the Protected Designation of Origin (PDO) status (Leclercq-Perlat, 2011). The manufacturing scheme of Camembert-type cheese production is discussed in the following sections with a simplified flow chart (Figure 1-1).

1. Camembert-type cheese making

1.1. Coagulation

The Camembert cheese is produced using a mixed coagulation method combining milk acidification and the activity of the rennet that result in a mixed coagulated curd. Coagulation time depends on the cheese type and varies from 30 to 90 min (Leclercq-Perlat, 2011). During coagulation, the temperature is maintained between 32-35 °C to promote both the rennet and the lactic acid bacteria activities. In general, in Camembert cheese, coagulation is performed using an inoculation rate around 5.70 log CFU/mL and the addition of 0.018- 0.022% (v/v) concentrated rennet (Leclercq-Perlat, 2011).

According to Tamime and Law (2001) as reported from Mietton (1986), during mold-ripened cheese manufacture, the pH of the curd at molding and unmolding is influenced by the renneting pH. The acidification controls the extent of curd demineralization and syneresis which induces several physicochemical and structural changes in cheese. When the acidification approaches the isoelectric point of casein (pH 4.6), the colloidal calcium phosphate (CCP) is extensively solubilized. This weakens the bonds between individual caseins which favors their dissociation from the micelle and reduces the water binding capacity of caseins (Fox *et al.*, 2004). Hydrophobic interactions between caseins are favored and as the pH approaches the isoelectric point of caseins, the para-casein network displays a more compact conformation (Fox *et al.*, 2004). These chemical changes result in the decrease of the protein matrix porosity and the increase of the serum expulsion. When acidification is limited (pH > 5.2), casein network porosity is higher and serum release is lower than in a more acidic curd (Fox, 2000; Lucey *et al.*, 1996). The final physicochemical and structural properties of cheese matrix also depend on other factors as temperature, and the equilibrium pH of the gel before drainage.

1.2. Draining of the curd

Draining takes place spontaneously at 26-28 °C as driven by the acidification and the rennet action on casein gel structure. Both acts simultaneously with antagonist effects on the final properties of the curd (Leclercq-Perlat, 2011). The syneresis of cheese curd depends on milk gel firmness at cutting and the surface area of the curd after being cut into cubes (Walstra *et al.*, 2005). The strong bonds between casein particles as a result of low renneting pH, limits the syneresis. While cutting the curd into smaller cubes increases the whey-curd interface and so the extent of syneresis. In the case of Camembert-type cheese, the brittle structure of the curd requires a moderate mechanical handling (i.e., cutting and stirring) to get a smooth final curd (Tamime and Law, 2001; Walstra *et al.*, 2005). This practice explains the final high moisture and moisture in non-fat basis of this cheese variety (Bylund, 2003). For traditional Camembert cheese, acidification continues during draining until a pH around 4.6 is reached within one day. To sum up, syneresis depends on the acidity, the temperature, and mechanical working in the vat of cheese curd during manufacturing. These factors modulate the final moisture content and textural properties of the cheese matrix.

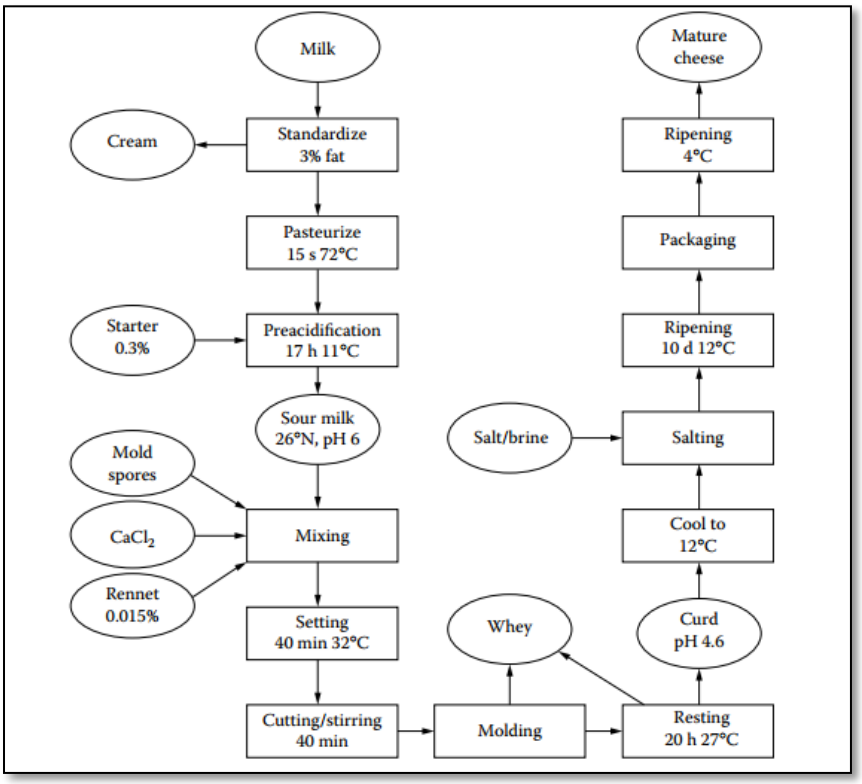

Figure 1-1: General diagram of mold surface-ripened cheese process (e. g Camembert-type)

Reproduced from Walstra et al. (2005)

1.3. Salting

In general, salting is done either using rubbing or brining techniques (Guinee, 2004). Brining is the most common practice in Camembert-type cheese making. Depending on the moisture content, shape and size of cheese curd, brining time might vary from 30 min, some hours to one day (Spinnler and Gripon, 2004; Walstra *et al.*, 2005). These factors also modulate the time required for salt-in-moisture (SM) equilibrium after salting. Due to the high moisture and the specific surface area of Camembert-type cheese salt absorption is fast. The brine solution does not only contain NaCl but also other solutes, notably lactic acid and salts that are leached out from the cheese (Walstra *et al.*, 2005). The targeted concentration of salt within soft surface-ripened cheese matrix is 1-2 % (g NaCl/100g of wet cheese) (Leclercq-Perlat, 2011; Walstra *et al.*, 2005) . For Camembert-type cheese, salt content influences the proteolysis and pH change. It also has a selective effect on the fungal microflora with salt sensitivity (Guinee and Fox, 1993). Overall brining allows further draining all with limiting pathogenic or spoilage microorganisms growth (Guinee, 2004).

1.4. Ripening

Cheese ripening starts before curd making is finished, and continues until the desired organoleptic and textural properties are reached (Figure 1-4). It includes the biochemical, physical and microbiological changes driven by enzymatic activities of the cheese microflora (Spinnler and Gripon, 2004). Camembert-type cheese ripening occurs in two steps (1) in a ripening chamber at a temperature between 10-14 °C, relative humidity 90 to 95%, during 9 to 14 days, and (2) ripening after packaging at a temperature between 4-6 °C using a specific wrapping (Leclercq-Perlat, 2011). The total ripening time varies between 12 and 35 days depending on the wanted organoleptic qualities (Spinnler and Gripon, 2004). The impact of the microbial activities on the physicochemical and structural properties of Camembert-type cheese are summarized in Figure 1-2 and will be detailed in sections I.4.3 & .

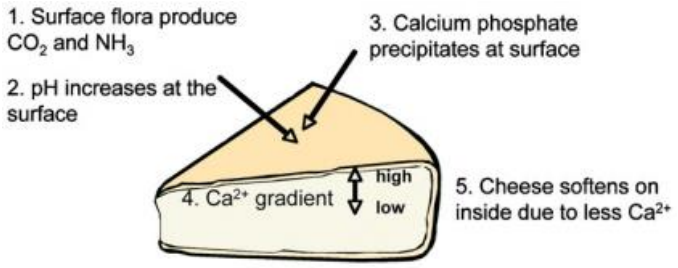

Figure 1-2: Cascade of physical and chemical reactions during ripening of Camembert cheese

Reproduced from Everett and Auty (2008)

2. Traditional vs. Stabilized Camembert cheese

Camembert-type cheese is manufactured through mixed coagulation by acidification and rennet action on casein gel structure. Rennet-mediated coagulation and acid-mediated coagulation provide two Camembert cheese categories (Tamime and Law, 2001). Processing dissimilarities result in physico-chemical, textural and microbiological differences. These are briefly listed in the following Table 1-1.

Table 1-1: *Manufacturing differences between traditional and stabilized Camembert-type cheese: physicochemical, textural and microbial consequences (Tamime and Law, 2001; Walstra et al., 2005)*

	Attributes	**Acid-mediated curd** **Traditional Camembert**	**Rennet-mediated curd** **Stabilized Camembert**
Main processing descriptors	**Milk acidification**	-Mesophilic bacteria 25 °C. -Extended pre-acidification.	-Thermophilic + negative proteases bacteria 35-39 °C. -Limited or omitted pre-acidification.
	Curd handling: cutting, stirring and washing	-Single knife cut (large lumps) -No stirring -No curd washing	-Cut into 0.7-1.0 cm cubes -Relatively high stirring -The curd is washed
Textural, physicochemical and microbial consequences	**Demoulding pH**	Low < 4.6	High > 5.2
	Buffering components	-Low calcium phosphate content -Low para-casein porosity	-High calcium phosphate content -High para-casein porosity
	Syneresis	Low	High
	Residual rennet/Lactose	High	Low
	Curd texture	Brittle	Soft
	G. candidum/ P. camemberti	Low/High	High/Low
	Shelf-life	Low	High

For acid-mediated or traditional Camembert, coagulation includes lactic acidification without vat working. Contrarily, stabilized Camembert is produced through a limited or omitted lactic acidification with a moderate vat working (Figure 1-4).

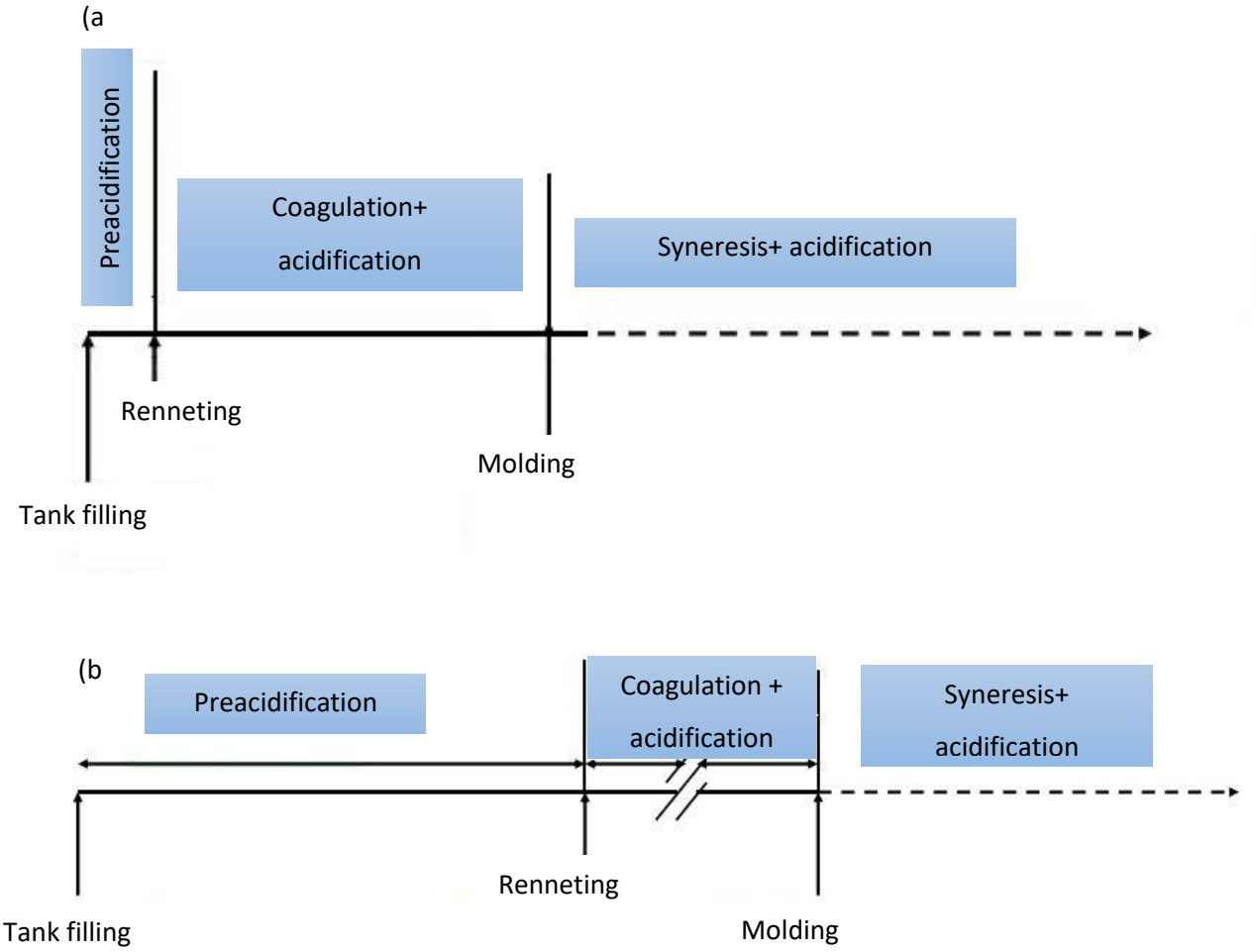

Figure 1-3: Description of (a) Stabilized curd versus (b) Traditional curd manufacture

For the acidification of the stabilized curd, proteinase negative strains such as thermophilic streptococci or the mixture of streptococci and lactococci are used below their optimal growth temperature 34-39 °C (Spinnler and Gripon, 2004). This procedure decelerates the lactic acid production to maintain the pH ≥ 5.2 which in turn reduces the demineralization of cheese matrix (Lawrence *et al.*, 1987). The curd of stabilized cheese is cut into 0.7 to 1.0 cm cubes, and then the whey-mixture is stirred leading to faster and higher syneresis and so lower moisture in the final curd (Spinnler and Gripon, 2004; Walstra *et al.*, 2005).

The resulting cheese has a firm and smooth texture (Figure 1-4) (Lortal *et al.*, 2004). Conventionally, during stabilized cheese making the initial level of rennet is reduced decreasing the residual rennet and increase its shelf life (Lawrence *et al.*, 1987).

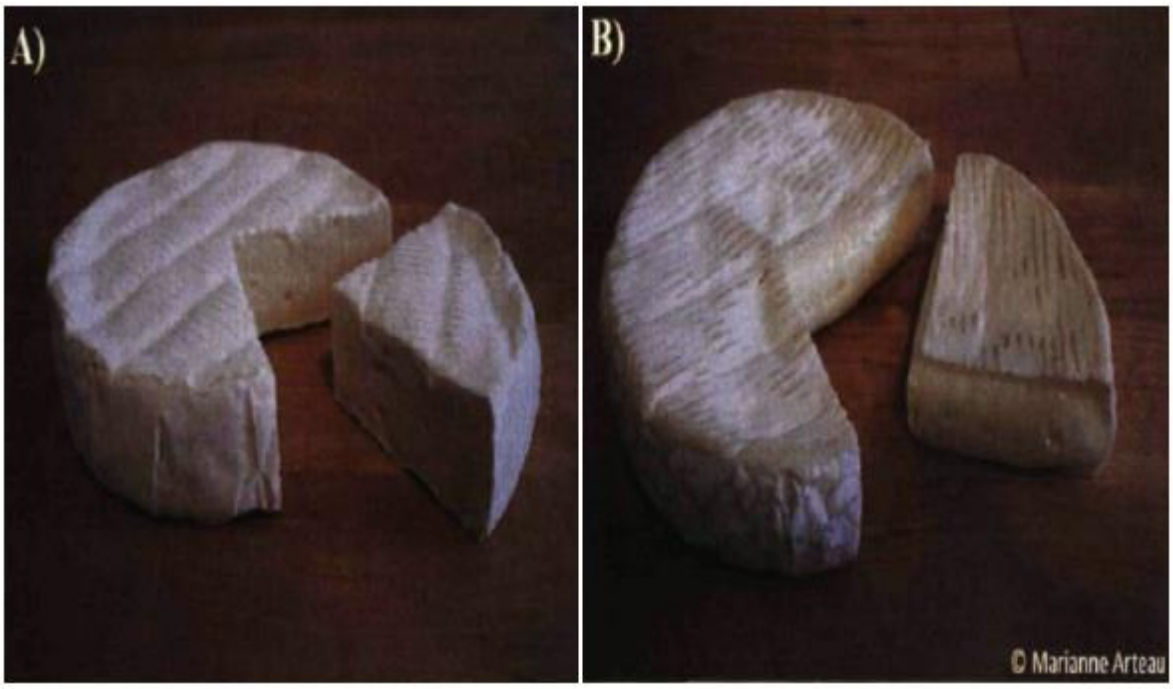

Figure 1-4: Stabilized (A) and traditional (B) Camembert-type cheese

Reproduced from Arteau (2009)

The traditional Camembert cheese is manufactured industrially with a mechanized system but could also be produced using a traditional 'moulage à la louche' (Tamime and Law, 2001). Inversely to stabilized curd, the extended acidification of traditional cheese requires the use of a mesophilic starter at 25-28 °C that decreases the curd pH to 4.6. These leads to a higher residual rennet activity and extensive colloidal calcium-phosphate solubilization (Lawrence *et al.*, 1987; Lucey *et al.*, 2000; Walstra *et al.*, 2005). The acid-mediated curd is merely cut without being stirred, which limits syneresis rate and provides a smooth, free 'pea' macrostructure (Tamime and Law, 2001; Walstra *et al.*, 2005). The cutting is followed by hooping or molding the curd to allow further whey drainage. The curd is not washed which induces a high residual lactose content within cheese matrix compared to stabilized Camembert (Walstra *et al.*, 2005).

3. Camembert cheese ecosystem

The diversity of Camembert microflora is reduced when cheese is made from pasteurized milk (Beuvier and Buchin, 2004; Skeie and Ardö, 2000). To compensate for the impact of heat treatment on the natural milk microflora, the manufacturer uses specific lactic acid bacteria (e.g., mesophilic and thermophilic species) and ripening microorganisms (e.g., *Brevibacterium aurantiacum*, *Penicillium camemberti, Geotrichum candidum*, *Debaryomyces hansenii* and *Kluyveromyces lactis*) (Spinnler and Gripon, 2004). The physicochemical and biochemical changes during Camembert cheese ripening promote the development of a complex microflora (Monnet *et al.*, 2015). Lessard *et al.* (2012), used an accurate real-time quantitative PCR to investigate the growth kinetics and the interactions between the fungal species in a Camembert model curd ecosystem during a 31-day ripening period. In a previous study, the same team showed that the diversity and distribution of the fungal microflora within Commercial Camembert cheese curd depend on cheese size, surface area, and cheese matrix properties as modulated during manufacturing (Arteau *et al.*, 2010).

3.1. Lactic acid bacteria and surface microflora

Depending on the Camembert cheese technology used, the lactic acid bacteria might include either mesophilic bacteria such as *Lactococcus lactis*, *Leuconostoc mesenteroides (Walstra et al., 2005)*, or protease negative thermophilic strains (Lawrence *et al.*, 1987). These lactic acid bacteria allow the acidification of cheese except for the *Leuconostoc mesenteroides* that reduces this phenomenon in the first six days of the cheese ripening (Leclercq-Perlat *et al.*, 2004a). The growth of lactic acid bacteria begins with the addition of the starter up to the salting stage to reach around 3-5x10^9 CFU/g of wet cheese. Afterward, the concentration of the microflora remains stable until the 10^{th} day of ripening, then decreases progressively to around 5x10^8 CFU/g of wet cheese on the 41^{st} day. The surface bacterial microflora is either sprayed on the cheese curd after ripening or inoculated in the cheese milk before renneting, simultaneously with the starters. The growth of these bacteria (e.g., *Brevibacterium linens)* starts when the pH of cheese surface is above 6.0 due to the cheese alkalinization by the fungal microflora (Leclercq-Perlat, 2011). This higher pH promotes the proteolytic activity of lactic acid bacteria to produce small peptides and free amino acids (Leclercq-Perlat, 2011). Overall, lactic acid bacteria represents a minor fraction of the surface community due to the competition with other microorganisms that consume a large proportion of the lactic acid, amino acids and lipids energy sources such as the yeast *G. candidum* (Leclercq-Perlat *et al.*, 2004a; Monnet *et al.*, 2015).

3.2. Yeasts and molds

Arteau *et al.* (2010) investigated the microbiota of Canadian Camembert cheeses made of pasteurized milk and identified nine fungal genera including *Cladosporium*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Mucor*,

Penicillium, Pichia, *Saccharomyces* and *Yarrowia*. According to this study, the source of such fungal microflora might be either the ripening starter, the native ecosystem of milk or processing conditions (reviewed in Irlinger *et al.* (2014)). In the core of Camembert cheese, yeast count is two-to-three log lower than that at the surface (Leclercq-Perlat *et al.*, 2006). Another study reported that the concentration of yeasts in the center of cheese is around 1% of that on the surface (Beresford *et al.*, 2001). The study of the microflora of Camembert cheese showed that *Geotrichum* sp., *Penicillium* sp. and *Debaryomyces* sp. are the main genus of the surface while, *Kluyveromyces* sp. and *Saccharomyces* sp. were typically found in the core (Arteau *et al.*, 2010; Beresford *et al.*, 2001; Lessard *et al.*, 2012). These studies also showed that during cheese-making, the growth and the enzymatic activities of the fungi depend on the pH, the water activity, and salt content of the cheese curd (Addis *et al.*, 2001; Leclercq-Perlat *et al.*, 2004a; Leclercq-Perlat, 2011; Roostita and Fleet, 1996). The fungal microflora is not affected at a salt concentration < 2 % (Tabla et al., 2015), however, at a decreased salt content, *G. candidum* growth predominates over *P. camemberti* which might cause the ‘’toad skin’’ defect (Fox *et al.*, 2004). Inversely, the very high salt concentration causes the ‘’ bitterness defect ’’ due to the excessive proteolytic activity of *P. camemberti* (Spinnler and Gripon, 2004). When comparing traditional and stabilized Camembert cheese microflora, Arteau *et al.* (2010) showed that cheese stabilization modifies the composition of the fungal community of the core. Specifically, *G. candidum* seems to predominate traditional curd while *P. camemberti* predominates in stabilized cheese.

The growth rate and the distribution of the fungus are significantly affected by the interaction with other members of the cheese microbiota (Mounier *et al.*, 2008; Roostita and Fleet, 1996). Using classic microbial count (Addis *et al.*, 2001; Arteau *et al.*, 2010; Leclercq-Perlat *et al.*, 2004a; Mounier *et al.*, 2008; Roostita and Fleet, 1996), terminal restriction-fragment length polymorphisms (Arteau *et al.*, 2010) and metagenomics and meta-transcriptomic analysis (Dugat-Bony *et al.*, 2015; Lessard *et al.*, 2014), authors showed that *K. lactis* and *K. marxianus* are the first yeasts to grow in cheese curd at the beginning of the ripening period. They are subsequently followed by *G. candidum*, *P. camemberti* and *D. hansenii*. The enzymatic activities of these species promote further cheese alkalinization and induces the growth of acid-sensitive species (Leclercq-Perlat *et al.*, 2004a). Simultaneously, this microflora displays a key ripening role. *D. hansenii* uses lactose and lactate, *G. candidum* does not metabolize lactose but uses lactate instead, and governs the proteolytic and lipolytic activities that occur during Camembert-type cheese ripening. These metabolic activities contribute in the development of typical flavors and textural properties (Leclercq-Perlat *et al.*, 2004a; Lessard *et al.*, 2014; Roostita and Fleet, 1996; Schlesser *et al.*, 1992). Because of this *D. hansenii*, *Y. lipolytica* and *G. candidum* are often inoculated as a starter culture (Boutrou and Guéguen, 2005; Ferreira and Viljoen, 2003; Van den Tempel and Jakobsen, 2000). However, proteases and peptidases activities are detected only after two to three weeks of ripening (Boutrou and Guéguen, 2005; Boutrou *et al.*, 2006a; Engel *et al.*, 2001; Leclercq-Perlat *et al.*, 2004a; Lessard *et al.*, 2014).

Camembert cheese fungal microflora produces several aromatic compounds during their growth, such as dimethyl disulfide (DMDS) by *G. candidum* (Demarigny *et al.*, 2000; Jollivet *et al.*, 1994; Leclercq-Perlat *et al.*, 2004b) and an ester of fruity flavor by *D. hansenii* (Gori *et al.*, 2012). To sum up, the microbial interaction in soft cheese ecosystems and related biochemical activities contribute to the development of desired organoleptic quality, shelf life and safety of ready-to-eat cheese (Addis *et al.*, 2001; Lessard *et al.*, 2014).

4. Geotrichum candidum: Biochemical and physicochemical changes during Camembert cheese ripening

Originally isolated from milk by Fresenius in 1850 (reported by (Wouters *et al.*, 2002)). *Geotrichum candidum* is an aerobic acid-tolerant and salt-sensitive yeast. Some authors cite it as a mold because the fungi arbor sometime a fluffy phenotype, but *G. candidum* is now correctly assigned as a yeast (Boutrou and Guéguen, 2005). The name *G. candidum* was attributed to anamorphic yeast species used specifically in dairy products such as surface-ripened cheese (Prillinger *et al.*, 1999). The selected strains of *G. candidum* provide different desirable sensorial properties to cheese (i.e., flavor and texture) through their lipolytic and proteolytic activities (Boutrou and Guéguen, 2005). Moreover, certain strains have shown a significant inhibition of undesirable microbes such as *Listeria monocytogenes* (Dieuleveux *et al.*, 1998). Currently, *G. candidum* is used as a starter and colonizes a large number of surface-ripened cheese varieties during the first stages of ripening (Berger *et al.*, 1999).

4.1. Taxonomy and strains diversity

Several synonyms have been attributed to this microorganism. The name *Geotrichum candidum*, known as the anamorph of *Galactomyces Geotrichum*, was associated to the specie Link (1809) then Link: Fries (1832) with CBS 772.71 the type strain of *Ga. geotrichum* and neotype strain of *G. candidum* (De Hoog *et al.*, 1998). The taxonomic position was revised by de Hoog et al. (2004). The anamorphic state has been characterized as follows; *Candidacae* (family) and *Geotrichum* (genus). Prillinger et al. (1999) assigned all isolated *Geotrichum* from dairy products to *Ga. geotrichum* and gave the name of *Geotrichum candidum* to anamorphic yeast species. A type strain of this yeast was defined in Brie cheese (France), CBS 615.84 (De Hoog *et al.*, 1998). In the last decade, molecular methods, allowed the identification of *G. candidum* at the species and strain levels (Gente *et al.*, 2006) and strain diversity (Alper *et al.*, 2013; Lessard *et al.*, 2014; Lessard *et al.*, 2012) which improved the characterization of dairy strains specifically in cheese industry (Alper *et al.*, 2011; Alper *et al.*, 2013; Leclercq-Perlat *et al.*, 2004a; Sacristán *et al.*, 2012; Sacristán *et al.*, 2013).

4.2. Morphology

G. candidum is a dimorphic yeast because it involves strains that can display three distinct colonial morphotypes (Table 1-2) (Gente *et al.*, 2002; Guéguen and Jacquet, 1982; Guéguen and Schmidt, 1992; Wyder and Puhan, 1999). According to Guéguen and Jacquet (1982), the three types of *Geotrichum candidum* morphotypes were well correlated to their physicochemical properties (i.e., lipolysis, proteolysis, and alkalinization). More details about yeast-like colony type characteristics are summarized in table 1-2.

Table 1-2 : Characterization of *G. candidum* yeast-like morphotype (Guéguen and Jacquet, 1982).

Characteristics	**Yeast-like**
Colonies color	Cream-colored
Pattern	Cloudy bottom and lowly developed mycelium
Arthrospore	Arthrospore predominance
Growth Media	Surface and core
Optimum Temperature	22-25 °C
Proteolytic/ lipolytic activities	High/ moderate
Alkalinization	High

4.3. pH change

During Camembert-type cheese ripening, the pH of the rind remains unchanged until about the 6th day. Hence, it increases from about 4.6 to 7.8 within six days and remains stable until the end of ripening (Leclercq-Perlat *et al.*, 2004a; Lessard *et al.*, 2012). This change was attributed to the growth of surface yeasts and their biochemical activities and mainly the presence of *G. candidum* (Aldarf *et al.*, 2004; Boutrou *et al.*, 2006b). *G. candidum* assimilates lactate produced by lactic acid bacteria and releases ammonia which reduces rind acidity. Given this, Leclercq et al. (2004) showed that the pH of the surface of Camembert cheese was positively correlated to NPN and ASN concentrations that are metabolized by *G. candidum* proteases. These proteolytic enzymes are produced on the rind and did not diffuse to cheese core (Noomen, 1978). Subsequently, ammonia is produced at the cheese surface, and a pH gradient occurs from the rind to the core due to the diffusion of the metabolites (Aldarf *et al.*, 2004; Leclercq-Perlat *et al.*, 2004a). Similarly, lactate was reported to diffuse from the core to the surface allowing a further rise of core pH. These phenomena (i.e., ammonia and lactate diffusion) contribute to the pH gradient between the rind and the core. Recently, two key studies confirmed these previous statements using metatranscriptome analysis

(Dugat-Bony *et al.*, 2015; Lessard *et al.*, 2014). Authors detected *G. candidum*'s transcripts confirming that *G. candidum* catabolises peptides, amino acids and lactate principally, and produces NH_3 to alkalinize cheese curd. These pH changes also occur during the simultaneous growth of *G. candidum* and *P. camemberti* (Figure 1-5).

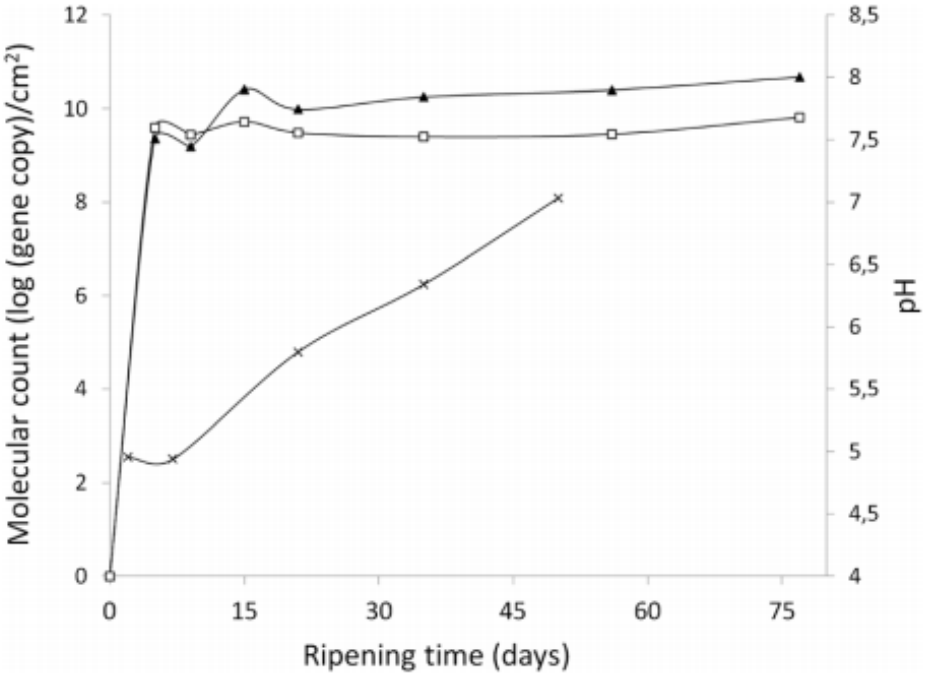

Figure 1-5: Evolution of pH and fungal growth during Camembert cheese ripening.

The ripening culture was a mixture of (□) G. candidum LMA-1028 and (▲) P. camemberti LMA- 1029. Each strain was quantified individually using a TaqMan real-time qPCR method pH (×) measures were taken weekly until day 50.

Reproduced from Lessard et al. (2014)

4.4. Flavor-formation

- Proteolysis

From the 8th day of ripening, the increase of pH promotes the proteolytic activity within Camembert cheese matrix. The proteolysis is mainly governed by the growth of *G. candidum* and *P. camemberti* on the surface of the Camembert cheese. For this reason, it is known to be more intensive near the rind than in the core. Particularly, *G. candidum* is considered to be the main proteolytic yeast, responsible for 85 % of peptidasic and proteolytic activity (Figure 1-6) (Baroiller *et al.*, 1990; Dugat-Bony *et al.*, 2015; Guéguen and Schmidt, 1992). To discriminate the technological features of *G. candidum* strains, Sacristan et al. (2012) focused on the enzymatic profile, including the proteolytic and amino-peptidase activity of several *G. candidum* strains. Among the 41 strains characterized only eight strains showed an extracellular proteolytic activity. These strains have been divided into two strains sub-groups, respectively with weak or strong proteolytic activity. Besides, among these strains, intracellular proteolytic activity was suggested to be higher than extracellular activity. Dugat-Bony *et al.* (2015) observed the same behavior and showed that during milk coagulation, lactic acid bacteria and rennet activities allow the release of casein fragments, which are catabolized by extracellular proteases or by vacuolar proteases and peptidases of *G. candidum* after being adsorbed by endocytosis.

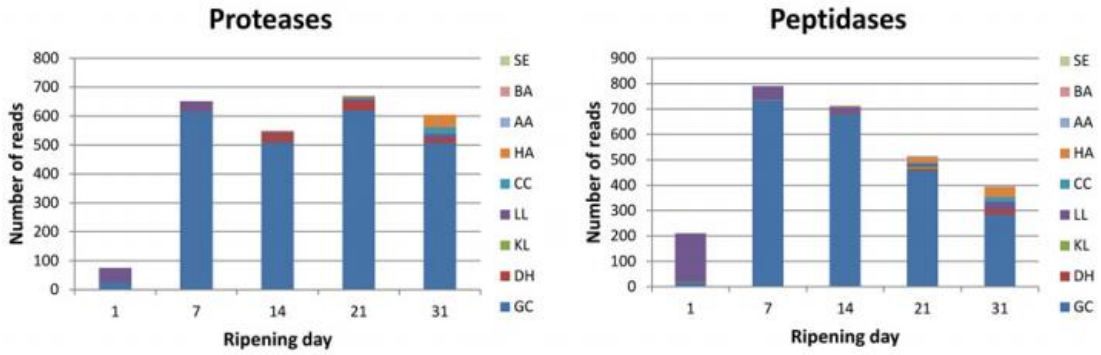

Figure 1-6: Protein degradation during surface-ripened cheese maturation: Expression data observed for genes encoding proteases (A) and peptidases (B).

SE: Staphylococcus equorum. BA: Brevibacterium aurantiacum. AA: Arthrobacter arilaitensis. HA: Hafnia alvei. CC: Corynebacterium casei. LL: Lactococcus lactis. KL: Kluyveromyces lactis. DH: Debaryomyces hansenii. GC: G. candidum

Reproduced from Dugat-Bony et al. (2015).

In another study, Boutrou et *al.* (2006) characterized the proteolytic activity of *G. candidum* in Camembert-type cheese. They used reverse-phase high-performance liquid chromatography to determine the contribution of *G. candidum* to the primary and secondary proteolysis at the surface of this soft mold-ripened cheese. Electrophoretic profiles showed a low intensity of the casein bands after the first week of ripening. The transcriptomic analysis indicated that the metabolic pathways responsible for ammonia production and amino acid metabolism are active. This is referred as the primary proteolytic activity of *G. candidum*, which is allowed due to the increase of cheese surface pH up to the *G. candidum* proteinase's optimal pH of 5.5-6.2 (Boutrou *et al.*, 2006b). Primary proteolysis results in the production of large- or medium-sized casein fragments and peptides. The generation rate of these products decreases by the third week making authors suggest a lower level of hydrolysis and/or the degradation of a part of primary proteolysis' products (Boutrou *et al.*, 2006b). After that, the secondary proteolysis takes place after the 15th day of ripening showing peptides decrease coupled with a FAA and small peptides increase on the cheese rind. Secondary proteolysis is referred to peptides degradation by *G. candidum* peptidases which are active at current cheese surface pH, but lactococcal peptidases were also functional when cheese surface pH increased to their optimal pH (6.5-9.5). Furthermore, casein fractions were hydrolyzed by *G. candidum*, particularly β-A_2 and α_{s1}-casein with preferred hydrolysis of β-casein (Dugat-Bony *et al.*, 2015).

Using meta-omics analyses Lessard *et al.* (2014) and Dugat-Bony *et al.* (2015) proved that *G. candidum* governs aroma compounds production through amino acids catabolism especially sulfured amino acids (i.e., methionine) and glutamate (Jollivet *et al.*, 1994) to produce further compounds that are important in the development of the aroma (Latrasse et al., 1987; Gripon, 1997). Among the volatile compounds generated during Camembert cheese-type ripening, the 3-methyl-butanol, DMS and isoamyl acetate were mainly associated to the *G. candidum* proteolytic activity. Likewise, thanks to its enzymatic properties, specifically carboxypeptidase and amino-peptidase activities, *G. candidum* reduces the bitterness provided by some hydrophobic peptides of low metabolic weight produced through the degradation of β-casein. This effect has been observed in Camembert cheese by several authors (Wyder and Puhan, 1999; Thammavongs et *al*., 2000; Marcellino et *al*., 2001, Boutrou et *al.,* 2006).

- Lipolysis

During soft mould-ripened cheese ripening, lipid hydrolysis may reach 5 to 20% (Fox *et al.*, 2004). As for proteolysis, lipolysis contributes to the development of cheese flavor. Lipolysis occurs intensively from the first to the 7th day of ripening then decreases slightly or remains stable until the 4th week (Dugat-Bony *et al.*, 2015). It has been correlated to the surface-fungal microflora including, essentially, *P. camemberti*, *K. lactis* and *G. candidum* (Molimard *et al.*, 1997). Also, the concentration of lipases has been reported to be twice higher under the rind when compared to the core due to the relatively high pH (<6.0) (Hassouna and Guizani, 1995). *G. candidum*'s lipases are responsible for

soft mould-ripened and Armada cheeses lipolysis during curd ripening when rind pH is between 5.5 and 7.5 (Fresno *et al.*, 1997; Tornadijo *et al.*, 1998). However, the intensity of the lipolytic activity, as well as lipase forms, are strain dependent (Baillargeon *et al.*, 1989; Boutrou and Guéguen, 2005). Lipases from *G. candidum* have been purified and characterized on relatively ''simple'' medium cultures (Baillargeon *et al.*, 1989; Jacobsen and Poulsen, 1992; Sugihara *et al.*, 1990). Authors identified six lipases forms (Lipase A, B, I, II, III and IV) with different substrate specificities. Nevertheless, *G. candidum* lipolysis was mainly due to the extracellular synthesized Lipases I and II (reviewed in Boutrou *et al.* (2006b)). These forms have unique specificity to *cis-9* unsaturated fatty acids (i.e., oleic acid) and unsaturated C18 fatty acids at the Sn2 position of the triglycerides, respectively (Bertolini *et al.*, 1995; Veeraragavan *et al.*, 1990). Fresno et al. (1997) correlated the low ratio of palmitic (16:0) to oleic acid (18:1) to the activity of *G. candidum* Lipase I whose affinity is to unsaturated fatty acids with a double bond cis-9 and cis-cis 9,12 position, with preference to 18:1. The screening of free fatty acids (FFAs) profile during the ripening of a traditional (Leclercq-Perlat *et al.*, 2007) and a freeze-dried Tibetan kefir co-cultured (Jun *et al.*, 2015) Camembert cheeses showed that the main liberated FFAs are long chain ones including palmitic (16:0), myristic (14:0) and stearic (18:0).

The specific lipolytic activity of *G. candidum* was not/barely characterized until the last ten years (Boutrou and Guéguen, 2005). Recently, Sacristàn et al. (2012) examined the extracellular and cell-bound lipases activities in Armada cheese and revealed significant differences between strains. About 40% of *G. candidum* strains showed high extracellular activity, and 10% presented cell bound lipase activity. Lessard et al. (2014), established the enzymatic activity (i.e., proteolysis, lipolysis) profile of *P. camemberti* and *G. candidum* during 77 ripening days of Commercial Canadian Camembert-type cheese. This study explored the lipolytic activity of both *P. camemberti* and *G. candidum*. The metabolic activities of pilot-scale made surface-ripened cheese ecosystem inoculated with *K. lactis*, *D. hansenii* and *G. candidum* ATCC 204307 showed that cheese lipolysis accounted, majorly, for *G. candidum* lipases activity (Dugat-Bony *et al.*, 2015). Most of the produced FFAs were detected in the rind and the core of Camembert-type cheese. Hence their diffusion phenomenon from the rind to the core has been highlighted (Leclercq-Perlat, 2011). Known as a major flavor precursor in Camembert-type cheese, *G. candidum* develops volatile compounds by metabolizing lipid and free fatty acids degradation. Indeed, it contributes to the production of several methyl-ketones through fat catabolism, which constitutes the main flavor component of Camembert-type cheese (Leclercq-Perlat *et al.*, 2004b; Leclercq-Perlat *et al.*, 2004c; Molimard and Spinnler, 1996).

II. Survival of cheese microbiota during *in vitro* and *in vivo* digestion

In addition to their nutrient values, fermented dairy products (e.g., yogurt, kefir and cheese) provide a diverse microflora that is composed of lactic starter culture and non-starter culture (Walther *et al.*, 2008). As detailed above, ripened cheese categories such as surface mould-ripened cheese comprise a wide variety of acidifying strains such as *Lactococcus lactis* and *Streptococcus thermophilus* and several flavoring yeasts and molds, like *Debaromyces hansenii, Geotrichum candidum*, *Kluyveromyces* spp., *Pichia* spp., *Rhodotorula* spp., *Saccharomyces* spp., *Trichosporon* spp., *Torulospora* spp., *Yarrowia* spp. and *Zygosaccharomyces* spp (Arteau *et al.*, 2010; Leclercq-Perlat, 2011; Lessard *et al.*, 2012). Cheese ecosystem provides about 10^8 – 10^9 CFU per gram of ready-to-eat cheese (Beresford *et al.*, 2001). Along with their technological role during cheese ripening, such abundant microbial diversity raises the question whether this microflora has a beneficial effect, such as an antimicrobial activity or a probiotic potential, on human microbiota when ingested (Hatoum *et al.*, 2012). Beforehand, the behavior of these microorganisms through the stressful gastrointestinal conditions should be investigated. To exert their beneficial properties the ingested microorganisms should resist the harsh physiological stress during digestion, such as the temperature, acidic pH of the stomach, intestinal bile salts, organic acids, gastrointestinal enzymes and secondary metabolism metabolites (e.g. H_2S, bacteriocins) (Fioramonti *et al.*, 2003). Since the tolerance of dairy yeasts to these stresses are barely investigated, some examples using lactic acid bacteria as models will be illustrated in the next sections.

1. Gastrointestinal stress

1.1. Gastric acid stress

Depending on the kinetics of food matrix disintegration, ingested microorganisms are exposed to a gastric pH ranging from 1 to 3 units during an average exposure time of 90 min (Kong and Singh, 2008b). The low acidity of the stomach induces a drop of the intracellular pH of the microorganisms which affects their cellular metabolism (i.e., inhibits cell growth and product formation) (Lohmeier-Vogel *et al.*, 1998; Matsushika and Sawayama, 2012). Eukaryotic cells control their intracellular pH and nutrients uptake by maintaining a proton gradient force over the plasma membrane (Madshus, 1988). Several yeasts can grow at pH 3.0 (Deak and Beuchat, 1994; Miller, 1979; Praphailong and Fleet, 1997; Walker, 1977) and resist to an acidic pH as low as 1.5 units (Czerucka *et al.*, 2007; Praphailong and Fleet, 1997). However, acidic pH tolerance and the metabolic response is strain-dependent (Fietto *et al.*, 2004). The tolerance of low pH depends on the activity of the plasma membrane H^+-ATPase that controls the intracellular pH through proton exchange (Eraso and Gancedo, 1987; Praphailong and Fleet, 1997). For instance, some *Saccharomyces* strains use a cell buffering capacity when exposed to acid stress or produce a particular protein

profile (Marešová *et al.*, 2010). Fietto *et al.* (2004). Given this, in the presence of lactic acid stress, recent studies performed metabolomics analysis using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) to investigate the metabolic tolerance mechanism of *S. cerevisae* (Nugroho *et al.*, 2015). They highlighted three metabolic responses interfered by low pH, namely the energy metabolism, redox balance, and amino acid composition. Authors reported the accumulation of ATP suggesting the reduction of ATPase activity under intensive acid stress. Furthermore, they observed the increase of the concentration of cysteine, glutamine and γ-amino-butyric-acid (GABA) and the decrease of glycine. Both induced the accumulation of glutathione and this has been suggested to improve the tolerance of cells to oxidative stress. Similarly, the accumulation of proline in cells was shown to protect yeast cells from an intracellular pH drop under acid stress.

1.2. Bile salts toxicity

Bile toxicity in the small intestine is a serious barrier to withstand by ingested microorganisms. Bile salts are known to be 'biological detergent' because they ''have a detergent action on particles of dietary fat which causes fat globules to break down or be emulsified into minute'' (Hofmann and Small, 1967). They are the conjugated form of bile acids secreted by the liver from cholesterol (Russell and Setchell, 1992). Due to microbial activities, these acids go through different chemical reactions such as de-conjugation and dehydrogenation (reviewed in (Prabha and Ohri, 2006)). Both de-conjugated and conjugated acids attack the lipid bilayer structure and the protein integrity of cellular membrane as a result of the cellular homeostasis disruption (Cabral *et al.*, 1987). In the neutral pH environment of the duodenum, the resistance to bile salts stress depends on the concentration of salts and the exposure time (Hill, 1993). The average concentration of bile salts in the intestine is around 0.3%. However, peaks of 1.5 to 2.0% might be found within the first hour of the intestinal digestion bile tolerance is often evaluated after microbial exposition to a range of 0.1 to 2.0% for probiotic bacteria selection (Noriega *et al.*, 2004). A microorganism is considered to have a good bile tolerance if it could withstand a concentration of 0.3% v/v of bile (Gilliland *et al.*, 1984; Gotcheva *et al.*, 2002; Lankaputhra and Shah, 1995). This concentration was selected to simulate the physiological conditions and is used to screen pure cultures or a mixture of bacterial strains for their bile tolerance (Gotcheva *et al.*, 2002). Many yeasts, more specifically dairy genera such as *Debaromyces hansenii* (Psani and Kotzekidou (2006)), *Kluyveromyces lactis, Yarrowia lipolytica* (Chen *et al.*, 2010), and *Kluyveromyces marxianus* ((Kumura *et al.*, 2004), have shown resistance to high bile salts concentration. Likewise, genetically unrelated *Saccharomyces* strains, isolated from dairy products, survived the simulated gastrointestinal stresses, specifically bile salts toxicity (Fietto *et al.*, 2004). The resistance to such 'biological detergents' includes mainly bile salts hydrolase activity(Smet *et al.*, 1995) that results from a ''detergent shock'' protein response. This response is displayed through the immunomodulation of the gastrointestinal tract (Buts *et al.*, 1990).

A similar immunomodulatory response of *G. candidum* strains had been reported by Plé *et al.* (2015) when investigating the immune effect of dairy products. Also, Adouard *et al.* (2015a) determined the viability of smear-ripened cheese microflora and their digestive stress response. *G. candidum* strains displayed a high resistance to the simulated gastrointestinal juices with a moderate immunomodulatory activity microflora (Adouard *et al.*, 2015a). The high viability of the species of cheese microflora through gastrointestinal stress might be attributed to their intrinsic tolerance or the properties of their ecosystem.

2. Protective effect of cheese matrix

Since the '90s, a full range of probiotic dairy products has been developed, e.g., pasteurized milk, ice cream, and fermented milk products (Boylston *et al.*, 2004; Fondén *et al.*, 2003; Ross *et al.*, 2002). Mainly, fermented dairy products are considered an excellent delivery system due to their structural features, physicochemical properties, and their extended shelf life (Buriti *et al.*, 2012; Tamime, 2008). Notably, the firm consistency (i.e., dense casein network), the high pH and the buffering capacity of cheese matrix of the soft-ripened cheese promote the development and the protection of its microflora (Ross *et al.*, 2002; Vinderola *et al.*, 2002). These advantages also allow the protection of probiotic microorganisms against the digestive stresses which made of cheese matrix a possible alternative probiotic carrier to yogurts and other fresh fermented dairy products (Dinakar and Mistry, 1994; Gardiner *et al.*, 1999; Karimi *et al.*, 2011; Plessas *et al.*, 2012; Possemiers *et al.*, 2010; Sharp *et al.*, 2008).

Particularly, fat protects microbes properly through the digestive tract by reducing their exposure to acid and bile acids stress (Karimi *et al.*, 2011; Possemiers *et al.*, 2010; Ranadheera *et al.*, 2012). The fat content of food matrix provides better protection than protein content. Given this, the viability of probiotic bacteria when ingested in milk chocolate vs. half-skimmed milk and skim milk vs. whole milk, with the same protein content, increased in high-fat dairies (Possemiers *et al.*, 2010; Tompkins *et al.*, 2011; Varcoe *et al.*, 2002). Furthermore, the addition of protein hydrolysates during the manufacture of Gouda-type cheese didn't appear to improve the viability nor did protect probiotic bacteria during cheese ripening (Champagne *et al.*, 2011; Gomes *et al.*, 1995; Ong *et al.*, 2006; Stanton *et al.*, 1998). Even-though cheese has been widely optimized as a probiotic carrier; few studies investigated the viability and the beneficial impact of its microflora on human metabolism and health.

3. Survival of microbial microflora of soft surface-ripened cheese

Diet is one among the important factors affecting the composition of human gut microbiota (Walker *et al.*, 2011; Wu *et al.*, 2011). Marteau *et al.* (1994) found *Bifidobacterium* (yogurt) and *Lactobacillus* (cheese) in human subject feces after dairy products intake and reported a possible beneficial effect on intestinal metabolism. Given this, Lay *et al.*

(2004) assessed the beneficial effect of the traditional Camembert-type cheese consumption on rat Human-associated microbiota using specific medium and PCR–temporal temperature gradient gel electrophoresis. Cheese microflora showed significant tolerance to harsh gastrointestinal conditions, particularly, *Streptococcus thermophilus*, *Lactobacillus* sp. and *G. candidum*. These resistant genera have shown potential enhancement of the intestinal metabolism. Subsequently, the same team investigated the survival of traditional Camembert-type cheese microorganisms in a small clinical trial. The study was conducted with twelve healthy volunteers who have consumed cheese during four weeks after two exclusion weeks, followed by a wash out period. Overall, the final analyzed fecal samples contain *Lactococcus lactic*, *Leuconostoc mesenteroides* and *G. candidum*. However, no intestinal metabolic changes have been shown after Camembert cheese consumption (Firmesse *et al.*, 2008). David *et al.* (2014), used 16S rRNA and ITS gene sequencing to study the effect of animal diet on human microbiome composition. The main bacterial (thermophilic strains) and fungal (*Penicillium* and *Candida* sp.) species of Camembert-type cheese survived the digestive stresses and were predominantly detected in the human distal gut. Adouard *et al.* (2015b), evaluated the survival of a mixture of nine microbial strains grown on a smear-ripened cheese during gastrointestinal transit. Interestingly, *G. candidum*, *K. lactis,* and *D. hansenii* showed high resistance to simulated digestive stress with less than 1.0 Log ($CFU.mL^{-1}$) of viability loss with a moderate immunomodulatory and anti-inflammatory effects (Adouard *et al.*, 2015a; Plé *et al.*, 2015). *G. candidum* displayed significantly high resistance during gastrointestinal transit specifically when tested as part of the cheese matrix microflora.

III. *In vitro* gastrointestinal digestion

In general, the digestive system is divided into four main sections, starting by the mouth, the stomach, the small intestine (duodenum, jejunum, and ileum) and finally the colon. Each of these digestive compartments is characterized by different physicochemical properties and a complex microflora. Since the human and the animal digestive systems aretime-consuming, ethically complicated, and expensive, *in vitro* digestion systems were developed to facilitate the study of the digestion process.

1. In vitro digestion

In vitro digestion models were developed as an alternative to animal and human subjects or to be complementary to them. Hur *et al.* (2011), reported about 80 studies using *in vitro* models of which seven were dedicated to dairy product digestibility and several for probiotic microorganisms' survival before human studies. *In vitro* digestion models are being used in different fields such as pharmacology, biotechnology or nutrition. In the latter case, it allows a rapid screening of the bioaccessibility and bioavailability of food nutrients' as a function of their composition, structure and functional properties. Likewise, simulated digestion models are used to investigate food microbiology

particularly, the optimization of delivery systems for probiotic microorganisms, and the viability of ingested strains through gastrointestinal transit as detailed in (section II). Digestive juices are prepared to simulate physiologic human conditions.

2. Models: study of microorganisms viability during in vitro digestion of dairy products

In vitro digestion models have been classified into 'batch' and 'dynamic' systems depending on whether the temporal profile of *in vivo* digestion are controlled\simulated or not (e.g., mechanical force, liquid flow, shear stress, dilutions by gastric secretions over time, gastric emptying and the removal of resulting digestion products) (Guerra *et al.*, 2012; Thomas *et al.*, 2007; Vieira *et al.*, 2014). A large number of *in vitro* digestion models has been reviewed by (Guerra *et al.*, 2012) and (Verhoeckx *et al.*, 2015). The physiological, chemical and enzymatic properties of simulated oral, gastric and duodenal steps will be detailed in the section III.3.3.

2.1. Static models

The static digestion system, also called 'batch' model, comprises a series of vessels (Figure 1-7), each simulating a digestive compartment (mouth, stomach, and small intestine: duodenum) as described by (Versantvoort *et al.*, 2005).

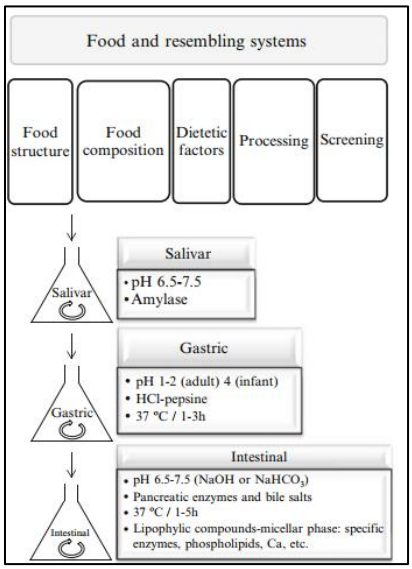

Figure 1-7: An overview of static digestion model

Reproduced from Verhoeckx et al. (2015)

Minekus *et al.* (2014) described the protocol of an international consensus for the standardization of the static digestion models. Authors detailed the recommendations for each biochemical process steps including their durations, volumes of digestive fluids secretions, enzymatic activity, bile salts concentration and pH adjustment (Table 1-3). Ortakci *et al.* (2012) used two compartments of this system to study the influence of the pH of simulated gastric and intestinal juices on the survival of microencapsulated probiotic bacteria when inoculated into Mozzarella cheese. These authors highlighted the importance of the selection of simulated gastric and intestinal digestion media for the prediction of the delivery carrier of probiotic bacteria. In a further study, the three digestion compartments have been simulated to assess the survival of *Lactobacillus acidophilus*, *Lactobacillus casei* subsp. *paracasei* and *Bifidobacterium lactis* when incorporated in a semi-hard goat cheese (Fernandes Garcia *et al.*, 2014). Based on the use of this simulation model parameters, results showed that goat coalho cheese matrix provided higher viability through harsh gastrointestinal conditions. Similarly, Sumeri *et al.* (2012) developed and validated a single vessel gastrointestinal tract simulator to investigate the viability of various lactic acid bacteria from semi-hard cheese. Furthermore, an optimized static model of (Oomen *et al.*, 2003) and (Versantvoort *et al.*, 2005), mono-step system, has been used to assess the survival of *Lactobacillus* in a semi-soft cheese (Mäkeläinen *et al.*, 2009). Despite the accurate provided results, this system does not simulate actions of mechanical forces, flow and mixing in real time which might influence the kinetics of digestion for complex matrices.

2.2. Dynamic digestion model

The dynamic system typically includes physical, mechanical processes, and temporal changes in luminal conditions to mimic *in vivo* properties. It has been used principally to simulate the physical conditions of the sample (i.e., the mixture of ingested food particles and fluids released during digestion) and changes over time such as particle size reduction, viscosity and some temporal effects like mixing, diffusion and formation of colloidal phases. This model includes the oral, gastric and intestinal steps (duodenum, jejunum, and ileum). Four systems have been developed under dynamic digestion model category as follows:

Dynamic mono-step systems itself includes **(1)** the Dynamic Gastric Model developed at the Institute of Food Research in Norwich UK by Mercuri *et al.* (2011), which comprises an apparatus that simulates gastric digestion using a conical flexible walled vessel and a cylinder that processes the food at typical shear rates. The Dynamic Gastric Model has been used and optimized to investigate the survival of probiotic bacteria such as *Lactobacillus rhamnosus* (Pitino *et al.*, 2010), as well as *Lactobacillus casei* subsp. *shirota, L. casei* subsp. *immunitas* and *Lactobacillus acidophilus* subsp. *johnsonii* in the upper gastrointestinal conditions (Lo Curto *et al.*, 2011) in milk matrix. Authors studied the main factors associated with digestion model that would affect the viability of probiotic bacteria and showed that the combination of computational and *in vitro* digestion model allowed more sophisticated

dynamic tracking of the viability of analyzed bacteria. Particularly, the control of gastric pH and its change during the gastric stage is a key factor to better investigate the resistance of these bacteria to digestive stress. The same model has been optimized to examine the viability of *Lactobacillus rhamnosus* after being inoculated into the cheese matrix (Pitino *et al.*, 2012). It has been shown that cheese matrix properties (i.e., structure, protein and fat contents) provided high viability rates through gastrointestinal transit. **(2)** The Human Gastric Simulator is the second mono-step system which is adapted from (Kong and Singh, 2010) and newly equipped with peristalsis function to get an improved direct observation and analysis of ingested food (Kozu *et al.*, 2014). This system was validated over the traditional *in vitro* tests when investigating the survival of ingested lactic acid bacteria within fermented milk matrices (Faye *et al.*, 2012)

Two multi-steps dynamic systems have been developed. To start with, **(1')** the simulator of the Human Intestinal Microbial Ecosystem known as SHIME (Molly *et al.*, 1993). It provides a relevant investigation of the intestinal microbiota. It comprises six reactors featured by their controlled pH, reactional volume and residence-time. However, it is inappropriate for neither the absorption of ingested products nor the progressive decrease of gastric pH. Several applications of this system have been made in different fi(Alander *et al.*, 1999)elds such nutrition, pharmacology, toxicology and microbiology and particularly to study probiotic bacteria viability under simulated gastrointestinal digestive stress (Alander *et al.*, 1999). The second multi-steps dynamic system is the **(2')** TNO Gastrointestinal Tract Model also called TIM. This is a computer-controlled *in vitro* digestive system that includes the stomach and the three intestinal sections (duodenum, jejunum, ileum, and colon) separated in different compartments.Today, two TIM models exist TIM-1 (stomach & small intestine) (Minekus *et al.*, 1995) and TIM-2 (large intestine) (Blanquet-Diot *et al.*, 2012; Minekus *et al.*, 1999).

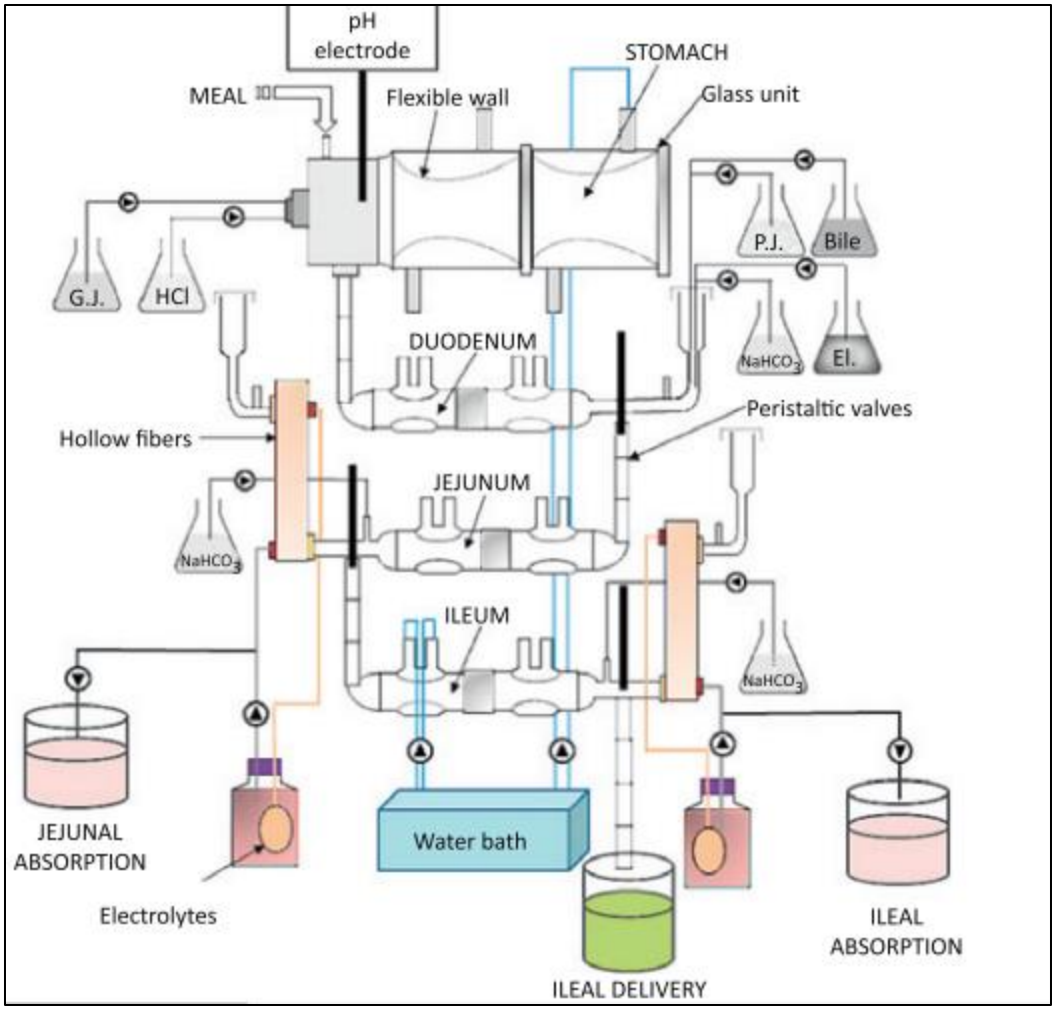

Figure 1-8: Schematic presentation of TIM-1 equipped with membranes to study nutrients bioaccessibility

Reproduced from Guerra et al. (2012)

TIM-1 is considered complete, satisfying and relevant digestive simulator (Minekus *et al.*, 1995). It allows the simulation of the different compartments all with considering the *in vivo* conditions such as mixing chyme, intestinal transit-time, gastric and intestinal emptying, the evolution of intestinal and gastric pH, sequential addition of digestive secretion and the passive absorption of water as well as digestive products with dialysis system. These models, allowed valuable investigation of the survival of lactic acid bacteria microbes during Saint-Paulin type cheese digestion (Kheadr *et al.*, 2011) and dairy yeast of Camembert cheese curd model (Hatoum *et al.*, 2013). However,

the static model developed by Versantvoort *et al.* (2005) is considered the most practical for such studies owing to the large number of samples that might be handled within a limited lap of time (Oomen *et al.*, 2003).

3. Disintegration kinetics of solid food during in vitro digestion

It is recognized that the behavior of food during digestion is modulated by 'the interaction food structure-digestion physiology' (Olthoff *et al.*, 1984). The degradation of ingested food starts with mastication process that allows the grinding and lubrication of food matrix (Figure 1-9). This facilitates its further disintegration during gastric digestion where enzymes and hydrochloric acid degrade the matrix and allow the breakdown of macronutrients. The last step of food disintegration is the intestinal phase through which pancreatic enzymes fulfill the complete release of nutrients increasing their bioavailability before absorption. A summarizing scheme of food matrix digestibility (i.e., disintegration, bioaccessibility, and bioavailability) through gastrointestinal digestion is described above Figure 1-9.

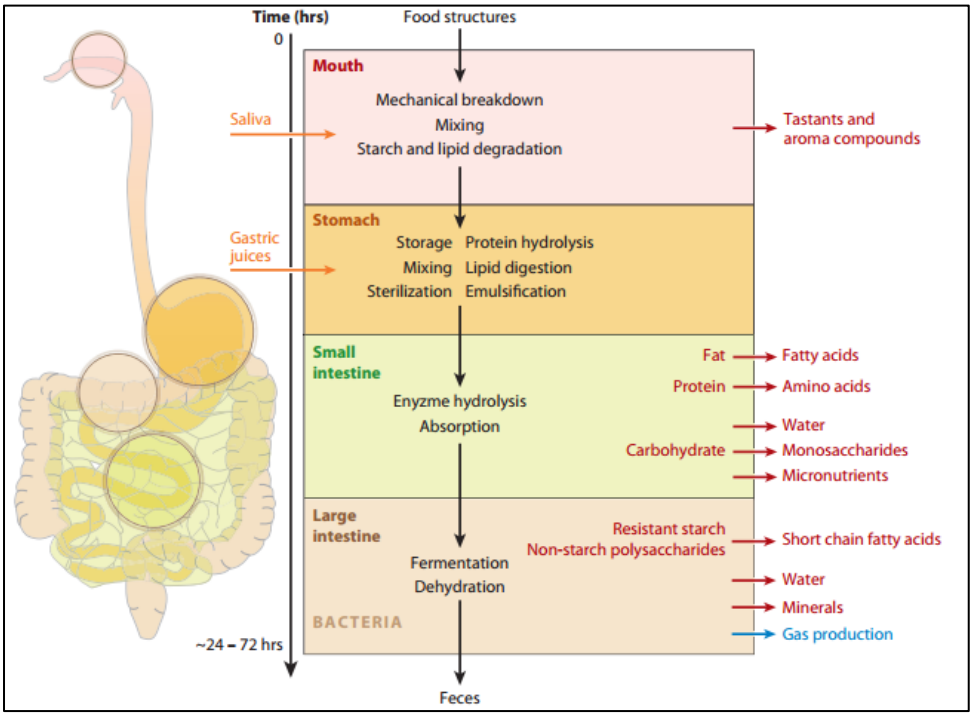

Figure 1-9: Scheme of the transit of food structures during digestion

Reproduced from Norton *et al.* (2014)

The simulated digestive fluids used in static digestion models (enzymes, electrolytes concentrations, minerals) and their dilution factors have been normalized by (Minekus *et al.*, 2014) to mimic human conditions (Table 1-3). Authors suggested that the use of mucin (oral phase) and gastric lipase could be omitted since their effects are barely known, since mucin is not very reproducible and gastric lipase with the same human lipase characteristics is commercially unavailable.

3.1. The process of oral digestion

The mastication of solid food is the first step of mouth-to-gastrointestinal digestion. Essentially, this step allows the mechanical breakdown and particle size reduction of the solid food matrix. The primary role of saliva is the hydrolysis of starchy components through amylase activity and the lubrication of ingested food with mucins at a neutral pH 6.8 at 37 °C (Minekus *et al.*, 2014). Both, the mechanical and biochemical actions favor the swallowing of the bolus. The size of reduced particle that results from *in vivo* oral disintegration varies from a 0.82 to 9.6 mm (Hoebler, 2000; Jalabert-Malbos *et al.*, 2007; Olthoff *et al.*, 1984; Peyron *et al.*, 2004). In general, when mimicking *in vivo* chewing with mechanical items, an average of 2 mm particle size is usually accepted with a recommended residence time of 2 min (i.e., static model) (Minekus *et al.*, 2014). Mastication rate depends on food properties such as composition, structure, texture, appearance and the size of ingested food (Foster *et al.*, 2006; Lenfant *et al.*, 2009; Togashi *et al.*, 2000; Woda *et al.*, 2006). Chen *et al.* (2013) investigated seven different food types masticated by ten healthy subjects and showed the crucial influence of solid matrix hardness on particle size distribution within the bolus. Several *in vivo* studies investigated the oral processing of different cheeses (Agrawal *et al.*, 1997; González *et al.*, 2002; Meullenet *et al.*, 2002). These studies showed that chewing cycles increase when cheese is harder and have lower moisture, and that the fat content of cheese is inversely correlated with chewing cycles number (Çakir *et al.*, 2012). Fang *et al.* (2016) evaluated the disintegration kinetics of five differently-structured Commercial cheese varieties (Camembert, smear cheese, young Cheddar, aged Cheddar, and Mozzarella). Results showed that the initial composition of the cheese matrix influenced its disintegration during the oral stage. After 2 min of oral digestion, the difference in the initial fat content within each cheese matrix induced different disintegration kinetics. Young Cheddar, Mozzarella and smear cheese showed lower disintegration compared to Camembert and aged Cheddar matrices. The higher disintegration of Camembert-type cheese (78%) during the oral step induced higher nutrients bioaccessibility through subsequent digestion steps.

3.2. The process of gastric digestion

After being swallowed the bolus is disintegrated due to peristaltic movements of the antral stomach, hydrochloric acid attack and pepsin diffusion into the ingested matrix (Kong and Singh, 2008b). The gastric stage consists of a biphasic emptying. Indeed, the stomach requires a lag phase to process solid foods and an equilibrium emptying phase (Siegel *et al.*, 1988). The first phase depends on the structure of food matrix, showing a striking difference between liquid and solid matrices. Once the solid matrix is reduced into particles of size small enough allow their passage through the pylorus. The equilibrium emptying time is the same for liquid or solid foods. In general, the half emptying time of stomach is between 60-70 min. The pyloric sphincter allows the release of food steadily into the small intestine when the particle size is reduced to 1-2 mm or less (Camilleri, 2006; Siegel *et al.*, 1988).

For dynamic and static digestion models the gastric compartment has been developed and optimized, essentially to investigate the disintegration of differently-structured food matrices (liquid, semi-solid and solid) (Kong and Singh, 2008b). These studies proved the crucial impact of the pH, temperature, mechanical motions (described in section II.3) and food matrix properties (section III.4) to modulate gastric juice diffusion and control the disintegration of meat, fruit, nuts, baked and fried food (Kong and Singh, 2009a) brown and white rice (Kong *et al.*, 2011), raw and cooked carrots and ham matrices (Bornhorst and Singh, 2013; Kong and Singh, 2008a). Overall, authors showed that the application of hydrodynamic forces in a dynamic gastric model enhanced gastric juice diffusion within food matrix and resulted in a greater solid release. In a later study, the addition of wave-like peristaltic movements improved the performance of dynamic gastric model (Kong *et al.*, 2011). Noteworthy, the increase of the disintegration process during the gastric stage results in a pH increase of the gastric content which is a function of the buffering capacity of the ingested food matrix. Hence, the pH is being maintained between 2-3 units through steady secretion of HCl all along gastric digestion. For this reason, the rate of the disintegration in the stomach cannot be predicted based on a static pH level (Van Wey *et al.*, 2014).

Particle size reduction could be due to either the acidic pH or the peptic activity. However, the kinetics of disintegration depends mainly on the efficiency of pepsin activity at optimal pH (Guo *et al.*, 2015). Ye *et al.* (2016a) evaluated the behavior of heated and unheated skim milk during gastric digestion using human gastric simulator developed by (Kong and Singh, 2010). Thereby, results showed that the degradation of coagulated casein is due to peptic activity since the hydrochloric acid did not contribute to the hydrolysis of protein clot. The disintegration is also a function of the intra-cohesive forces holding the matrix together known as ‘‘the critical stress’’. When the mechanical stress of the stomach is higher than the critical stress, fragmentation controls the disintegration profile of solid foods otherwise erosion is dominant (Kong and Singh, 2009b).

In static digestion models, pH is being adjusted from about 6.5 to 3.0 then maintained between 2.0-3.0 pH units using 12 M hydrochloric acid. Regarding the buffering capacity of ingested food, pre-tests are always done to preview the time and volume of acid addition (Minekus *et al.*, 2014). In the case of these models, the peristaltic motions are simulated simply using a rotating water-bath (Minekus *et al.*, 2014), a head-over-heal rotator (Oomen *et al.*, 2003) or a beaker equipped with a rotating paddle (Ferrua *et al.*, 2011). Overall the temperature is kept at 37 °C for 1 to 3 hours of gastric digestion. In the same way, the dynamic models mimic the physiological conditions of digestion. However, these models simulate the mechanical movement of the stomach effectively with ''a fixed outer cylinder with a movable inner cylinder to crush foods in between'' (Wickham *et al.*, 2012). On the contrary to static models, the dynamic systems are functional at the real time.

3.3. The process of intestinal digestion

After being emptied through the pyloric sphincter, the chyme is transferred steadily to the small intestine to allow nutrients release and absorption. The small intestine comprises three segments (i.e., duodenum, jejunum and ileum). However, macronutrients digestion takes place intensively in the duodenum (Borgström *et al.*, 1957). The intestinal digestion starts by the neutralization of chyme's pH from 2.0-3.0 to 7.0 by pancreatic bicarbonate. This launches the enzymatic activity of pancreatic juice (i.e. proteases, peptidases, lipases, esterase, and amylases), bile salts and phospholipids reactions (Borgström *et al.*, 1957). The digestion of carbohydrate occurs in the duodenum through the activity of pancreatic amylases (Borgström *et al.*, 1957; Hall, 2015). Pancreatic proteases and peptidases (i.e., trypsin, chymotrypsin) allow the further breakdown of proteins that have been partially hydrolyzed by gastric pepsin. Pancreatic lipases and bile salts allow the conversion of ingested fat into 2-monotriglycerides and available free fatty acids. Bile salts have an emulsifying function that reduces fat's surface tension to break fat globules in addition to the formation of lipid micelles complex that favor further fatty acids absorption (Hall, 2015).

The food particles delivered from the stomach are mixed with intestinal secretions and transported in the intestine due to segmentations and peristaltic contractions during 3-4 hours variable transit time (Davis *et al.*, 1986). McIntyre *et al.* (1997) investigated the transit time of rice pudding in the human small intestine when added to coarse bran or plastic particles and showed the crucial impact of particle size on intestinal transit. Intestinal physiological conditions have been simulated using either static (Minekus *et al.*, 2014) or dynamic models (Guerra *et al.*, 2012) both allowing nutrients bioaccessibility investigation. In the first case, the transit time has been fixed for 2 hours with a neutral pH 6.5-7.0, and mechanical movements are simulated using a rotating water-bath. Otherwise, the TIM-1 system (TNO, Netherlands) mimics the interaction digestive juice-meal mixture, all with reproducing peristaltic movement by applying external hydrodynamic forces (Guerra *et al.*, 2012). Wright *et al.* (2016) designed a separate Human duodenal model (HDM) including segmentation function and sigmoidal shape with ascending and descending

sections that allow mimicking human intestinal digestion and useful for food digestibility. It is to note that solid disintegration is minor in the intestinal tract. However, non-digested gross-particle might remain in the large intestine where the bacterial activity of gut microbiota may contribute to its digestion (Norton *et al.*, 2014).

1-3: Preparation of stock solutions of simulated digestion fluids a final volume of 500 mL).

Reproduced from (Minekus et al., 2014)

			SSF		**SGF**		**SIF**	
			pH 7		pH 3		pH 7	
Constituents	Stock concentration		Volume of stock	Concentration in SSF	Volume of stock	Concentration in SGF	Volume of stock	Concentration in SIF
	$g\ L^{-1}$	$mol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
$KH_2\ PO_4$	68	0.5	3.7		0.9		0.8	
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	_	_	11.8	47.2	9.6	38.4
$MgCl_2\ (H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2\ CO_3$	48	0.5	0.06	0.06	0.5	0.5	_	_
For pH adjustment								
	$mol\ L^{-1}$		mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$	mL	$mmol\ L^{-1}$
NaOH	1		_	_	_	_	_	_
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids, see details in legend								
	$g\ L^{-1}$	$mol\ L^{-1}$		$mmol\ L^{-1}$		$mmol\ L^{-1}$		$mmol\ L^{-1}$
$CaCl_2(H_2O)_2$	44.1	0.3		1.5 (0.75*)		0.15		0.6 (0.3*)

(*) In brackets is the corresponding Ca^{+2} concentration within final digestion mixture.

4. Nutrient bioaccessibility: dairy matrices

Functional food design requires the understanding of the structural and physicochemical properties of food matrix since this governs the rate of nutrients release (McClements *et al.*, 2008b; Parada and Aguilera, 2007; Turgeon and Rioux, 2011). Nutrient release or nutrient ''bioaccessibility'' refers to the fraction of ingested nutrient that is released from food matrix during gastrointestinal transit (Versantvoort *et al.*, 2005). Several studies showed that the different applied unit operations during food processing play a key role in modulating food matrices attributes, thus their nutrients bioaccessibility during digestion (Sensoy, 2014). The importance of food matrix properties in modeling nutrients release kinetics and how technological processing enhance or limit their bioaccessibility will be discussed in the following sub-sections.

4.1. Interest in matrix properties

Aguilera (2006) defined the food matrix as a ''complex multiphase'' with a unique microstructure. Several studies showed that nutrients' release depends on food matrix properties (Aguilera and Lillford, 2007; Chen *et al.*, 2006; Norton *et al.*, 2007; Parada and Aguilera, 2007). Noteworthy, compositional properties have shown an important impact on nutrients' bioaccessibility (Turgeon and Rioux, 2011). The hydrolysis of protein is an essential step to initiate food matrix disintegration in the stomach. Though, protein bioaccessibility is modulated by the extent of hydrochloric acid, pepsin, trypsin and chymotrypsin diffusion to cleavage site as well as the flexibility of the molecule owing to unfolding (Mackie and Macierzanka, 2010). These parameters are determined by the protein concentration (Thévenot *et al.*, 2017), its microstructure and its interaction with others food matrix components (Lundin *et al.*, 2008). For instance, in the case of milk proteins, the microstructure of casein facilitate trypsin and pepsin accessibility at cleavage site whereas β-lactoglobulin, globular protein showed a high resistance to pepsin (Guo *et al.*, 1995). The increase of the casein concentration in dairy matrices hinders pepsin diffusion into dairy gels network, hence delays protein hydrolysis (Thévenot *et al.*, 2017).

When fat is entrapped within a hydrogel or solid matrix, the release of lipids depends on the kinetic and rate of surrounding components breakdown (McClements, 2007). The main factors influencing lipid bioaccessibility have been reviewed by (McClements *et al.*, 2008a). These authors highlighted the effect of food matrix's composition (e.g., fiber, protein, polysaccharide, and minerals: Ca^{2+} Mg^{2+}), the synergy between its constituents (electrostatic, hydrophobic, covalent) and digestive fluid diffusion (porosity, enzymes, and acid accessibility) on lipid bioaccessibility during digestion. Furthermore, the structure of the interface of the oil droplets have shown a significant effect on the bioavailability of fatty acids during digestion (Zhang *et al.*, 2015). Using *in vivo* digestion tests Armand *et al.* (1999) showed that the bioaccessibility of lipid increases when the initial fat globule size decrease, which in turn increases

lipid/water interface and so the accessibility of enzymes to the fat proportion (Fave *et al.*, 2004). Additionally, authors reviewed the significant impact of the partition of lipid molecules between the core and the surface of an oil droplet, the structure of the droplet surface (i.e., layers, ultrastructure, and protein) and the molecular structure of the triglycerides and phospholipids on lipid bioaccessibility. Several studies highlighted the impact of milk fat globule membrane (MFGM) structure on milk fat bioaccessibility during *in vitro* digestion (Gallier *et al.*, 2012; Garcia *et al.*, 2014; Ye *et al.*, 2010). Specifically, Fardet *et al.* (2013) reported that when milk is clotted at gastric acid (pH 2.0-3.0), the released casein and whey proteins (i.e., due to pepsin action), tend to adhere to fat globules surface which limits the release of oil droplets (Armand *et al.*, 1996; Michalski *et al.*, 2005). Under these conditions, milk fat coalesces in the digest and within protein clots (Ye *et al.*, 2016a).

Dairy matrices are the result of distinct conformations of three major ‘’building blocks’’ (i.e., milk fat, casein micelles and whey proteins: β-lactoglobulin) (Aguilera, 2006) (Figure 1-10). Both β-lactoglobulin and casein have shown fast and ‘slow’ appearance of their amino acids in the blood plasma and were then defined as fast and slow proteins, respectively (Boirie *et al.*, 1997). Once these proteins arrive in the stomach their behaviour is different with caseins forming a coagulum more slowly digested and released from the stomach to the duodenum. *In vitro* experiments also showed differences in the disintegration of two dairy gel matrices coagulated either by acid or rennet method (Barbé *et al.*, 2014). Although the significant similarities of physicochemical and microstructural properties of both gels, breakdown kinetics were different during gastric digestion (Barbé *et al.*, 2014). Rioux and Turgeon (2012) used *in vitro* static digestion model to evaluate the disintegration of yogurt matrix prepared with different casein: whey protein ratios. Yogurt with higher casein content displayed higher viscosity and delayed matrix disintegration; however, the bioaccessibility of amino acids was similar. Thévenot *et al.* (2017) investigated the coefficient of pepsin diffusion within dairy gels with different casein concentrations during simulated gastric digestion. The concentration of casein has been shown to influence the microstructural parameters of the network of casein rennet gels that in turn modulated the diffusion of pepsin. The increase of casein concentration of rennet gels reduced the coefficient of pepsin diffusion and so protein clot breakdown.

The study of the disintegration of more complex dairy matrices (i.e., milk, yogurt and cheese) has shown the influence of composition on breakdown kinetics. In this perspective, Ayala-Bribiesca *et al.* (2016) compared the disintegration of three Cheddar cheese varieties with different calcium content through *in vitro* simulated digestion. Authors have shown that the increase of calcium content within cheese matrix, increased its firmness which in turn delayed its disintegration. Moreover, the very high-calcium Cheddar showed lower free fatty acids release compared to other Cheddar cheese since calcium content reduces the available interface lipid-water. In other words, the released free fatty acids bind to calcium easily at 37 °C and a neutral pH during duodenal digestion and limits lipases accessibility to lipid (Hu *et al.*, 2010). Recently, the comparison of Mozzarella and aged, fresh mild and light Cheddar

cheese types, smear cheese, Camembert-type cheese showed how the protein-to-fat ratio modulated the disintegration of these cheeses (Fang *et al.*, 2016). A high-fat content increased the disintegration whereas, a high protein content limited the hydrolysis and so delayed the disintegration (Van Wey *et al.*, 2014). Particularly, the state (i.e., resistance to pepsin, brittleness, and initial proteolysis degree) and concentration with which milk proteins act to entrap milk fat globules was suggested to have a significant impact on nutrient release.

Le Feunteun *et al.* (2014) evaluated the gastric emptying of differently structured dairy matrices (raw and heated milk, two rennet gels corresponding to each milk, acid gel, and a mixed gel) when ingested by mini-pigs. The rennet gel have shown lower disintegration than acid gels due to their lower rigidity. Likewise, independently of physicochemical properties. Using *in vitro* static digestion model, Rinaldi *et al.* (2014) studied the disintegration of liquid matrices: sterilized and pasteurized skimmed milk and semi-liquid: yogurt. Authors showed that the difference in the microstructure of these dairy matrices resulted in different disintegration kinetics and nutrients bioaccessibility. Lamothe *et al.* (2012) and Fang *et al.* (2016) investigated the disintegration of aged cheddar a fresh mild Cheddar, light Cheddar and Mozzarella after 2 h of gastric digestion. Light Cheddar cheese displayed lower gastric degradation (25%), compared to Mozzarella. The porous structure of Mozzarella favored a full-fast disintegration within 180 min of duodenal digestion. This has resulted in a higher fatty acids release at the end of duodenal digestion. The disintegration of cheese matrices ended on their initial texture attributes, particularly, the elasticity, firmness, and cohesiveness of those cheeses.

4.2. Impact of some manufacturing steps on food bioaccessibility

The bioaccessibility of food nutrients depends on the physiological parameters of digestion, the native structure of food matrix (raw plant, fruit, vegetables and raw milk) and essentially, how food processing modulates the intramolecular and intermolecular properties of food matrix (Aguilera and Stanley, 1999; Fernández-García *et al.*, 2009; Kong *et al.*, 2013; Parada and Aguilera, 2007). Betoret *et al.* (2015) reviewed the impact of homogenization, and drying operations on the bioaccessibility of nutrients and functional compounds, such as β-carotene, lycopene or polyphenols. The release of these components was enhanced and/or allowed due to either heat treatment or mechanical processing disrupting cells wall (Aguilera and Lillford, 2007). Dairy matrices include a wide range of products whose physicochemical and structural attributes are modulated by heat treatment, acidification, fermentation, pressing and shear (Aguilera, 2006). These practices are expected to affect their disintegration and their nutrients release through gastrointestinal transit (Norton *et al.*, 2014).

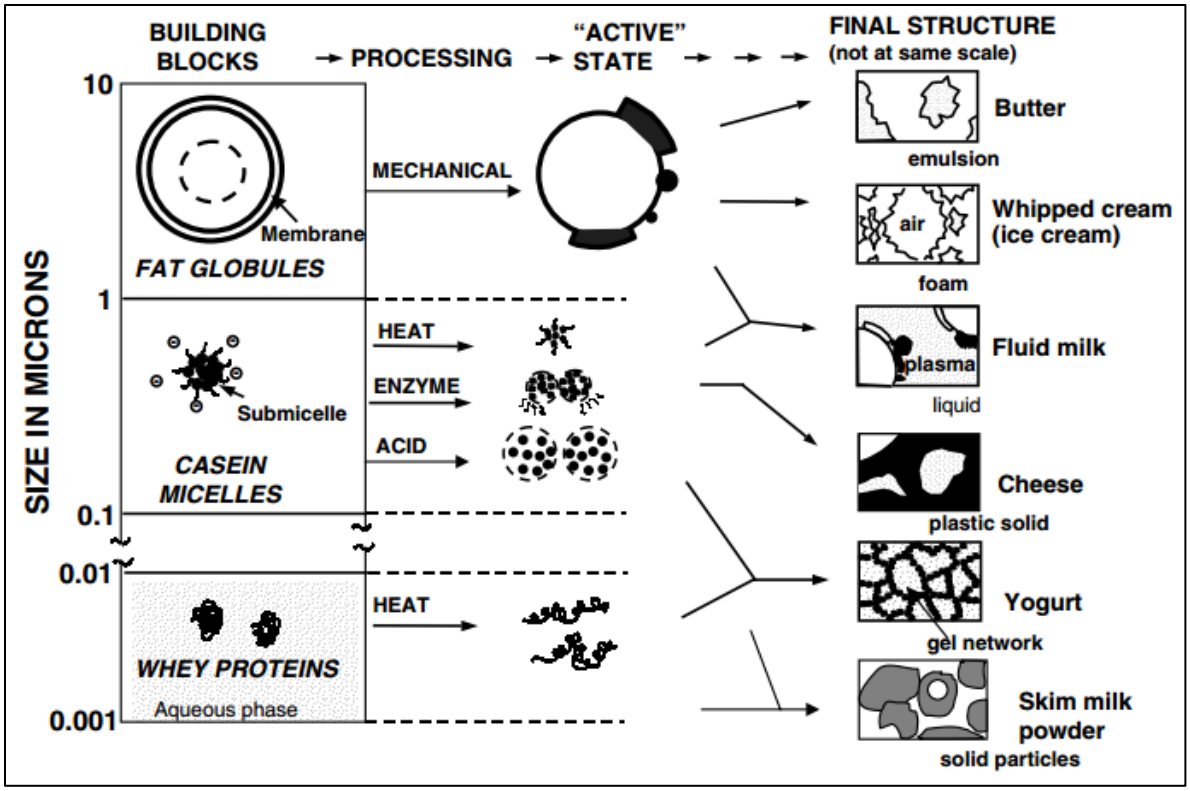

Figure 1-10: The structure of produced dairy products during processing by non-specific association at the sub-micron level of three major components of milk matrix

Reproduced from Aguilera (2006)

Guo *et al.* (1995) showed that heating β-lactoglobulin solution (> 80 °C, 10 min) had increased the brittleness of the gel. This enhanced β-lactoglobulin susceptibility to pepsin and trypsin activity. Likewise, during simulated *in vivo* digestion of heated skimmed milk, milk proteins hydrolysis, and amino acids release have increased in comparison with unheated samples (Barbé *et al.*, 2013; Rinaldi *et al.*, 2014; Ye *et al.*, 2016a).

Similarly, milk coagulation results in differently-structured gels. The final structure of these protein gels is a function of several factors including the pH, calcium concentration and milk processing history. To investigate the impact of these practices on the digestibility of differently coagulated milk gels, Barbé *et al.* (2013) evaluated the disintegration of heated and unheated milk and of rennet and acid coagulated gels. Heat treatment influenced both the bioaccessibility and the bioavailability of amino acids. However, gelation method (rennet vs acid coagulation) showed a more significant impact. In other words, the difference in the kinetics of matrix disintegration and the amino acid appearance in the serum were more influenced by the macrostructure (i.e., rigidity) than the microstructure (i.e., the conformation of protein network) of these dairy matrices (Barbé *et al.*, 2014). Besides, the fermentation occurring

during yogurt production with the enzymatic activity of lactic acid bacteria (i.e., *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus salivarius* subsp. *thermophilus*) is suggested to increase the release of amino acids compared to heated milk (Chandan and Kilara, 2013; Rinaldi *et al.*, 2014)

The analysis of the disintegration of Mozzarella and three Cheddar cheeses with different fat contents and ripening stage issued from different cheesemaking processes revealed the impact of different cheeses microstructures (Fang *et al.*, 2016; Lamothe *et al.*, 2012). Different proportions and release kinetics of fatty and amino acids were obtained depending on cheese characteristics.

As mentioned previously, cheese ripening governs the final physicochemical and structural properties of cheese. During this stage, the fungal community of surface-ripened cheese showed significant proteolytic and lipolytic activities. Particularly, *P. camemberti* and *G. candidum* contribute to the breakdown of Camembert-type cheese matrix. Their metabolic activities promote amino acid, and free fatty acids production in the ready-to eat cheese matrix (Dugat-Bony *et al.*, 2015; Lessard *et al.*, 2014).

Hypothèse et objectifs

Geotrichum candidum is a key yeast during soft mould-ripened cheese ripening. Specifically, it contributes to the development of textural and flavor profiles of Camembert cheese thanks to its biochemical attributes (e.g. lipolysis, proteolysis). In addition to technological processing, cheese microflora establishes the structural and physicochemical properties of cheese matrix which in turn govern the disintegration rate of the ingested amount of cheese as well as the bioaccessibility of its nutrients. Several studies showed an increase of the viability of the ingested probiotic microorganisms, when they are grown on a dairy products. Particularly, cheese matrix has been highlighted due to its pH, high buffering capacity and nutrient composition (i.e. lipid and protein). Concurrently, few studies investigated the survival of *G. candidum* during *in vivo* digestion of lactic Camembert cheese matrix. Among several yeast species, *G. candidum* has been detected within feces samples and showed high resistance to gastric acid and bile stress. In this perspective, the following hypothesis has been formulated:

Camembert cheese matrix properties enhance the viability of *G. candidum* LMA-1028 during *in vitro*simulated gastrointestinal digestion.

For this purpose our study focused on the additional protection provided to *G. candidum* LMA-1028 by Camembert cheese matrix during its *in vitro* gastrointestinal disintegration.

The approach used in this study aims respectively to:

1st Objective: Characterize the physicochemical and microbiological (cheese) attributes of milk, traditional and stabilized Camembert-type cheeses.

2nd Objective: Evaluate the disintegration and nutrients behavior during simulated gastro-intestinal digestion of milk, traditional and stabilized Camembert-type cheeses.

3rd Objective: Determine the viability of *G. candidum* LMA-1028 under digestive stress function of milk and both Camembert cheeses during *in vitro* digestion.

Chapter 2 : Survival of *Geotrichum candidum* in Camembert-type cheese during simulated gastro-intestinal transit

Abstract

During *in vivo* digestion of Camembert cheese, *G. candidum* previously showed high resistance to digestive stress. The protection of probiotic microorganisms through digestive passage was enhanced in cheese matrix. Our study focused on the effect of Camembert cheese matrix to protect *G. candidum* during *in vitro* digestion using a static digestion model. For this purpose, four matrices have been investigated including traditional and stabilized Commercial Camembert-type cheeses, a pasteurized milk (3.25 % fat content) and a culture medium used as a control matrix. All matrices have been inoculated with *G. candidum* LMA-1028. Matrices disintegration was evaluated during 4 hours of simulated digestion (Oral: 2 min; Gastric: 120 min; Duodenal 120 min), sequentially. The counts of *G. candidum* LMA-1028 viable cells were evaluated after 2 min of oral digestion, at 60 min and 120 min through exposure to simulated gastric juice and at 60 min and 120 min during duodenal digestion. The structure of Camembert cheeses matrix appears to modulate the accessibility of digestive juice to these matrices during gastric digestion and so the bioaccessibility of their nutrients. Similarly, the fat content seems to have an impact on the rate of disintegration of these matrices during gastric and duodenal digestion. The difference in the original structure of both Camembert cheese matrices led to different rates of gastric disintegration and also resulted in differences in a fat layer (obtained by centrifugation of the chyme). When comparing viability counts, *G. candidum* LMA-1028 showed a significant intrinsic resistance to simulated gastrointestinal stresses. Camembert cheese matrices as well as milk didn't bring additional protection to the studied strain LMA-1028. These results suggest that *G. candidum* LMA-1028 showed a high stress response.

Introduction

Functional food designing requires the understanding of the structural and physicochemical properties of food matrix since this governs the rate of nutrients release (McClements *et al.*, 2008b; Parada and Aguilera, 2007; Turgeon and Rioux, 2011). In particular, this depends on the microstructure of food matrix and the synergy between its components, as modulated by processing practices. Nutrient release or nutrient ''bioaccessibility'' is defined as the aptitude to release a nutrient from its matrix in the digestive tract to be available for intestinal absorption (bioavailability) (Fernández-García *et al.*, 2009). The bioaccessibility and bioavailability of nutrients are determined by physiological conditions including enzymatic reactions, gastric emptying and/or oral degradation of food (Parada and Aguilera, 2007). This topic is interesting and being well investigated in order to develop functional foods with health benefits, as well as better nutrients and bioactive molecules (Sensoy, 2014). Main nutrients as protein, lipids, and complex carbohydrates are the major building blocks of the food matrix. These components have shown a relevant effect on digestibility kinetics, nutrients bioavailability and so, on the nutritional attributes of food matrix (Betoret *et al.*, 2015; Mackie *et al.*, 2012).

Cheese is a dense matrix of casein within which fat globules and serum are entrapped. It is gaining attention to better understand its digestibility as a 'functional food' (Walther *et al.*, 2008). This efficient nutritive matrix is not only an excellent source of high-quality protein (i.e., includes unique significant amino acid composition), but also comprises beneficial conjugated linoleic acid (CLA). Cheese is a good source of minerals (e.g. calcium and phosphorus) and vitamins (e.g. vitamin B_{12}, vitamin A, niacin, riboflavin and folate) (reviewed in (López-Expósito *et al.*, 2012; Preedy *et al.*, 2013). The final composition, physicochemical and textural properties of cheese matrix are influenced by the cheese-making process (i.e., coagulation, maturation, whey draining, salting and ripening microflora). Depending on cheese type, the destabilization of the casein micelles occurs either through K–casein degradation when rennet is used or by colloidal calcium-phosphate depletion using lactic acid producing bacteria, and often by a mixed coagulation. Surface mold-ripened cheeses such as Camembert-type cheese are manufactured using mixed coagulation. Two categories are industrially produced: traditional and stabilized Camembert-type cheeses. For the traditional curd production, acidification requires the use of mesophilic bacteria 25 °C, whereas for the stabilized curd the acidification is mainly due to thermophilic species below their optimal growth temperature 34-39 °C (Lawrence *et al.*, 1987). This decelerates lactic acid production to maintain the pH at values ≥ 5.2 which reduces the demineralization of cheese matrix (Lawrence *et al.*, 1987). Moreover, Walstra *et al.* (2005) reported different mechanical curd handling practices during traditional and stabilized Camembert cheese manufacture that also impact moisture content of cheese.

In general, the fungal microflora of Camembert-type cheese comprises *P. camemberti* and *G. candidum*, and less frequently *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* (Arteau *et al.*, 2010; Leclercq-Perlat *et al.*, 2004a; Spinnler and Gripon, 2004). Arteau *et al.* (2010) investigated the occurrence of the fungal microflora into Canadian stabilized and traditional Camembert-type cheeses. For the first curd type *P. camemberti* was predominant while for traditional Camembert the major strain was *G. candidum*. *G. candidum* is an aerobic acid-tolerant and salt-sensitive yeast. It is a key microorganism for the development of organoleptic properties of surface-ripened cheese due to its lipolytic and proteolytic activities (Boutrou *et al.*, 2002). *G. candidum* consumes lactate produced by LAB and contributes to the alkalinization of the Camembert cheese surface. It also produces ammonia which allows further alkalinization of the cheese curd (Boutrou *et al.*, 2006b; Leclercq-Perlat *et al.*, 2004a).

Previous work demonstrated that the physicochemical and structural characteristics of cheese matrix may enhance a long-term survival of probiotic microorganisms and cheese was proposed as a favorable carrier for probiotic (Ouwehand *et al.*, 2010; Stanton *et al.*, 1998). For instance, the low acidity, the high buffering capacity and the dense matrix of protein and fat, when compared to other more acidic fermented dairy foods, have been reported to improve the protection of ingested microorganisms during gastrointestinal transit (Champagne *et al.*, 2011; Plessas *et al.*, 2012). These attributes also promote the development of a diverse microflora within cheese matrix providing about 10^9 CFU microorganism / g of ready-to-eat cheese (Beresford *et al.*, 2001). The diversity of the soft mould-ripened cheese ecosystem including indigenous and added bacteria, yeasts and molds raised the question whether this microflora exerts a beneficial effect on human microbiota after cheese consumption. In this perspective, Lay *et al.* (2004) showed that as for fermented milk, the consumption of traditional Camembert cheese consumption did not greatly modify the microbiota profile or the major metabolic activities of human microbiota-associated rats. However, an increase of mucolytic activity and a decrease of azoreductase activity were observed. Interestingly, these authors observed that *G. candidum* (Anonymous strain) was able to survive through the *in vivo* gastrointestinal transit. Subsequently, the same team further investigated the effect of a daily consumption of traditional Camembert cheese (during eight weeks), on the metabolic activities of human subjects' microbiota and confirmed the decrease of nitrate reductase activity (Firmesse *et al.*, 2008). In accordance with the animal study, *G. candidum* was detected in the feces of the human subjects during the study period and thus this strain was suggested as a "good marker" of Camembert cheese consumption. These observations demonstrated a good resilience of *G. candidum* toward the digestive gastric acid and bile salts stresses when ingested within cheese matrix. Recently, Adouard *et al.* (2015b) compared the viability of microbial mixture, including *G. candidum* ATCC 204307, when inoculated as pure culture, rennet gel or smear-ripened cheese, using *in vitro* gastric and duodenal batch challenges. On the contrary to previous studies, authors observed, that pure culture allowed a better survival rate than the cheese matrix (Livarot cheese). However, no study evaluated the possible impact of the disintegration of ingested matrices on the survival

rate of microorganisms. Consequently, the aim of this paper was to study the viability of *G. candidum* LMA-1028, used for Canadian Camembert-type cheese production, during the disintegration of whole pasteurized milk (3.25 % fat content) and two Camembert-type cheeses with different protein and fat contents through gastrointestinal transit using an *in vitro* static model. This may allow to understand the potential influence of structure and physicochemical properties of a dairy matrix on *G. candidum* during digestion.

Materials and methods

1. Material

Freeze-dried culture of *G. candidum* strain LMA-1028 (commercial starter used for Canadian cheese ripening) was confidentially provided. The ingredients of the defined culture medium (YEGP): Yeast Extract Glucose Peptone, were Yeast Extract (BD, Mississauga, ON, Canada), Glucose (EMD Chemicals, Darmstadt, Germany) and Peptone (Bacto™, BD, Mississauga, ON, Canada).

The composition of digestive fluids were described by Rinaldi *et al.* (2014). The used enzymes of *in vitro* digestion were purchased from Sigma-Aldrich (Oakville, ON, Canada). Oral digestion: α-Amylase (A3176), gastric digestion: pepsin (P7000) and duodenal digestion: lipase (L3126), bile (B8631) and pancreatin (P7545). The activities of the enzymes used (unit/g sample) were described by (Rioux and Turgeon, 2012). The analyzed dairy matrices (Milk and cheese) were purchased from local grocery store Métro (Québec, QC, Canada).

2. Sample preparation

Four different matrices have been investigated including two liquid matrices (i.e. Milk and culture medium and two cheese matrices a traditional and a stabilized Camembert-type cheese). Matrices differed by nutrient contents (lipid and protein) and by structure (liquid *vs* solid) and by proteolysis degree.

Culture medium and Milk: For the liquid matrices, *G. candidum* LMA-1028 was rehydrated cultured overnight at 4°C in either 150 mL of (YEGP) culture medium (CM); Yeast Extract 30 g/L, glucose 30 g/L, peptone 1 g/L) or 150 mL of commercial whole pasteurized milk with 3.25 % fat content (Milk), in order to obtain 10^6 CFU of *G. candidum* LMA-1028/mL of matrix.

Commercial Camembert-type cheese: Traditional (CTRAD) and stabilized (CSTAB) Camembert-type cheeses have cylindrical shape with curved sides, are of 2 cm and 4 cm high and weigh 170 g and 1200 g, respectively. Both cheeses were bought about two weeks before *in vitro* digestion and kept at (4 °C), to be analyzed on 42^{th} ±3 ripening day. The estimation of the ripening stage was based on 77-days complete ripening period of Commercial Canadian Camembert-type cheese.

3. Matrices characterization

The cheeses were analyzed without their curved sides. The curved sides were removed uniformly using a round cutter. Cheese samples were grounded and mixed to get quasi-homogenate paste sample and kept at 10 °C 1 h before the disintegration study and microbial analysis.

3.1. Compositional and biochemical analysis

In general, matrices composition was determined in triplicate according to AOAC or ISO methods. Cheese moisture (CM) was measured gravimetrically after drying approximately 1.5 g in a forced-air oven at 100 °C for 6 h (AOAC, 2008). Solids of Milk and CM were determined in a forced-convection oven at 105 °C for 2 h until a constant weight was reached. Fat content was determined by Mojonnier extraction method (ISO/IDF, 2004) on wet basis and fat content in dry matter (FDM) of cheese matrices was calculated as shown in Equation 1. Total nitrogen (TN) content was measured using the Kjeldhal (ISO/IDF, 2008) method. Ash content was measured through sample ignition at 550 °C overnight (AOAC, 2000). Moisture in non-fat substance (MNFS) (Equation 2) is equal to the percentage of moisture divided by (100- fat in dry matter). This is basically a ratio of water to protein.

$$Fat\backslash \mathrm{DM}_{cheese} = \frac{Fat}{100 - moisture} * 100 \quad (1)$$

$$MNFS = \frac{Moisture}{100 - Fat} * 100 \quad (2)$$

The proteolysis degree of CTRAD and CSTAB was evaluated by the measurement of the water soluble nitrogen content (WSN) (IDF, 1991a). Nitrogen content of WSN was determined using Kjeldhal method. Proteolysis was expressed as a percentage of the cheese TN content (Equation 3).

$$Proteolysis\ (\%) = \frac{WSN}{TN} * 100 \quad (3)$$

pH

pH measurements of cheeses were done in triplicate, following the method of (Touchette, 2016). Cheese samples were frozen at -30 °C the day of the digestion. They were unfrozen 3 h before pH measure. The white layer on the surface was removed, then the rind was separated from the core and about 4-cm diameter ball was made with each part. When cheese balls attain 20 °C, pH values were taken using an electrode (InLab cool, Mettler Toledo, Columbus, USA) calibrated with freshly prepared pH 4.0 and 7.0 standard buffers.

3.2. Cheeses textural properties

Texture Profile Analysis (TPA) test allowed the characterization of cheese texture. This test simulates mechanical chewing during mastication through double compression. For this purpose, eleven (11) standardized cylindrical samples (rind and core) (10 mm x 10 mm) were prepared at 10 °C, then put 30 min in an airtight petri dish to prevent moisture loss and at room temperature to reach 22 ± 2 °C. TPA was done using texture analyzer TA-XT_2 (Texture Technologies Corp., Hamilton, MA, USA) equipped with a 25 mm diameter acrylic cylinder probe. Double compression test was performed at 50 % (deformation) and 1 mm s^{-1}. Because Camembert cheese is considered a semi-solid food due to its high moisture and proteolysis degree (Gunasekaran and Ak (2002) from Schlesser *et al.* (1992). 5 cheese texture parameters of the TPA results were used: hardness, adhesiveness, cohesiveness, gumminess and resilience (Kfoury *et al.*, 1989; O'Callaghan and Guinee, 2004; Tunick, 2000), Results presented in Table 2-3 are the means of 9 samples measured for each repetition, for which the difference is < 2 x standard deviations.

3.3. G. candidum LMA-1028 enumeration

G. candidum count in ripened cheese was done one day before *in vitro* digestion. A cheese slice (rind + core) was grounded with a Hamilton-Beach coffee grinder (Ontario Canada). Four grams of the mixture were transferred to a sterile 50 mL conical tube containing 2 g of glass beads and 36 g of sterile sodium citrate solution (2 %) preheated at 45 °C. The mixture was obtained using a vortex at maximum speed for 5 min in order to obtain an homogenized solution. The efficacy of this method was evaluated in preliminary tests. The sample is homogenized, then filtrated, the remaining particles were weighed. Overall, 99% of the added cheese is being homogenized in the solution. Serial dilutions were performed in peptone water (0.1 %) and plated (10^{-2}, 10^{-3}) in triplicate. Viable cells counts of *G. candidum* LMA-1028 were done on YEG agar and incubated for 24 h at 25 °C.

4. Survival of *G. candidum* during *in vitro* simulated gastrointestinal disintegration of dairy matrices

4.1. Static in vitro digestion model

The simulated gastrointestinal digestion was done following the procedure developed by Versantvoort *et al.* (2005) and adapted for cheeses by Fang *et al.* (2016). This *in vitro* model simulates the main digestion steps (i.e. oral, gastric and duodenal). Different digestion parameters such as (enzyme solutions and concentration, stirring intensities and digestion time at each digestive stage and pH values) are described in Table 2-4 (Annex). Enzyme

solutions were prepared freshly and were kept in an ice bath prior to use. For each digestion step, the temperature was maintained at 37 ± 1 °C.

Overall, the same conditions of *in vitro* digestion have been conducted for liquid (CM, Milk) and solid matrices (CTRAD, CSTAB). Before digestion, cheese matrices (rind and core) were ground with a coffee-grinder to obtain an homogenous paste. The obtained cheese paste and the overnight prepared CM and Milk media were kept at 10 °C prior to digestion. Each matrix was weighted in a 150 mL beaker (9.0 ± 0.5 g) to which glass beads (2.0 ± 0.05 g) were added. For the oral step, the matrices were mixed at 75 rpm for 2 min with 6 mL of oral solution using a Caframo (Georgian Bluffs, ON, Canada) equipped with a straight blade paddle (1.0 cm high, 3.8 cm diameter). Then, the gastric solution containing pepsin was added to the beaker and transferred to an orbital stirring water bath adjusted at 200 rpm. At this step, the pH was reduced and maintained between pH 2.0 and 3.0 with HCl 12 M. Acid and pepsin solution were added twice during gastric digestion (i.e. 0 min and 60 min). After 120 min of gastric digestion, the duodenal solution (e.g. pancreatin, bile salts and lipase) was added. The pH was increased and maintained between 6.5 and 7.05 using 1.0 M $NaHCO_3$ solution. Duodenal pH adjustment of cheese matrices required also the use of dehydrated $NaHCO_3$. The duodenal digestion was performed under the same stirring conditions (200 rpm) for 120 min.

During *in vitro* digestion, the digests were collected as follows: (O) 2 min after oral digestion, (G60 and G120) after 60 and 120 min gastric digestion and (D60 and D120) after 60 and 120 min duodenal digestion with O: oral, G: Gastric and D: Duodenal. Five independent digestions were realized and stopped at a specific digestion stage (O, G60, G120, D60 and D 120) to study the survival of *G. candidum* LMA-1028. Matrices' disintegration was evaluated at O, G120 and D120 steps. Once recuperated, the pH of digested samples was adjusted to 7.0 ± 0.5 using NaOH 5 M to stop pepsin activity. Samples used for the tracking of survival were analyzed immediately as illustrated in Section 2-4.4, while disintegration samples were centrifuged 20 min x 4 °C x 9800g, Eppendorf-580F, Hamburg, Germany). Three different layers were obtained (i.e. fat, liquid and pellet) and have been weighed then frozen at (– 20 °C) until further analysis (Mass balance and disintegration: Section 2-4.2 & 2-4.3). For the liquid layer, the activity of trypsin and chymotrypsin is stopped using an inhibitor of trypsin-chymotrypsin (Sigma-Aldrich, Oakville, Ontario, Canada) (126 µL/ 4 mL of liquid) before freezing.

4.2. Matrices mass balance during in vitro digestion

The mass balance of each layer was determined for the four matrices after each digestion step (O, G120 and D120) and was calculated using the following formula:

$$Mass\ balance_i(\%) = \frac{Layer_i}{(W_0 + J)} * 100\ (5)$$

Where $Layer_i$ represents the weight of separated phase (i: Fat, pellet or liquid), W_0 is the weight of ingested matrix and J is the weight of digestive juice.

4.3. Milk and soft-ripened cheese disintegration

To evaluate the disintegration of dairy matrices, the tubes containing the pellet layers were put on ice and transferred at fridge temperature 8 °C to slowly unfreeze overnight. The following day, pellets were vortexed to obtain an homogenate mixture. For accurate results, total solids of each pellet were measured in duplicate except for milk and culture medium samples due to the small quantity of separated pellets. Dry matter is determined gravimetrically, pellet was dried in a forced-convection oven at 105 °C overnight. The disintegration (i.e. the proportion of cheese solids dispersed into the water phase) is calculated with the following formula (Equation 6). Milk and CM disintegrations were evaluated following the same protocol.

$$Matrix\ desintegration\ (\%) = \frac{W_0 - W_p}{W_0} * 100(6)$$

Where W_0 is the dry matter of weighed matrix and W_p is the dry matter of separated pellet.

4.4. Viability of G. candidum LMA-1028 during in vitro digestion

Samples were collected at the end of each step (O, G60, G120, D60 and D120) to evaluate the count of *G. candidum* LMA-1028 viable cells. The whole digested sample was transferred to a sterile conical tube and diluted with sodium citrate solution, previously heated to 45 °C. The concentration of sodium citrate is adjusted to 2 % based on a total final volume (digested sample + Na-citrate solution) of 48 mL /9 g of matrix. The mixture was mixed using vortex for 5 min and the obtained homogenate, described in 3.3, then was serially diluted in triplicate. With respect to the initial count of *G. candidum* in Camembert cheese matrix, dilution were plated on YEG agar in triplicate, then incubated 24 h at 25 °C.

5. Statistical analysis

The experimental study was done following a completely randomized design. For all the statistical tests, the significant difference between means values was based on a 95 % confidence level ($p < 0.05$). The presented means values and standard deviations of each physicochemical and initial microbial counts were obtained from four

independent repetitions. The significant difference between these values was assessed using a one-way-ANOVA and a Tukey's test.

G. candidum LMA-1028 counts were evaluated at (O, G60, G120, D60, and D120) while matrices disintegration and mass balance were measured at (O, G120, and D120). Outliers of *G. candidum* counts were identified with (Proc Univariate in SAS, version 9.4) then have been omitted. The difference between survival counts of *G. candidum* of each matrix over digestion time were assessed with repeated measures mixed model ANOVA with fixed factors: (treatments (matrices)= CM, Milk, CTRAD, CSTAB; period= O, G60, G120, D60, D120). This allowed the evaluation of the effect of (Time, matrix, Time x matrix). In the same way the disintegration of matrices was compared at three periods of time. It is to note that, repeated measures tests were validated when the type of covariance structure (unstructured (UN), autoregressive structure (AR (1) or compound-symmetry (CS)) allowed the smaller calculated AIC (Akaike Information Criterion) which is recommended to get a better model of analysis. All analyses were performed using SAS (SAS version 9.4, SAS Institute, Cary, NC, USA).

Results and discussion

1. Physicochemical and biochemical properties of dairy matrices

CM, Milk and Camembert cheeses (CTRAD, CSTAB) chemical composition is shown in Table 2-1. CM composition had lower fat and protein contents compared to dairy matrices, with less than 1.0 % protein and negligible amount of fat. Ash content was also lower than in dairy matrices. Milk and cheese composition were in accordance with the average compositions of whole pasteurized cow's milk and Camembert-type cheese reported in literature (Walstra *et al.*, 2005).Compositional characterization showed significant difference between CTRAD and CSTAB, particularly for protein, fat (in dry matter), ash and moisture contents. The moisture content of CTRAD and CSTAB was 51.16 and 47.43 %, respectively. Since MNFS is the same in both cheeses, the difference in moisture explains the different fat proportions.

The two cheese matrices studied had different fat-in dry matter ratios. It is to note that the fat content is generally compared on dry matter to overcome cheese moisture variation during storage and between manufacturing batches (Bylund, 2003). FDM was 50.60 and 57.43 % in CTRAD and CSTAB, respectively.

Table 2-1: Chemical composition (%) of CM, Milk, CTRAD and CSTAB

	CM	Milk	CTRAD	CSTAB
Moisture	96.56 ± 0.04 [a]	86.50 ± 0.04 [b]	51.16 ± 1.70 [c]	47.43 ± 1.36 [d]
Fat	0.0026 ± 0.0005 [a]	3.10 ± 0.078 [b]	24.70 ± 0.74 [c]	30.19 ± 0.91 [d]
Fat in dry matter	-	-	50,60 ± 0,55 [b]	57.43 ± 0.73 [a]
Protein	0.845 ± 0.027 [a]	3.13 ± 0.044 [b]	19.98 ± 0.34 [c]	18.74 ± 0.38 [c]
Protein in dry matter	-	-	40.95 ± 0.90 [b]	35.40 ± 1.51 [a]
Ash	0.16 ± 0.013 [a]	0.67 ± 0.04 [b]	3.45 ± 0.15 [c]	3.09 ± 0.10 [d]
MNFS	-	-	67.94± 1.64[a]	67.49± 0.98[a]

Values are means ± standard deviation of each analyses (n =4). a, b, c, d, e Means in the same line with different letters are significantly different by Tukey‘s test ($p < 0.05$).

Protein content of CTRAD and CSTAB on dry basis was 40.95 and 35.40 %, respectively. CSTAB has a higher fat content entrapped into a less dense protein matrix than CTRAD considering its lower protein content. Due to low renneting pH usually found in the traditional Camembert cheese process it may be hypothesized that CTRAD casein dissociation is higher than in CSTAB matrix. Acidification of stabilized Camembert is limited using protease negative strains below their optimal growth temperature (Lawrence *et al.*, 1987). CTRAD showed higher proteolysis degree than CSTAB, respectively 36.64 and 26.99% (Table 2-2). This may be attributed to a higher content of residual rennet within CTRAD matrix due the low pH during whey draining. Conventionally, during stabilized Camembert cheese making the quantity of rennet added is reduced in order to decrease the residual rennet in cheese and increase the shelf life of stabilized variety (Lawrence *et al.*, 1987; Lucey *et al.*, 2003). The range of ash content of both cheeses is typical of Camembert-type cheese as reported in the literature (Walstra *et al.*, 2005). However, CTRAD have higher ash content than CSTAB (Table 2-1). This is contradictory to the expected effect of the low pH of whey at drainage of CTRAD which should result in a cheese with a lower mineral content. Though, the difference in ash content is relatively small. Recently, Batty *et al.* (2017) reported a difference of more than 50% in calcium content between traditional and stabilized Camembert. However, this is not an analytical error as buffering capacities of CSTAB and CTRAD were not similar (results not shown).

Table 2-2: Proteolysis degree (%) and pH values[1] of Camembert-type cheese (CTRAD and CSTAB)

	CTRAD	**CSTAB**
Proteolysis	36.64 ± 2.48 ^{a}	26.99 ±2.70 ^{b}
pH-surface	6.66 ± 0.02 ^{aA}	6.44 ± 0.16 ^{aA}
pH-core	5.96 ± 0.26 ^{aB}	5.53 ± 0.26 ^{bB}
pH-gradient	0.70^{b}	0.91^{a}

1) Values are means ± standard deviation of each analyses (n =4). Different letters in the same row (a, b) and (A, B) indicate significant statistical difference (Tukey's test ($p < 0.05$)).

Surface pH was higher than core pH in both cheeses (Table 2-2). In the case of Camembert cheese, this is explained by the proteolytic activity of surface microflora (i.e., *G. candidum* and *P. camemberti*) (Boutrou *et al.*, 2006b; Leclercq-Perlat *et al.*, 2004a) (Noomen, 1978). In general a pH change within the cheese matrix depends on ammonia and lactate concentrations. *G. candidum* consumes lactate and releases ammonia, which contributes to the alkalinization of cheese rind (Leclercq-Perlat *et al.*, 2004a). In fact, the proteolytic enzymes are mainly produced on cheese rind and do not diffuse to the core (Leclercq-Perlat *et al.*, 2004a; Noomen, 1983) whereas, ammonia and lactate diffusion have been suggested. Both phenomena are known to contribute to a pH gradient between the

surface and core of cheese curd. As mentioned above, *G. candidum* governs surface pH change (Boutrou *et al.*, 2006b). When comparing the surface pH of CTRAD and CSTAB there was no significant difference. This may be explained by similar *G. candidum* cell counts (Table 8 Annex). However the pH at the core of CTRAD was higher than that of CSTAB. This may be attributed to the higher residual lactose that is retained in the unwashed traditional cheese curd (Walstra *et al.*, 2005). Lactose is transformed into lactate by the activity of lactic acid bacteria. Thereafter, the core pH of CTRAD may reach higher values than CSTAB after the lactate is consumed by the fungal community. The differences in buffering capacity (Data not shown) may also explain the differences of the core pH between both cheeses.

2. Camembert Cheese texture

Results showed no significant difference between CTRAD and CSTAB texture parameters (Table 2-3). A different textural behavior was observed in one of our CTRAD cheese batches which was softer and did not allow textural measurements with the current method (data not shown). It was considered as a missing data in texture statistical analysis. It is to note that this sample behaved similarly as the other batches during *in vitro* digestion.

The texture attributes of CTRAD and CSTAB were similar and did not reflect the differences observed between the two cheeses in moisture content, FDM, PDM and proteolysis. Possibly, the high fat content of CSTAB compensated the low proteolysis and high ash content. The similar textural behavior of CSTAB might be attributed to an increased softening of its matrix to reach similar properties of CTRAD. This might be related to a higher Ca^{2+} migration possible due to a lower pH at the core of the cheese. It was reported to increase the softness of stabilized Camembert cheese matrix (Fox *et al.*, 2004; Spinnler and Gripon, 2004). Moreover, Gunasekaran and Ak (2002) reported from Lelievre and Gilles (1982) that the MNFS is an important factor affecting cheese rheological properties. Different MNFS can result in differences in textural quality (Olson and Johnson, 1990). Walstra *et al.* (2005), reported that for the same type of cheese, the one with higher FDM has a higher MNFS, and so the lower modulus E (i.e. hardness). Even though, this factor was not controlled in our study, compositional results showed different FDM percentages but equal MNFS between CTRAD and CSTAB. This might explain the similar textural parameters as suggested by (Lawrence *et al.*, 1987; Lucey *et al.*, 2003).

Table: 2-3 Texture profile Analysis of Camembert cheese (CTRAD; CSTAB) at room temperature

Cheese	Hardness	Cohesiveness	Gumminess	Resilience	Adhesiveness
	N		J		J
CTRAD	1,49 ± 0,67[a]	0,60 ± 0,14[a]	0,84 ± 0,28[a]	0,19 ± 0,05[a]	(-) 0,31 ± 0,07[a]
CSTAB	1,51 ± 0,34[a]	0,52 ± 0,06[a]	0,78 ± 0,2[a]	0,17 ± 0,034[a]	(-) 0,22 ± 0,15[a]

Values are means ± standard deviation of each analyses (*n* =4 CSTAB; n=3 CTRAD). [a,] Means in column with different letters are significantly different by LSD 's test ($p < 0.05$).

3. Dairy matrices behavior during *in vitro* digestion

The disintegration of CM, Milk, CSTAB and CTRAD at the end of each digestion step is presented in Figure 2-1. The digestion process involves addition of digestive fluids under controlled shearing conditions allowing to compare the behavior of these different matrices. Liquid matrices and solid matrices showed different behaviors.

Both liquid matrices were almost completely soluble at the end of the oral and gastric step. After 120 min of gastric digestion, the percentages of CM and Milk degradation were 99.78% and 99.01%, respectively. Ye *et al.* (2016b) also reported the complete sample solubilization by hydrolysis of casein and whey proteins of heated whole fat milk by pepsin after 220 min of gastric digestion. A small but significant difference between CM and Milk arose at the end of the duodenal digestion step. Milk disintegration decreased from 99.01% to 97.08% ± 0.67 between gastric and duodenal steps. Proteins should be completely digested at the end of the duodenal step. However, the calcium content of milk allows the interactions with free fatty acids available after fat hydrolysis by duodenal lipase. The formation of insoluble calcium fatty-acid soaps has been reported for cheeses using similar in vitro model (Ayala-Bribiesca *et al.*, 2016; Lamothe *et al.*, 2012)

After the oral step, cheeses disintegration was lower than liquid matrices with values near 50%. Digestion pH changes and enzymes actions contribute to cheeses degradation and at the end of the duodenal digestion more than 90% of the matrices is disintegrated. Although the physicochemical and textural properties of both CTRAD and CSTAB only showed slight differences (Section 1, 2), both cheeses displayed a small but significant difference in disintegration during *in vitro* gastrointestinal digestion (Figure 2-1).

Due to the semi-solid texture of Camembert cheese, the simulated mastication resulted in a kind of creamy paste after 2 min of oral digestion. At this step, the disintegration of CTRAD and CSTAB was significantly different at

49.44% and 53.61%, respectively. When gastric juice was added to the oral bolus, CSTAB displayed a slightly higher degradation percentage than CTRAD. At the end of gastric digestion CSTAB disintegration increased by 23.5% while CTRAD rose by 13.88% (Figure 2-1). Finally, the addition of the duodenal juice to the chyme resulted in a similar increase of disintegration of both cheeses averaging 22%. The final disintegration values were of 90% and 95%, CSTAB still slightly higher than CTRAD by about 5%. The composition and physicochemical properties of both cheese curds might explain their different behavior during digestion. First, the fat content of cheeses is different with a lower fat content for CTRAD compared to CSTAB (24.7 vs 30.19% respectively (Table 2-1). Fat contributes to disrupting the continuous casein matrix and may favor cheese disintegration. Many studies showed the significant impact of structure and fat content on the digestibility of different food matrices and some nutrients release such as protein as reviewed by (Boland *et al.*, 2014). During digestion, fat can melt at physiological temperature and be released from the casein matrix causing gaps that facilitate enzyme access and solubilization of the cheese matrix. Furthermore, duodenum conditions with free fatty acid release by the action of lipase is known to favor lipid-calcium interaction to produce insoluble fatty acid calcium salts (Hu *et al.*, 2010). The higher mineral content of CTRAD (Table 2-1), may have contributed to increase insoluble fat due to interaction with calcium and may explain its lower disintegration.

Another difference in cheese composition which may influence disintegration is the proteolysis extent of each cheese matrix before digestion. The proteolysis degree of CTRAD is higher than CSTAB (Table 2-2). Peptides have been reported to be adsorbed more easily on the lipid interface than intact proteins and may delay lipid hydrolysis and release (Fardet *et al.*, 2013). This could contribute to delay in CTRAD disintegration. The viable fungal flora has been also reported to promote the breakdown of Camembert cheese matrix during ripening in terms of lipolysis and proteolysis (Dugat-Bony *et al.*, 2015; Lessard *et al.*, 2014). *G. candidum* governs the proteolysis and the lipolysis of soft-cheese matrix. In our study, both cheeses showed similar *G. candidum* LMA-1028 viable cells count (Table 2-4), and cannot account for differences in proteolysis and disintegration.

Texture is also a factor to consider as it reveals the cheese matrix organization. However, both cheeses showed similar textural properties. Finally, due to differences in the cheesemaking process, the cheeses had different core pH values ranged from pH 5.96 (CSTRAD) to 5.53 (CSTAB). A difference in pH may reveal a different protein matrix structure (density, etc.). A difference in structure between an acidic cheese (pH 4.6) and a stabilized cheese (pH > 5.2) has been reported, the former having a more compact protein matrix (Fox, 2000; Lawrence *et al.*, 1987; Lucey *et al.*, 1996). Considering the small difference in pH values between CSTAB and CTRAD and the similar textural properties, the results are consistent with the small difference of disintegration values observed in this study.

The difference in fat content is probably the most influential factor influencing cheese disintegration. CSTAB displayed higher fat content and is the most disintegrated. A high fat content is associated with a looser casein matrix which is more easily accessible to the enzymes and consequently more solubilized. Hence, a dense casein network is expected to be more resistant to gastric pepsin (Boland *et al.*, 2014). The higher protein content of CTRAD may give a denser protein matrix which may have limited pepsin proteolysis at gastric acidic pH. These observations have been supported by a recent study showing that pepsin diffusion in rennet gel depends on casein concentration and microstructure (Thévenot *et al.*, 2017). The next section will look into the comparison of the repartition of digested samples after centrifugation and may contribute to better understand the origin of these differences.

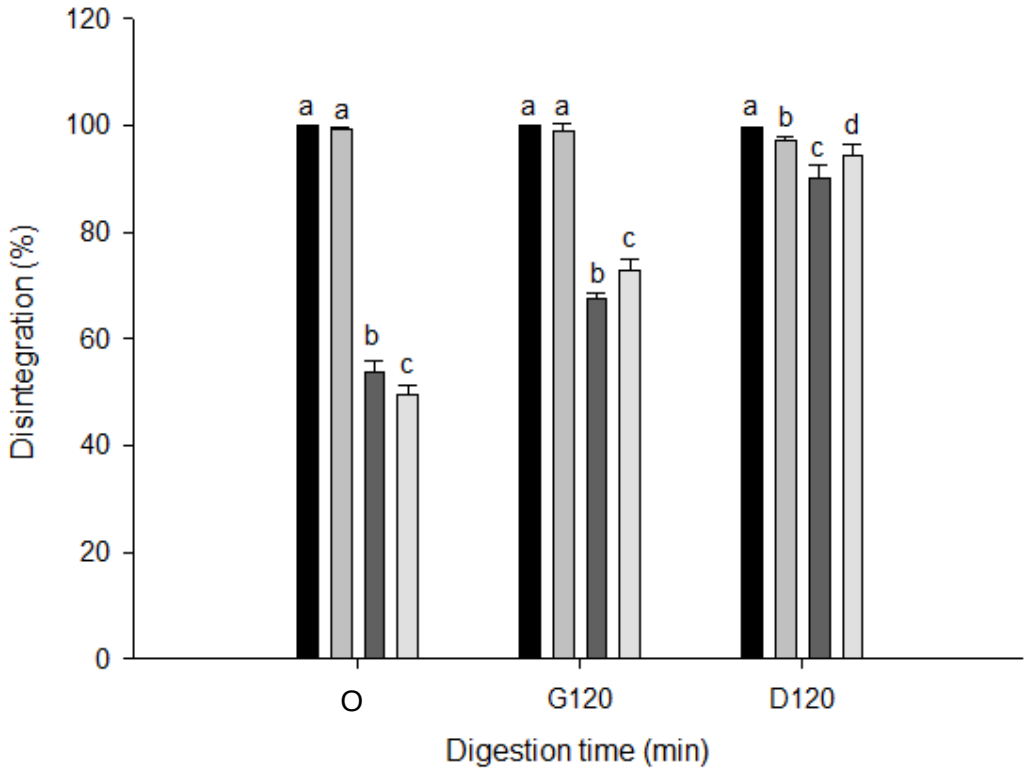

Figure 2-1: Disintegration rate of CM (■), Milk (■), CTRAD (■) and CSTAB (■) during static in vitro digestion. [ab] denotes significant differences ($p < 0.05$) between disintegration of each matrix at the same period of in vitro digestion.

The repartition of each matrix into three distinct layers after centrifugation of samples at the end of oral, gastric and duodenal steps during digestion is presented in Figure 2-2. A pellet layer was found at the bottom of the tube, a fat layer on the top and in between a liquid phase containing water-soluble components.

3.1. Liquid matrices

Upon oral digestion of CM and Milk, the pellet layers were very small, compared to those of the cheese matrices. No difference was observed between CM and Milk pellet layers (Figure 2-2). This is consistent with the high disintegration of liquid matrices (Figure 2-2). On the contrary, a difference in the amounts of fat and liquid layers was observed. Fat value for CM is near zero while it was 2.78% ± 0.37 for milk. This is, obviously, attributed to the fat content of Milk (Table 2-1). During oral stage at physiological temperature (37 °C) and at neutral pH (6.93), milk fat is in a liquid state and is easily separated through centrifugation to obtain a fat layer. The amount of the fat layer of the Milk matrix was reduced from 2.78% ±0.37 to 1.39% ±0.47 at the end of gastric digestion. This cannot be due to the lipolysis of triglycerides since no gastric lipase was added in our simulated gastric juice as there is no lipase available commercially. However, the gastric digestion including protein hydrolysis by pepsin and acidic pH favored coalescence of fat and its entrapment within a protein matrix (Lopez, 2011; Michalski and Januel, 2006). Proteins and peptides may cover milk fat globules and cause its entrapment in a casein network limiting fat separation during centrifugation (Fardet *et al.*, 2013; Ye *et al.*, 2016b). Our results are in agreement with those reported by (Ye *et al.*, 2016b). They quantified the release of fat from heated whole milk during 220 min of gastric digestion. They observed a decrease of fat release and an increase of the entrapment of the coalesced fat within the clotted protein matrix as a function of digestion time. They hypothesized that this may be due to the presence of peptides at the surface of the fat globule. This is in accordance with our results and explains the reduction of released fat between the oral and gastric steps in our study where only part of the fat can be isolated by centrifugation while some are being trapped in the pellet.

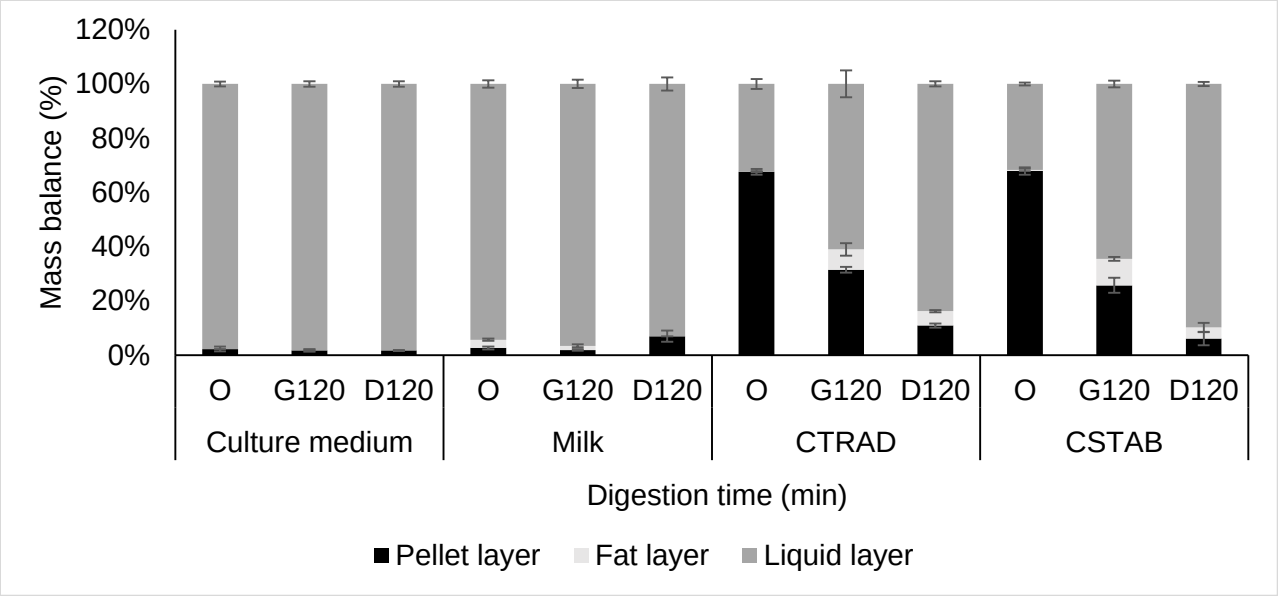

Figure 2-2: Mass balance of pellet (■), fat (■) and liquid (■) layer through in vitro gastrointestinal digestion of CM, Milk, CTRAD and CSTAB.

When duodenal juice (*i.e.* bile salts, lipases, trypsin and chymotrypsin) was added, a difference between the amount of pellet layers of CM and Milk was observed (Annex-2; Annex-3). The quantity of insoluble components of Milk increased by about fivefold from 1.74% ± 0.26 at end of gastric digestion to 6.48% ±1.91 at the end of duodenal phase. This corresponds to the observed decrease in disintegration (Figure 2-2). In literature, this phenomenon was also observed for whole and partially skimmed milk samples and was attributed to changes in the composition and the physicochemical properties of the fat globule interface (Berton *et al.*, 2012; Singh and Sarkar, 2011; Singh and Ye, 2013). This has been previously argued by Devraj *et al.* (2013); Hu *et al.* (2010), our result is consistent with their observations and may be explained by fat milk globules properties. Further details about the phenomena will be discussed in the section 4.3.

3.2. Solid matrices

Both cheeses showed a similar behavior after oral digestion (Figure 2-2) but when exposed to gastric juice differences were observed between layers heights. The fat layer of CSTAB is higher than CTRAD which may be explained by the difference in the initial fat content of each matrix (Table 2-1). As discussed previously, the denser casein network of CTRAD may have delayed the release of fat globules (Thévenot *et al.*, 2017) and it would also

require a longer exposure to gastric enzymes to reach the same level of disintegration (Figure 2-1). Since CSTAB contains more fat than CTRAD, the cheese matrix is expected to be less compacted and interrupted by more fat pools. Thus, following lipid diffusion out of the cheese matrix, the accessibility of pepsin to the casein matrix would be increased enhancing the degradation of CSTAB matrix. Correspondingly, the mass balance of CTRAD pellet was higher at the end of gastric digestion suggesting a lower disintegration rate of its matrix compared to CSTAB. Lastly, CSTAB exhibited the lowest pellet and fat layer mass balances during duodenal digestion corresponding to a higher disintegration (Figure 2-2), while CTRAD displayed a higher fat layer. As mentioned previously several factors linked to the composition and physicochemical properties are involved in this behavior (section 3).

3.3. Liquid vs solid matrices

In this section, the mass balance of the three layers will be compared between Milk and a cheese matrix. CSTAB will serve for this comparison. In general, a difference was observed between the layers (pellet, fat and liquid) of Milk and CSTAB after each digestion step (Annex-2). The proportion of digested sample that was found in the pellet layer was lower for Milk than for cheese (CSTAB) which was expected due to the lower solid content of milk. The liquid state of milk also facilitated a fast disintegration of the Milk matrix compared to a gelled matrix as a yoghurt (Rinaldi *et al.*, 2014). Besides, the amounts of the fat layer upon oral and gastric digestion of Milk and CSTAB were different. As observed in Figure 2-2, Milk showed higher fat release than CSTAB after 2 min of oral digestion. Inversely CSTAB displayed the higher fat release at the end of the gastric step. This can be explained by the fat content of each ingested matrix, their characteristics (size of fat globules, milk fat globule membrane structure) and the structure of the casein matrix within both dairy matrices and how it changes upon digestion conditions (Singh and Gallier, 2016). In fact, as oppose to Milk which may release fat easily, when a semi-solid matrix as CSTAB was exposed to oral juice at physiological temperature, the fat remained entrapped within the casein matrix.

During duodenal digestion, differences were observed between the amount of fat layers of Milk and CSTAB. There is no fat layer at the end of Milk digestion (Figure 2-2). According to previous studies, lipolysis is restrained by the end of duodenal digestion due to the reduction of available substrates and the accumulation of long-chain FFAs at the fat globule membrane (Liang *et al.*, 2017; Porter *et al.*, 2007) . Free fatty acids can be either found in the liquid or in the pellet layer. Free fatty acids are soluble but long chain saturated fatty acids may interact with calcium to form insoluble calcium-free fatty acids soap in the duodenal sample (Hu *et al.*, 2010). These insoluble fatty acid soaps will be found in the pellet layer at D120. It is to note that, the exact proportion of lipid in each fat layer has not been measured in this study.

In summary, the disintegration of three dairy matrices at the end of digestion was nearly 100%. Although the semi-solid structure of Camembert cheese delayed nutrients release, the complete disintegration makes the nutrients of these two differently structured dairy matrices similarly bioaccessible within 240 min of gastrointestinal transit. The overall disintegration is driven by the liquid or solid state of the matrix, its composition and physicochemical properties during digestion and interactions between their components. The fast disintegration of milk might be useful to increase the bioaccessibility of bioactive substances once their resistance to digestive stresses is confirmed. Noteworthy, the slow disintegration of Camembert cheese may reduce the degradation of bioactive ingredients by digestive enzymes or acidic pH. It also can promote the protection of probiotic micro-organisms or beneficial intrinsic microflora to remain viable and active through the gastrointestinal stress. This will be presented next as the viability of *G. candidum* LMA-1028 during *in vitro* digestion of Milk and traditional and stabilized Camembert cheese matrices have been investigated.

4. Survival of *Geotrichum candidum* to simulated gastro-intestinal stress

The comparison of the *G. candidum* strain LMA-1028 counts on traditional and stabilized cheese showed similar results indicating a similar level of development during cheese ripening; 5.58 (CTRAD) and 6.11 (CSTAB) log $CFU.g^{-1}$ Table 2-4. Overall, there was no significant difference in the loss of viability between oral and subsequent gastric (G60, G120), and duodenal (D60, D120) steps. After digestion, it was found that *G. candidum* LMA-1028 in the culture medium didn't show any significant loss of viability. Indeed, only 0.02 log of *G. candidum* LMA-1028 were lost during the gastric phase while no significant decrease was noted after the duodenal phase, either.

2-4: Viability of *G. candidum* LMA-1028 during *in vitro* digestion (viable cells count CFU g-1 of matrix)1

Matrix			Viable count		Loss of viability (G120)	Viable count		Loss of viability (D120)
	Initial count	O	G60	G120		D60	D120	
CM	6.0	6.33 ± 0.12	6.27 ± 0.11	6.25 ± 0.11	0.08	6.29 ± 0.13	6.26 ± 0.12	0.03
Milk	6.0	6.56 ± 0.06	6.50 ± 0.06	6.44 ± 0.06	0.11	6.50 ± 0.11	6.46 ± 0.15	0.09
CTRAD	5.5 ±0.38	5.95 ± 0.55	5.93 ± 0.57	5.88 ± 0.62	0.07	5.87 ± 0.60	5.87 ± 0.54	0.07
CSTAB	6.1 ±0.41	6.06 ± 0.36	6.01 ± 0.42	5.99 ± 0.37	0.06	5.98 ± 0.41	5.89 ± 0.41	0.16

1) Values are means ± standard deviation of (n=4).

Because of this intrinsic resistance of *G. candidum* LMA-1028 to digestion stresses, the experiments did not permit to validate the hypothesis and to demonstrate a protective effect of the cheese matrix and counts remained the same independently of the matrix used. This result is in accordance with previous studies that evaluated the survival of cheese microflora during gastrointestinal transit. Authors reported specific high resistance of several dairy yeasts (Diosma *et al.*, 2014; Hatoum *et al.*, 2012; Kumura *et al.*, 2004), such as *G. candidum* (Adouard *et al.*, 2015a; Adouard *et al.*, 2015b; Firmesse *et al.*, 2008; Lay *et al.*, 2004). A recent study of Adouard *et al.* (2015a) determined the viability of smear-ripened cheese microbiota and their digestive stress response. Authors showed that *G. candidum* strains have high resistance to the simulated gastrointestinal stress. In a further work Adouard *et al.* (2015b) studied the viability of smear-ripened cheese's isolated *G. candidum* strain ATCC 204307 that has grown into two carriers (pure culture: Brain Heart Infusion and surface ripened-cheese model: Livarôt). For cheese matrix, a decrease of viability by 2.0 and 2.8 log $CFU.mL^{-1}$ was observed after gastric and duodenal digestion, respectively. While for the pure culture the viability remained unchanged during all digestion time. The obvious differences in the rate of *G. candidum* ATCC 204307 viability in both matrices compared to our results arise either from the yeast strain properties, the *in vitro* digestion model or the different matrices characteristics. In order to determine which parameter induced the differences served, it would be interesting to compare both strains in each cheese matrix using a common digestion model.

Conclusion

Overall, our study allowed a better understanding of the factors influencing dairy food matrix disintegration. This seems to be determined, at least partly, by the composition and structure of dairy matrices. The structure of the casein networks of Milk and Camembert cheeses appears to modulate the accessibility of digestive juice to these matrices during gastric digestion and, possibly, the bioaccessibility of their nutrients. Similarly, the fat content seems to have an impact on the rate of disintegration of these matrices during gastric and duodenal digestion. The difference in the original structure of both Camembert cheese matrices, as modulated during manufacturing, led to different rates of gastric disintegration and different proportions of separated layers of fat. Noteworthy, the difference in Camembert cheese manufacturing resulted in two different curds with relatively slight different physicochemical properties and digestibility rates. This is appealing for further understanding of the effect of processing stages on the digestibility of the several cheese categories. The study of the synergy between casein matrix and fat globules within dairy matrices is also of potential importance to enable an effective nutrients bioavailability, thus efficient food designing. The fast and complete gastric disintegration of milk renders its nutrients available. The low gastric disintegration of Camembert cheeses is relevant for the protection of probiotic microorganisms against gastric acid and peptic activity stresses. However, it should be emphasized that a complete duodenal digestion showed that all the nutrients will be released at the end of the digestion and are expected to be bioavailable.

The viability of *G. candidum* LMA-1028 during function of the disintegration of dairy matrices was stable. Interestingly, Camembert cheese matrix did not contribute to the protection of this strain through gastrointestinal stress. *G. candidum* LMA-1028 was resistant to digestive stresses independently of matrices properties. The high resistance of *G. candidum* LMA-1028, in our study, is particular among fungal and bacterial species, but similar to other *G. candidum* strains.

General conclusion

The main objective of this thesis was to investigate the effect of cheese matrix on the viability of *G. candidum* LMA-1028 during the *in vitro* digestion of Canadian Camembert-type cheeses. The hypothesis stated that the structural and physicochemical properties of cheese matrix may contribute to the protection of *G. candidum* strains against digestive stresses. Our experimental approach started by the physicochemical and textural characterization of three industrial dairy matrices differing by their structure (liquid *vs* semi-solid), composition (fat and protein contents) and their proteolysis degree (i.e. whole milk (3.25 % fat content), traditional and stabilized Camembert-type cheese). Afterward, the impact of matrix properties on its disintegration was determined at the end of oral, gastric and duodenal steps. Eventually, regarding the slow disintegration of cheese matrix compared to milk and control culture medium, we evaluated the viability of *G. candidum* LMA-1028 through gastric acid and bile salts stresses in order to emphasize a potential additional protection provided by the dense matrix of Camembert cheese.

Disintegration of milk and Camembert cheese matrices during *in vitro* digestion

In order to evaluate the digestibility of milk and Camembert cheese matrices, we determined the disintegration percentage and the mass balance of three layers, separated through centrifugation, which are fat, pellet and liquid layers. This has been performed after oral, gastric and duodenal steps in order to understand differences between the disintegration of the different matrices. The proportion of the separated layer of fat depended on the composition of the dairy matrix. The separation of the fat layer from a milk matrix was faster than from Camembert cheeses due to its liquid behavior. Inversely, the fat layer increased gradually during gastric and duodenal digestion of cheese, suggesting a slow release of fat from the semi-solid matrix. This has been attributed to the coagulated casein matrix in cheese, entrapping fat globules within its network, which appears to be degraded gradually by pepsin and pancreatic proteases activity. Our study, therefore, showed the major role of the structure of casein network to modulate the matrix disintegration of dairy matrices. The comparison of differently-manufactured Camembert-type cheese with different moisture, fat and protein contents allowed us to better identify the factors that affect the disintegration of cheese matrix. Although, traditional and stabilized Camembert cheese didn't show any significant difference in their textural attributes, the stabilized cheese matrix displayed more rapid and higher fat layer after gastric and duodenal steps. Our results suggest that the microstructure of the cheese matrix controls the kinetics of disintegration consequently may modulate some nutrients release. In fact, we propose that the higher protein concentration in traditional Camembert may hinder the access of pepsin and pancreatic proteases, thus slightly delayed the disintegration of cheese matrix. We observed that the high fat content of stabilized Camembert promoted the disintegration of its matrix, probably due to a disruptive role of fat interrupting the continuous casein matrix. As for mineral content or protein concentration, the increase of fat within cheese or milk matrix influences the disintegration

and possibly the bioaccessibility of these matrices nutrients. A further investigation of the synergy between casein network and fat globule within differently processed dairy product, will enable an effective nutrients bioavailability, thus efficient dairy food designing. The low gastric disintegration of Camembert cheese matrix reinforce its usefulness for the protection of bioactive elements or probiotic microorganisms sensitive to gastric stress.

Viability of *G. candidum* LMA-1028 during *in vitro* digestion of milk and Camembert cheese matrices

The low gastric disintegration of Camembert cheeses raises the question whether this feature will promote the viability of *G. candidum* LMA-1028 through simulated gastrointestinal transit compared to milk and culture medium. Our study investigated the fate of *G. candidum* LMA-1028 of Canadian Camembert-type cheese during *in vitro* digestion function of the disintegration of Milk, traditional and stabilized Camembert cheeses. Contrarily to our hypothesis, it was not possible to differentiate the contribution of a Camembert cheese matrix to the protection of this strain through gastrointestinal stress. *G. candidum* LMA-1028 was resistant to digestive stresses independently of matrices properties. The high resistance of *G. candidum* LMA-1028, in our study, is particular among fungal and bacterial species, but similar to other *G. candidum* strains. Additional studies should be conducted to understand the contribution of *G. candidum* during its transit in the gastrointestinal tract.

Annex

Annex-1: Typical parameter settings during static in vitro digestion

Parameter /Matrix	CM	Milk	CTRAD	CSTAB
		Common attributes		
Texture	Liquid	Liquid	Semi-solid	Semi-solid
Meal size (g)	9	9	9	9
Digestion temperature (°C)	37	37	37	37
		Oral digestion		
Volume (mL)	6	6	6	6
Duration (min)	2	2	2	2
Stirring agitation (rpm)	75	75	75	75
		Gastric digestion		
Volume (mL)	(60 min: 16.5); (120 min: 18.0)	(60 min: 16.5); (120 min:18.0)	(60 min: 16.5); (120 min: 18.0)	(60 min: 16.5); (120 min: 18.0)
HCl addition (time: min, volume: mL)	(min 0, 0.019)	(min 0, 0.057)	(min 0, 0.15); (60 min, 0.10)	(min 0, 0.155); (60 min, 0.085)
Duration (min)	120	120	120	120
Orbital agitation (rpm)	200	200	200	200
		Duodenal digestion		
Volume (mL)	38	38	38	38
Bicarbonate 1.0 M addition (volume or/and weight)	(2.0 mL, 0.0 g)	(1.0 mL, 0.0 g)	(2.0 mL, 0.3 g)	(2.0 mL, 0.35 g)
Duration (min)	120	120	120	120
Orbital agitation (rpm)	200	200	200	200

Annex-2: Different letters in a column (A, B, C, D); indicate significant difference of the same layer between different matrices at the same step.

Matrix\ Layer	**Mass balance of fat**			**Mass balance of pellet**			**Mass balance of liquid**		
	O	**G120**	**D120**	**O**	**G120**	**D120**	**O**	**G120**	**D120**
CM	B	C	B	B	C	C	A	A	A
Milk	A	B	A	B	C	B	B	A	B
CTRAD	B	B	A	A	A	A	C	B	C
CSTAB	B	A	B	A	B	B	C	B	BC

Annex-3: Different letters in a row (a, b, c, d); indicate significant difference of the same layer of the same matrix between different steps.

Matrix\ Layer	**Mass balance of fat**			**Mass balance of pellet**			**Mass balance of liquid**		
	O	**G120**	**D120**	**O**	**G120**	**D120**	**O**	**G120**	**D120**
CM	A	A	a	A	a	A	a	a	A
Milk	A	B	c	B	c	A	a	a	A
CTRAD	C	A	b	A	b	C	c	b	A
CSTAB	c	A	b	a	b	C	c	b	A

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