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# Novel insights into starch digestion and the glycaemic response: from *in vitro* digestions to a human study using magnetic resonance imaging (MRI)

Thèse de doctorat de l'Université Paris-Saclay préparée à  
AgroParisTech (l'Institut des sciences et industries du vivant et de  
l'environnement)

École doctorale n°581 Agriculture Alimentation Biologie  
Environnement Santé (ABIES)  
Spécialité de doctorat: Sciences de la Nutrition

*Thèse soutenue à Paris le 21 Novembre 2018.*

**DA SILVA ROSA FREITAS, Daniela**

Composition du Jury :

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M. Furio Brighenti Professor, Università di Parma (Dipartimento di Scienze degli Alimenti e del Farmaco)	Rapporteur
M. Luca Marciani Associate Professor, University of Nottingham (Faculty of Medicine and Health Sciences)	Rapporteur
Mme Valérie Micard Professor, Montpellier SupAgro (UMR IATE – Ingénierie des Agro-polymères et Technologies Émergentes)	Rapporteur
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M. François Boué Directeur de Recherche, CNRS/INRA (UMR GMPA – Génie et Microbiologie des Procédés Alimentaires)	Directeur de thèse
M. Steven Le Feunteun Chargé de Recherche, INRA (UMR GMPA – Génie et Microbiologie des Procédés Alimentaires)	Co-encadrant



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## LIST OF PUBLICATIONS, PRESENTATIONS, CLINICAL RESEARCH PROTOCOL AND GRANT PROPOSALS PREPARED DURING THE PHD

### Peer reviewed articles

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1. Freitas, D., & Le Feunteun, S. (2018). Acid induced reduction of the glycaemic response to starch-rich foods: The salivary  $\alpha$ -amylase inhibition hypothesis. *Food & Function*.
2. Freitas, D., & Le Feunteun, S. (2019). Oro-gastro-intestinal digestion of starch in white bread, wheat-based and gluten-free pasta: Unveiling the contribution of human salivary  $\alpha$ -amylase. *Food Chemistry*, 274, 566-573.
3. Freitas, D., Le Feunteun, S., Panouillé, M., & Souchon, I. (2018). The important role of salivary  $\alpha$ -amylase in the gastric digestion of wheat bread starch. *Food & Function*.
4. Bohn T., Carriere F., Day L., Deglaire A., Egger L., Freitas D., Golding M., Le Feunteun S., Macierzanka A., Menard O., Miralles B., Moscovici A., Portmann R., Recio I., Remond D., Sante- Lhoutelier V., Wooster T.J., Lesmes U., Mackie A.R., Dupont D. (2017) Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static *in vitro* digestion models? *Critical Reviews in Food Science and Nutrition*.  
*Collaboration with Steven Le Feunteun with section "1. Estimation of the digestion of starch" (the submitted version is provided in annex 1)*

### Posters and oral presentations

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1. **Freitas, D.**, Le Feunteun, S., Lutton, E., Benamouzig, Jourdan, L., R., Darrasse, L., Maître, X., Boué, F., (2017) Monitoring the digestion of starch-rich meals by Magnetic Resonance Imaging and their glycaemic response. Seminar on the Non-invasive exploration and imaging: Gut – brain axis Dijon, France (2017-11-13 – 2017-11-14). [Oral conference](#)
2. **Freitas D.**, Lutton E., Panouillé M., Souchon I., Boué F., Le Feunteun S. (2017). How can the glycaemic response to starchy foods be improved? The forgotten role of salivary alpha-amylase. Keystone Symposia on Molecular and Cellular Biology - Gastrointestinal Control of Metabolism (Z6), Copenhagen, Denmark (2017-05-09 – 2017-05-13). [Poster](#)
3. **Freitas D.**, Lutton E., Panouillé M., Souchon I., Boué F., Le Feunteun S. (2017). The forgotten role of salivary alpha-amylase on starch digestion: a dynamic *in vitro* study. 5th International Conference on Food Digestion, Rennes, France (2017-04-04 – 2017-04-06). [Poster – 3<sup>rd</sup> place in the poster competition for PhD students](#)

### Clinical research protocol

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1. **DECOUVRIR-M** - Digestion: CONstruire une meilleure santé et mieux comprendre les processus digestifs grâce à l'Imagerie par Résonance Magnétique. Submitted for evaluation on July 2017, ethical clearance obtained on November 2017 (approximately 103 pages).

## Grant proposals Funded applications

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1. ABIES international mobility program – [Funded mobility grant](#) for a 3-month research visit at the University of Copenhagen (2016)
2. Application of Magnetic Resonance Imaging to the understanding of model gels' digestion: unraveling opportunities for improving nutrition through controlled nutrient release. [Funded PhD grant](#) - IDEX (Excellence Initiative) Paris-Saclay (2015)

## Other applications

1. Project : DéCouvriR-M - Digestion: CONstruire une meilleure santé et mieux comprendre les processus digestifs grâce à l'Imagerie par Résonance Magnétique. Competition: L'Oréal-UNESCO For Women in Science Awards (2016), selected in the [Top 100 out of 1000 applications](#)
2. Project: Food for health: how to build effective solutions to optimize physiological responses to carbohydrate-rich foods and preserve our wellbeing? Competition: BCFN YES! Application (2016)
3. DéCouvriR-M - Digestion: CONstruire une meilleure santé et mieux comprendre les processus digestifs grâce à l'Imagerie par Résonance Magnétique. Competition : Bourses de recherche de la Fondation Nestlé France (2017)

## Résumé en Français

### PRÉSENTATION DU PROJET

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La nutrition est un phénomène complexe pour lequel non seulement le type et la quantité de macro-/micro- nutriments, mais aussi le taux auquel ces nutriments sont apportés à l'organisme ont été considérés comme des facteurs importants, en particulier dans le cas des glucides. L'importance du taux de digestion pose un nouveau défi : développer des stratégies de promotion de la santé pour atteindre des régimes alimentaires équilibrés sur le plan nutritionnel, mais ayant aussi des profils de digestion adéquats. En parallèle, ce défi ouvre de nouvelles opportunités au niveau scientifique et a été le point de départ de mon projet de thèse. Ce projet pluridisciplinaire associe des compétences dans les domaines des sciences de l'alimentation, de la nutrition, de la santé et de l'imagerie médicale, dans le cadre d'un travail conjoint entre deux groupes de recherche français : (1) GMPA, unité mixte de recherche d'AgroParisTech et de l'INRA (Grignon), et (2) IR4M, unité mixte de recherche de l'Université Paris Sud et du CNRS (Orsay). Au total, 5 études ont été menées (4 études *in vitro* et 1 étude à deux branches chez l'homme). Trois de ces études ont déjà été publiées dans des revues à comité de lecture.

### ÉTAT DE L'ART

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Nous passons tous plus des trois quarts de notre vie dans l'état postprandial (Vors, Nazare, Michalski, & Laville, 2014). Cependant, si le lien entre le métabolisme à jeun et la santé a fait l'objet d'études approfondies, moins d'attention a été accordée à l'étude de l'état postprandial. Bien que les recherches sur l'état postprandial attirent de plus en plus l'attention de la communauté scientifique depuis les années 1970, au moment de la rédaction de ce manuscrit, il existe encore deux fois plus d'articles traitant de sujets liés au métabolisme à jeun. L'état postprandial est un état dynamique résultant de l'ingestion et de la digestion d'un repas qui dure de 4 à 5 heures selon le type de nutriment. Il se distingue de l'état post-absorptif (qui correspond à la période de 6h suivant l'état postprandial) et de l'état à jeun (qui suit l'état post-absorptif, 10-12 heures après le dernier repas). Ces états se succèdent et peuvent aussi se superposer (Vors, Nazare, Michalski, & Laville, 2014).

Dans notre société actuelle, la consommation de 3 à 4 repas par jour signifie que nous quittons rarement l'état postprandial (Vors, Nazare, Michalski, & Laville, 2014). De plus, comme conséquence des déséquilibres entre besoins nutritionnels et la consommation d'aliments,

l'alimentation est le deuxième facteur de risque de décès au niveau mondial (Gakidou, et al., 2017). Il est également devenu clair que non seulement le type et la quantité de nutriments ingérés sont importants, mais aussi leurs taux de digestion et d'absorption. Ceci est valable pour les protéines, par exemple, car leur résistance à la digestion et leur taux de digestion peuvent influencer les mécanismes qui déclenchent les allergies alimentaires (Bøgh & Madsen, 2016) et l'efficacité du métabolisme protéique post-prandial (Dangin, Boirie, Guillet, & Beaufrère, 2002; Koopman, et al., 2009; Walrand, et al., 2016). Il en va de même pour les glucides, avec de nombreux travaux qui mettent en évidence un lien entre l'augmentation du taux de glucose postprandial et un risque accru de diabète de type 2 (Bhupathiraju, et al., 2014), les maladies cardiovasculaires chez les femmes (Dong, Zhang, Wang, & Qin, 2012; Mirrahimi, et al., 2012), le cancer du sein (Mullie, Koechlin, Boniol, Autier, & Boyle, 2016), ainsi que les complications liées à l'obésité (Schwingshackl & Hoffmann, 2013).

Les glucides jouent un rôle clé dans l'alimentation humaine, fournissant jusqu'à 45% des besoins énergétiques dans les pays développés et jusqu'à 85% dans les pays en développement. On les trouve sous forme de sucres (monosaccharides et disaccharides) et polysaccharides (amidon ou polysaccharides non-amylacés) (Stylianopoulos, 2012). L'amidon est un glucide clé dans l'alimentation humaine. Comme l'amidon est exclusivement composé de résidus de glucose et représente 20 % à 50 % de l'apport énergétique total (Stylianopoulos, 2012), le glucose dérivé de l'amidon est un déterminant majeur des concentrations sanguines postprandiales de glucose (glycémie postprandiale) suscitées par nos régimes alimentaires. Il existe des évidences scientifiques qui démontrent que la prise en compte de l'impact glycémique des aliments consommés, plutôt que seulement leur teneur en glucides, est un élément essentiel pour atteindre un régime alimentaire optimal pour la santé (Blaak et al., 2012; Livesey, Taylor, Hulshof, & Howlett, 2008). Des exemples concrets sont fournis dans des études récentes soulignant l'impact bénéfique de la réduction de la réponse glycémique sur la diminution du risque (Miao, Jiang, Cui, Zhang, & Jin, 2013) et l'amélioration du contrôle (Russell et al., 2016) du diabète de type 2, son potentiel à promouvoir la satiété (Miao et al., 2013) et éventuellement la perte de poids (Juanola-Falgarona et al., 2014). Le taux d'hydrolyse de l'amidon pendant la digestion semble avoir un impact prédominant sur la réponse glycémique provoquée par les aliments riches en amidon. En effet, la relation positive entre la vitesse à laquelle l'amidon des aliments est hydrolysé dans des conditions contrôlées *in vitro* et le degré auquel il augmente la glycémie a été établie plusieurs fois (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Granfeldt,

Hagander, & Björck, 1995; Jenkins et al., 1982). Toutefois, le processus de digestion de l'amidon reste à bien comprendre car la contribution de chaque phase digestive n'est pas claire.

Deux enzymes clés participent à la digestion de l'amidon : l' $\alpha$ -amylase salivaire humaine (HSA) et la  $\alpha$ -amylase pancréatique. L'HSA initie le processus amylolytique dans la cavité buccale (Bornhorst & Singh, 2012) et peut rester actif dans l'estomac (Bornhorst & Singh, 2012; S. S. Gropper & J. L. Smith, 2013) jusqu'à son inactivation due à l'acidification gastrique, entre pH 3.0 et 3.8 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. H. Meyer, 1987). L' $\alpha$ -amylase pancréatique et les enzymes de la bordure en brosse terminent ensuite la digestion de l'amidon dans l'intestin grêle, où le glucose, le produit final de la digestion de l'amidon, est finalement absorbé (S. S. Gropper & J. L. Smith, 2013). Le rôle de l'HSA pendant la digestion orale de l'amidon est relativement bien établi, par contre, son impact pendant la phase gastrique de la digestion est méconnu. De nombreux travaux ont été menés *in vitro* afin de mieux comprendre le processus digestif de l'amidon. (Berti, Riso, Monti, & Porrini, 2004; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; Jenkins, et al., 1982; Woolnough, Bird, Monro, & Brennan, 2010), mais la phase orale est soit ignorée (Berti, Riso, Monti, & Porrini, 2004; Goñi, Garcia-Alonso, & Saura-Calixto, 1997) soit suivie d'une phase gastrique à un pH acide, généralement entre 1,5 et 3 (Yvonne Granfeldt, Hagander, & Björck, 1995; Jenkins, et al., 1982; Minekus, et al., 2014a; Woolnough, Bird, Monro, & Brennan, 2010). Par conséquent, même lorsqu'elle est prise en compte dans ces protocoles, l'HSA est immédiatement inactivée au début de la phase gastrique, et la plupart de l'amidon est donc hydrolysé pendant la phase intestinale. Par ailleurs, comme ces études sont généralement en très bon accord avec les données *in vivo* (Bohn, et al., 2017), une analogie entre la cinétique de l'hydrolyse intestinale et la réponse glycémique est souvent établie, renforçant ainsi l'idée que la grande majorité de l'amidon est hydrolysée par l'amylase pancréatique (des Gachons & Breslin, 2016). Toutefois, la simulation de la digestion gastrique à un pH acide fixe n'est pas représentative de la dynamique *in vivo*, car il a été observé que l'acidification postprandiale du contenu gastrique peut prendre entre 60 et 200 min (Dressman et al., 1990; Gardner, Ciociola, & Robinson, 2002; Kalantzi et al., 2006; Malagelada, Go, & Summerskill, 1979; Troost, Steijns, Saris, & Brummer, 2001). Il semble donc qu'il y ait une grande fenêtre de temps durant laquelle l'amylase d'origine salivaire peut rester active pendant la digestion gastrique. Ceci corrobore des études selon lesquelles l'action du HSA peut continuer longtemps après la phase orale, et même atteindre la lumière intestinale sous sa forme active (M. Fried, S. Abramson, & J. H. Meyer, 1987; Skude & Ihse, 1976). Ces observations suggèrent que l'HSA peut jouer un rôle plus important dans la digestion de l'amidon que celui qui lui est couramment attribué. La littérature

scientifique à ce sujet est rare, mais des évidences que l'HSA peut hydrolyser en maltose jusqu'à 76% de l'amidon de la purée de pommes de terre et 59% de celui du pain, dans l'estomac, remontent au début du 20ème siècle (Bergeim, 1926).

L'inhibition des amylases digestives peut ralentir la digestion de l'amidon, ce qui constitue une opportunité pour retarder l'absorption du glucose dérivé de l'amidon et améliorer les réponses glycémiques postprandiales. Une stratégie diététique simple, visant à réduire la réponse glycémique par des effets d'inhibition pourrait être bénéfique tant pour les personnes en bonne santé que pour celles qui ont une régulation altérée de leur glycémie. En effet, cette stratégie a été jugée efficace dans des contextes où le contrôle de la glycémie représente un défi encore plus grand que dans des populations saines. Un exemple est le traitement du diabète de type 2 par l'administration d'acarbose, qui inhibe les enzymes amylolytiques dans le tube digestif (Salvatore & Giugliano, 1996). Une stratégie diététique, visant à associer des aliments ou repas riches en amidon à des produits alimentaires susceptibles d'exercer un effet inhibiteur pourrait donc constituer une approche intéressante.

Les différents sites d'action des enzymes amylolytiques dans le tube digestif sont déterminants pour comprendre leur vulnérabilité. L'HSA, par exemple, est présente pendant les phases orale et gastrique. Elle est donc directement exposée aux aliments tels qu'ils sont ingérés, ce qui inclue leur pH et leurs caractéristiques structurelles. Quant à l' $\alpha$ -amylase pancréatique, elle est principalement susceptible d'être affectée par la composition des aliments, puisque le pH du chyme est rapidement neutralisé dans le duodénum et la structure des aliments a déjà été largement modifiée à ce stade. En toute logique, la contribution réelle de chaque enzyme au processus digestif influencera également l'ampleur des effets qui peuvent être obtenus.

## OBJECTIFS

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L'objectif principal de cette recherche a été la clarification du processus digestif des aliments riches en amidon et la proposition de solutions efficaces et faciles à mettre en œuvre pour améliorer l'impact glycémique des aliments riches en amidon en ralentissant leur cinétique digestive. Cinq sous-objectifs spécifiques peuvent être distingués :

1. Examiner des boissons (thé, café, vin, jus de citron) et des condiments (vinaigre) couramment consommées, pour évaluer leur capacité à inhiber l'amylase salivaire et pancréatique.
2. Vérifier si l'effet de la consommation d'aliments riches en amidon avec une boisson acide (jus de citron) ou une boisson riche en polyphénols (thé) peut réduire le taux de digestion de l'amidon in vitro comparativement à l'eau.
3. Déterminer si le jus de citron et le thé peuvent atténuer efficacement la réponse glycémique au pain chez l'homme.
4. Déterminer si le fait d'abaisser la réponse glycémique d'un repas sans en modifier la composition nutritive peut influencer les perceptions liées à la satiété et à l'appétit et/ou à la consommation énergétique lors du repas suivant.
5. Étudier les caractéristiques de la digestion gastrique (surtout la vidange gastrique) d'un repas de pain avec de l'eau, du jus de citron ou du thé, par imagerie par résonance magnétique.

Un résumé des principales étapes expérimentales est présenté dans la Figure 1.

La première partie du travail a consisté en une série d'expériences *in vitro* qui ont été réalisées pour :

- déterminer l'impact des niveaux de pH rencontrés par l'amylase salivaire dans l'ensemble du système digestif sur son activité ;
- étudier les mécanismes de digestion de l'amidon à différents stades du processus digestif ;
- déterminer l'effet des boissons et condiments de consommation courante sur l'activité des amylases digestives et sur la digestion de l'amidon.

Ces travaux ont nécessité l'élaboration et l'optimisation de protocoles de laboratoire.

Les connaissances issues des études *in vitro* ont été appliquées à la conception d'une étude chez l'homme visant à évaluer l'impact de 3 repas riches en amidon sur la réponse glycémique, l'appétit, la consommation calorique et aussi la vidange gastrique (étudié par Imagerie par Résonance Magnétique). La planification, la préparation et la mise en œuvre de cette étude ont été assurées grâce aux collaborations avec l'unité de recherche IR4M (Imagerie par Résonance Magnétique Médicale et Multi-Modalités, UMR8081 Université Paris Sud - CNRS, Orsay, France), le Service Hospitalier Frédéric Joliot à Orsay et le CEFRED (Centre d'exploration fonctionnelle et de rééducation digestive) de l'Hôpital Avicenne. Un protocole de recherche clinique a également été préparé et soumis pour approbation éthique.

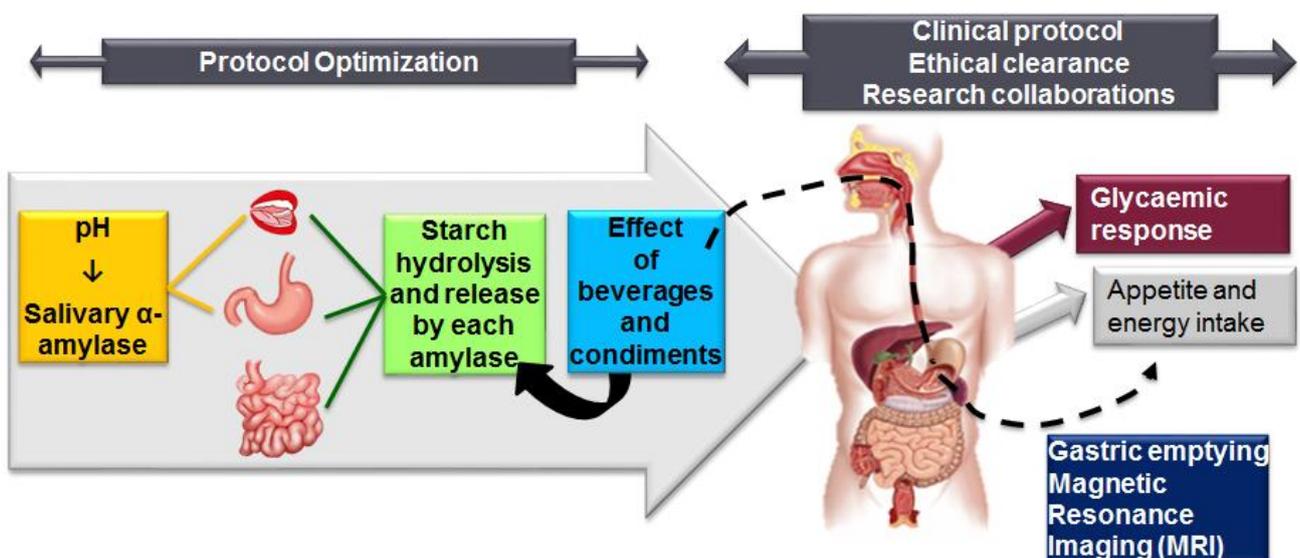


Figure 1 - Approche expérimentale.

### I. RÉÉVALUATION DE LA CONTRIBUTION DE CHAQUE ÉTAPE DIGESTIVE À L'HYDROLYSE DE L'AMIDON À PARTIR D'ALIMENTS ASSOCIÉS À UNE FORTE VS. UNE FAIBLE RÉPONSE GÉCÉMIQUE

Pour comprendre la contribution de l'HSA à la digestion de l'amidon, il est important de déterminer dans un premier temps son comportement dans la gamme de pH qu'elle rencontre pendant la digestion. L'évolution de l'activité de l'HSA en fonction du pH est présentée à la Figure 41. L'activité amylolytique la plus élevée correspondait à  $352 \pm 41$  U/mL et a été observée entre pH 6 et 7. Aux alentours de pH 4, 50 % de l'activité maximale était encore observée et l'inhibition complète a eu lieu entre pH 3,0 et 3,5.

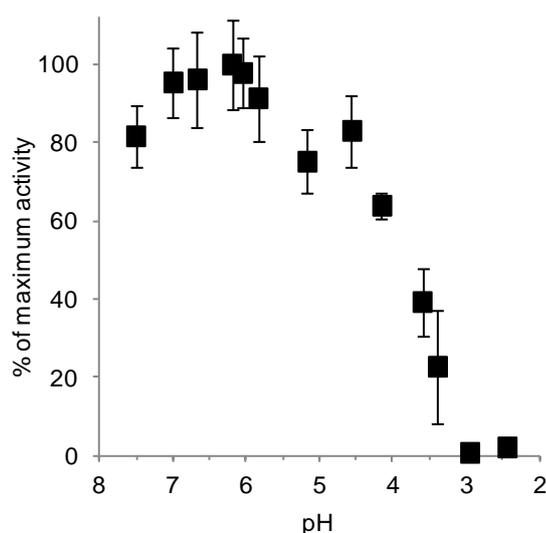


Figure 2 - Activité amylolytique de la salive en fonction du pH. L'activité maximale, mesurée à pH 6,2, a été de  $352 \pm 41$  U/mL de salive (1 unité a libéré 1,0 mg de maltose de la féculose de pomme de terre en 3 minutes à pH 6,9 à 20°C).

Ces résultats sont bien en accord avec la littérature sur la salive et les propriétés de l'HSA. Le pH optimal de l'HSA (à la fois dans la salive et sous forme purifiée) a été rapporté entre 6,5 et 7,0 (Peter Bernfeld, 1955; P Bernfeld, Staub, & Fischer, 1948; Walker & Whelan, 1960) et l'activité amylolytique maximale de 352 U/mL obtenue pour la salive est très proche des 410 U/mL précédemment rapportée pour de la salive centrifugée (Peter Bernfeld, 1955) en utilisant le même protocole de dosage que dans cette étude.

Au total, 6 aliments riches en amidon ont été étudiés : 3 pains blancs au blé (2 baguettes et 1 pain sandwich) et 3 types de pâtes (pâtes perlées, spaghetti au blé et spaghetti sans gluten). Le pain blanc au blé suscite généralement une réaction glycémique élevée, tandis que les pâtes alimentaires suscitent habituellement une réaction glycémique faible. Toutes les digestions ont comporté une phase orale avec de la salive humaine suivie d'une phase gastrique et, dans certains cas, d'une phase intestinale. Les phases gastrique et intestinale ont été réalisées dans un système dynamique (DiDGI®) et les conditions digestives ont été définies en fonction de la littérature publiée rapportant des observations d'études humaines. Trois cinétiques d'acidification gastrique ont été reproduites in vitro : de 6 à 2 en (1) 30 min, (2) 60 min ou (3) 90 min. Les résultats obtenus dans chaque condition sont présentés dans la Figure 76 A, B et C, respectivement, et reflètent un large spectre de conditions digestives allant d'une collation légère à un repas plus copieux. L'amidon libéré lors de la digestion des trois types de pains est présenté dans la Figure 76. Le premier point de chaque graphique correspond à la fin de la phase orale qui, malgré sa courte durée, montre que près de 20% de la teneur totale en amidon a été libérée à ce stade. Les points suivants correspondent à la phase gastrique. Quel que soit le type de pain ou la cinétique de réduction du pH, la même cinétique de libération d'amidon a été observée, avec un plateau final entre 70 et 85%, atteint pendant les 15-20 premières minutes de la digestion.

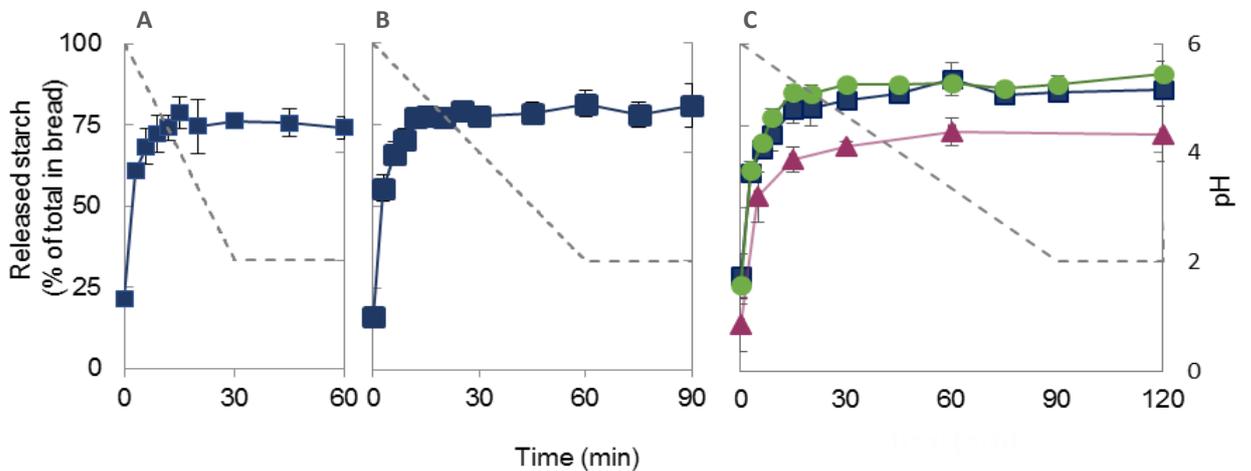


Figure 3 – Digestion oro-gastrique du pain in vitro. Relargage d'amidon (symboles complets) et pH du chyme (lignes pointillées) lors de la digestion de trois pains. Le premier point de chaque graphique correspond à la phase orale, les autres correspondent à la phase gastrique. Différents symboles illustrent les résultats obtenus avec différents pains. Deux baguettes (■ and ●) et un pain de mie ont été étudiés (▲). Moyenne  $\pm$  SD, 3 rpt.

D'autres analyses ont été effectuées pour déterminer le niveau d'hydrolyse de l'amidon, en particulier la proportion d'amidon qui avait été hydrolysée en oligosaccharides pendant la digestion des trois pains étudiés dans les mêmes conditions expérimentales. Les résultats sont présentés dans la Figure 77. On a observé que l'hydrolyse en oligosaccharides suivait également une progression exponentielle, avec un plateau atteint quelques minutes plus tard que pour le relargage de l'amidon (Figure 77 vs. Figure 76C). Plus important encore, la quantité d'oligosaccharides libérés au plateau était remarquablement élevée, représentant 60 à 65% de l'amidon libéré pour tous les pains.

Pour ce qui concerne les pâtes (données non présentées), la digestion de l'amidon était plus lente. Le plateau de libération de l'amidon était atteint après 45 à 50 minutes de digestion et était, en moyenne, environ 2,5 fois inférieur à celui du pain (environ 30%). Néanmoins, 60 à 70 % de l'amidon libéré l'était de nouveau sous la forme d'oligosaccharides.

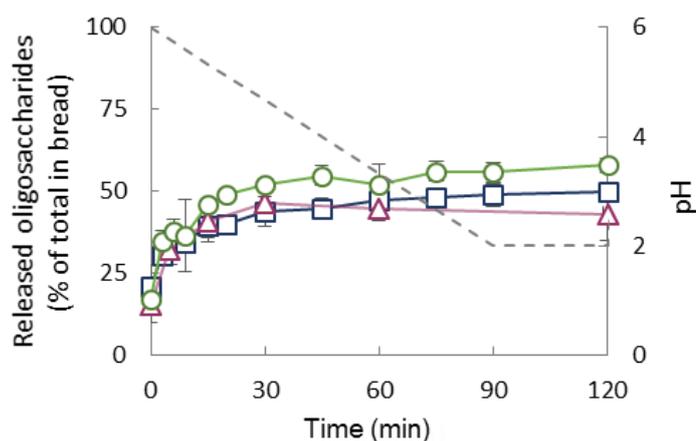


Figure 4 - Digestion dynamique in vitro du pain. Oligosaccharides libérés (symboles ouverts) et pH du chyme (ligne pointillée) au cours de la digestion oro-gastrique de trois pains. Le premier point de chaque graphique correspond à la phase orale, les autres correspondent à la phase gastrique. Différents symboles illustrent les résultats obtenus avec différents pains. Deux baguettes (□ and ○) et un pain de mie (△) ont été étudiés. Moyenne  $\pm$  SD, 3 rpt.

Dans certains cas, les digestions oro-gastriques ont été suivies d'une phase intestinale. Comme la plupart de l'amidon du pain avait déjà été libéré pendant la phase gastrique, quand une phase intestinale a été incluse dans les expériences, les augmentations supplémentaires de l'amidon et des oligosaccharides libérés n'étaient que d'environ 5-8% et 15-20%, respectivement. Inversement, comme seule une fraction de l'amidon des pâtes alimentaires avait été libérée lors

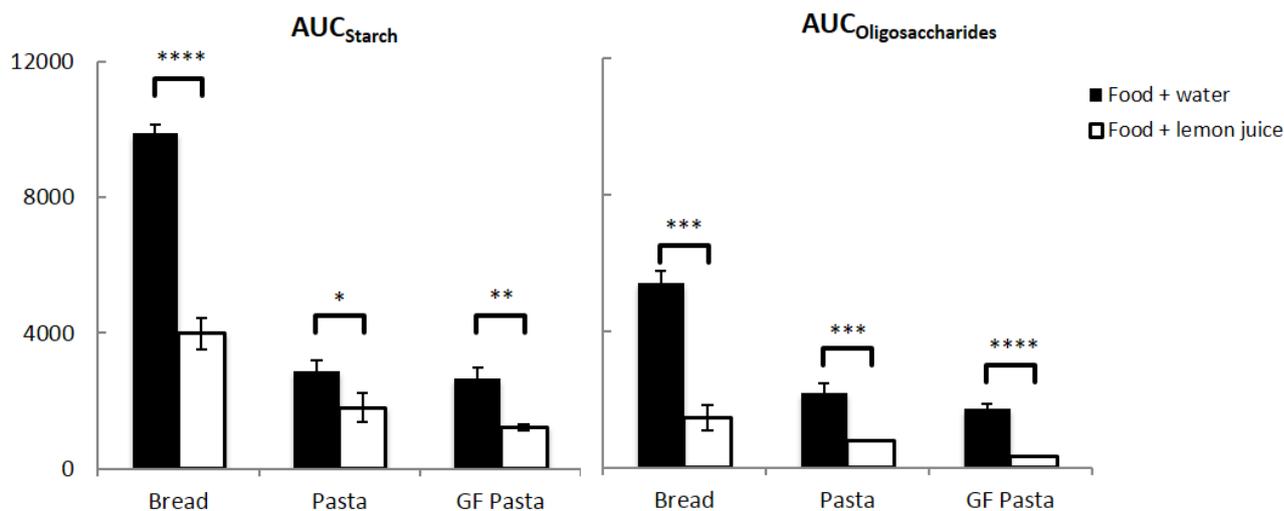
de la digestion gastrique, la majeure partie de sa libération et de son hydrolyse s'est produite pendant la phase intestinale.

Bien que l'amylase pancréatique ait été très efficace pour poursuivre l'hydrolyse de l'amidon pendant la phase intestinale, nos études *in vitro* ont démontré que le rôle de l'amylase salivaire pendant les phases digestives précédentes ne doit pas être négligé. Ceci est d'autant plus vrai que sa contribution à l'hydrolyse de l'amidon pendant la phase gastrique semble plus élevée pour des aliments associés à une réponse glycémique élevée, comme le pain.

Étant donné le rôle important de l'HSA dans la digestion de l'amidon, il est fort probable que des facteurs influant sur sa performance amylolytique, autres que les propriétés structurelles d'un aliment, puissent se traduire par une réponse glycémique modifiée. Cela pourrait mener à de nouvelles possibilités et stratégies pour atténuer la réponse glycémique élevée de certains féculents.

## II. L'EFFET DE BOISSONS COURAMMENT CONSOMMÉES (THÉ, CAFÉ, VIN, JUS DE CITRON) ET DE CONDIMENTS (VINAIGRE) SUR L'ACTIVITÉ AMYLOLYTIQUE DES AMYLASES SALIVAIRES ET PANCRÉATIQUES, SUR LA DIGESTION DE L'AMIDON *IN VITRO*

Sur la base de nos premiers résultats, nous avons émis l'hypothèse qu'il y avait une forte probabilité que des facteurs influençant la performance amylolytique de l' $\alpha$ -amylase salivaire, autres que les propriétés structurelles d'un aliment, puissent se traduire en une réponse glycémique modifiée. C'est pourquoi nous avons mené des expériences préliminaires simulant la digestion oro-gastrique de pain blanc au blé, et de pâtes alimentaires (au blé et sans gluten) avec du jus de citron ou de l'eau. Comme prévu, le jus de citron a provoqué une acidification prématurée du chyme. Par conséquent, la digestion gastrique a été initiée à un pH inférieur au seuil d'inactivation de la  $\alpha$ -amylase salivaire ( $< \text{pH } 3,5$ ) et l'amylolyse a été complètement interrompue après la phase orale. La proportion d'amidon et oligosaccharides libérés a été significativement plus faible pour tous les aliments en présence de jus de citron, comme présenté dans la **Figure 48**. Un effet plus accentué a été observé avec le pain, pour lequel le rôle de l'amylase salivaire lors de la digestion gastrique est plus prépondérant, que pour les pâtes.



**Figure 5-** Surface sous les courbes de l'amidon (AUC<sub>Starch</sub>) et des oligosaccharides (AUC<sub>Oligosaccharides</sub>) libérés lors de la digestion gastrique *in vitro* de pain blanc au blé et des pâtes alimentaires au blé (Pasta) et sans gluten (GF Pasta) avec de l'eau ou du jus de citron. Moyenne ± SD, 3 rpt. Les astérisques (\*) indiquent des différences statistiquement significatives : \* p < 0,05, \*\* p < 0,01, \*\*\* p < 0,001, \*\*\*\* p < 0,0001.

Des expériences ultérieures visaient à mieux comprendre ces mécanismes et à étudier l'impact d'autres aliments sur l'activité des amylases salivaires et pancréatiques.

Du jus de citron, des thés, des cafés, des vins et des vinaigres ont été étudiés pour leurs capacités d'inhibition amylolytique avec des tests enzymatiques à pH neutre. Les résultats sont présentés dans la Figure 49. La réduction de l'activité amylolytique de la salive par les aliments à pH neutre a augmenté dans l'ordre suivant : cafés (5-10%), thés verts (20-45%) et noirs (30-70%) (Figure 49A). Des niveaux similaires d'inhibition ont été observés pour l'activité amylolytique de la pancréatine dans chaque catégorie de produits (Figure 49B). Des niveaux similaires d'inhibition ont été observés pour l'activité amylolytique de la pancréatine dans chaque catégorie de produits (figure 6B). L'effet des aliments acides (vins, vinaigres et jus de citron) sur l'activité amylolytique de la salive a également été étudié à leur pH naturel (Figure 49A). Dans ces conditions, les aliments acides étudiés ont montré une capacité inhibitrice quasi totale (90-100%).

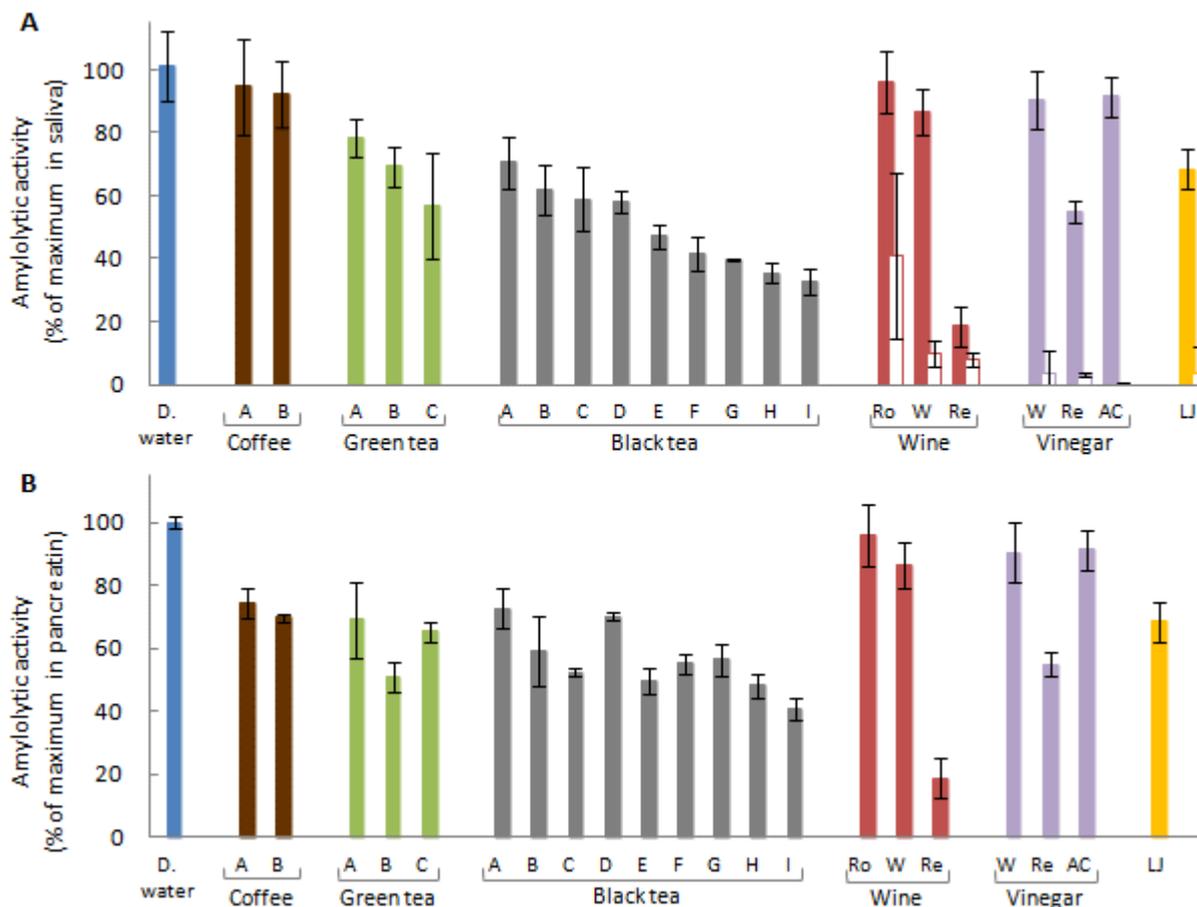


Figure 6 - Impact des boissons et des condiments. Activité amylolytique (% du maximum) de la salive (A) et de la pancréatine (B) en présence d'eau distillée, de cafés, de thés verts et noirs, de vins, de vinaigres et de jus de citron (LJ) à leur pH naturel (barres colorées) et neutralisé (barres blanches). Des lettres différentes dans chaque catégorie de produits correspondent à des marques différentes. L'activité amylolytique maximale, mesurée avec de l'eau distillée, a été de  $345 \pm 37$  U/mL pour la salive et de  $24 \pm 1$  U/ mg pour la pancréatine. Moyenne  $\pm$  SD, 3 rpt.

Sur la base de ces résultats, une nouvelle série d'expériences de digestion in vitro a été conçue pour évaluer l'impact de la présence d'aliments à fort pouvoir inhibiteur sur la digestion de l'amidon du pain et des pâtes. Des digestions in vitro de pain ont été reproduites pour simuler sa consommation avec de l'eau, du jus de citron et avec le thé noir ayant le plus fort pouvoir inhibiteur (thé I de la Figure 49A). Les résultats de ces expériences sont résumés à la Figure 52.

En présence de thé, l'hydrolyse de l'amidon en oligosaccharides a atteint un plateau inférieur d'environ 10% à celui observé avec l'eau pendant la phase gastrique. Pendant la phase intestinale, l'hydrolyse en oligosaccharides est restée limitée, stagnant à environ 50% de l'amidon total. L'impact limité du thé lors des expériences de digestion, comparé à la forte capacité inhibitrice observée avec les tests d'activité enzymatique, est très certainement dû aux différentes conditions employées dans ces expériences telles que le rapport amidon/thé supérieur. Comme l'effet du thé est, en partie, dû à une inhibition compétitive, il est très probable qu'il ait été partiellement inversé par la concentration plus élevée de substrat. Si l'on en juge par la plus faible proportion d'amidon hydrolysé en oligosaccharides dans la phase intestinale, il semble que le thé ait quelque peu limité les derniers stades de l'amylolyse, mais il reste à déterminer si cela suffit à produire un effet *in vivo*.

En présence de jus de citron, l'hydrolyse en oligosaccharides a été totalement interrompue après la phase orale. Les résultats étaient en fait comparables à ceux obtenus lors d'expériences témoins sans amylase salivaire (données non présentées). Pendant la phase intestinale, l'hydrolyse en oligosaccharides est restée proche de celle obtenue en présence de thé, c'est-à-dire légèrement inférieure au niveau observé avec l'eau.

Dans l'ensemble, les résultats de nos expériences *in vitro* ont fourni une base biochimique robuste pour le développement de nouvelles stratégies qui pourraient être efficaces pour améliorer la réponse glycémique induite par les aliments riches en amidon.

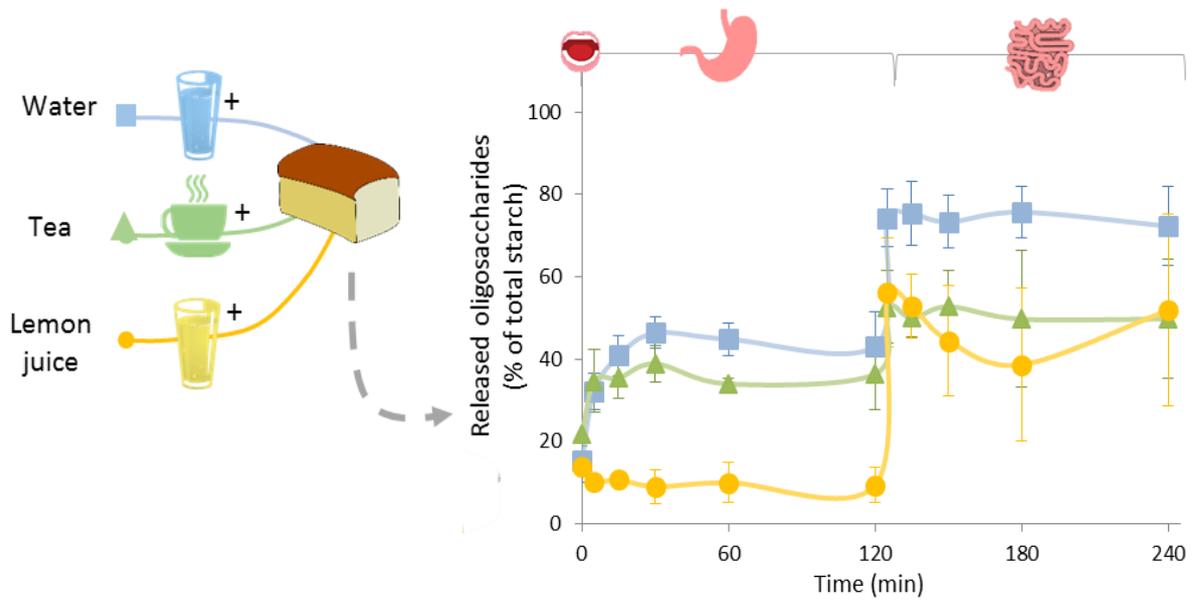


Figure 7 - Digestion *in vitro* du pain. Oligosaccharides libérés au cours des digestions oro-gastro-intestinales de portions égales de pain avec de l'eau (■) du thé (▲) ou du jus de citron (●). Le premier point de chaque courbe correspond à la fin de la phase orale et au début de la phase gastrique. Les phases gastrique et intestinale ont duré 120 min chacune. Toutes les valeurs sont moyenne  $\pm$  SD, 3 rpt.

### III. TESTER LES STRATÉGIES QUI SE SONT MONTRÉES EFFICACES LORS D'ÉTUDES DE DIGESTION IN VITRO CHEZ L'HOMME

L'objectif suivant était de tester ces stratégies dans le cadre d'une étude humaine, composée de deux parties, comme le montre la Figure 8. Dans les deux parties de l'étude, les volontaires ont consommé des portions égales de pain avec de l'eau (témoin), du thé noir ou du jus de citron.

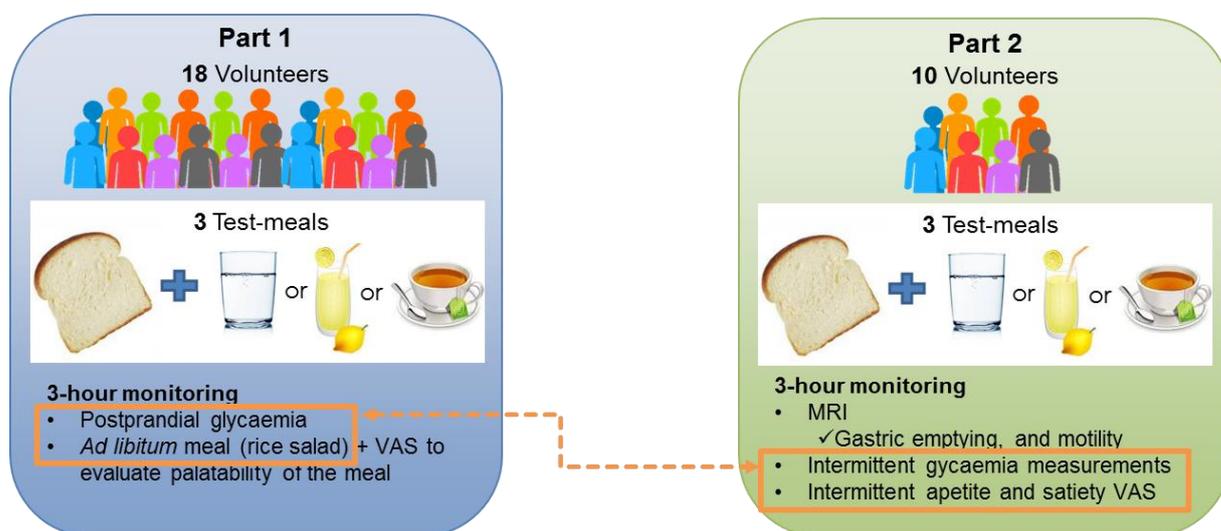


Figure 8 - Récapitulatif schématique de l'étude humaine.

#### PARTIE 1:

L'inhibition des enzymes digestives qui participent à la digestion de l'amidon pourrait constituer une opportunité pour ralentir la libération du glucose de l'amidon et d'améliorer l'impact glycémique des aliments riches en amidon. Des approches diététiques simples consistant à associer ces aliments à des boissons ayant la capacité d'inhiber ces enzymes pourraient être une stratégie facile à mettre en œuvre. L'objectif de cette partie des études chez l'homme était de tester l'impact de l'ajout de thé noir ou de jus de citron à un repas riche en amidon sur la glycémie postprandiale et sur la consommation énergétique chez les sujets sains.

Une étude croisée randomisée a été menée. Des portions égales de pain (100 g) plus 250 mL d'eau, de thé noir ou de jus de citron ont été consommés au petit-déjeuner après un jeûne de 10 heures dans un ordre aléatoire. Les concentrations de glucose dans le sang capillaire ont été auto-surveillées par piqûre de doigt à des moments prédéfinis pendant 180 minutes. Dans cette partie de l'étude, les volontaires étaient libres de suivre leur quotidien normal pendant les 3

heures suivant la consommation du repas test. Après cette période, la consommation énergétique *ad libitum* a été évaluée.

Les réponses glycémiques postprandiales suite à la consommation des repas associés à de l'eau, du thé et du jus de citron sont présentées à la Figure 58. Les données se rapportent à 17 participants (les données d'un participant ont été exclues pour des problèmes de non-conformité avec le protocole expérimentale). Il n'y a pas eu de différence significative dans les concentrations glycémiques à jeun entre les trois repas testés ( $p = 0,81$ ). Aucune différence significative n'a été observée entre l'eau et le thé, mais la consommation de pain avec du jus de citron a entraîné une baisse de la glycémie, avec des différences statistiquement significatives entre 15 et 45 minutes. Le jus de citron a réduit de 33% ( $p = 0,03$ ) les concentrations maximales de glucose dans le sang. De plus, il a aussi retardé significativement ( $P < 0,01$ ) de plus de 30 min (78 contre 41 minutes, en moyenne) le temps pour atteindre le maximum de concentration de glucose dans le sang. La surface sous la courbe pour le repas de jus de citron a été significativement plus faible jusqu'à 90 min ( $p = 0,03$ ), mais pas après 3 heures ( $p = 0,76$ ), ce qui suggère une absorption retardée, plutôt qu'incomplète, du glucose du repas. En raison de la nature semi-contrôlée de cette étude, il est suggéré que la combinaison d'une boisson acide avec un repas riche en amidon pourrait être une stratégie efficace pour réduire la réponse glycémique aux aliments riches en amidon dans la vie quotidienne.

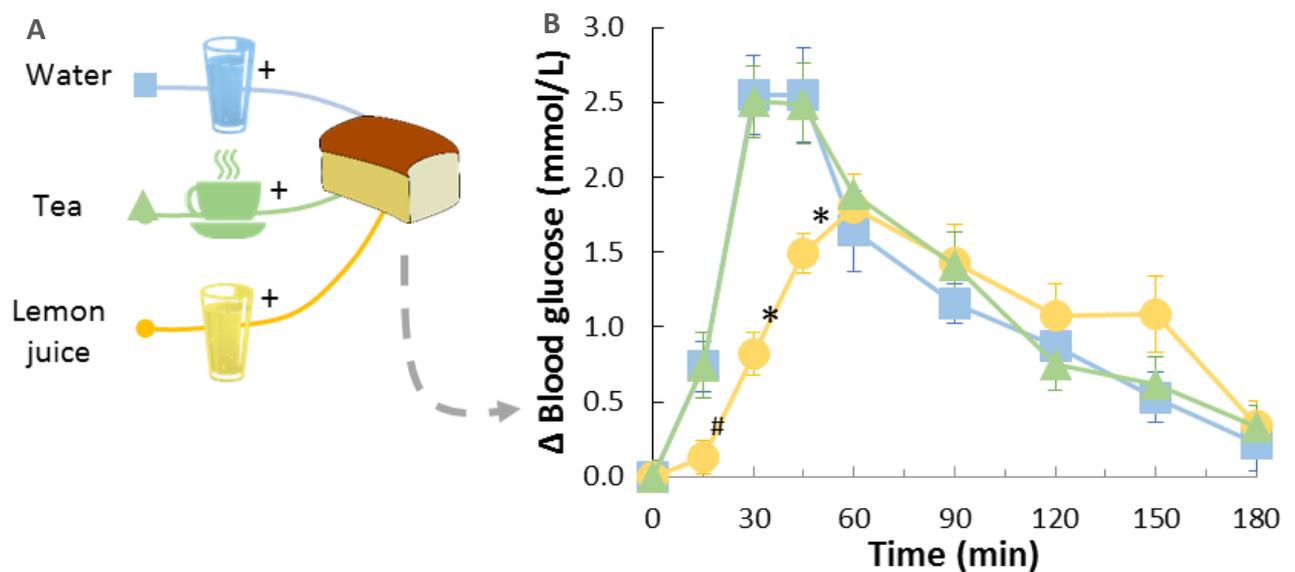


Figure 9 – (A) Repas test. (B) Réponses glycémiques postprandiales. Changements incrémentiels ( $\Delta$ ) moyens ( $\pm$ SEM) des concentrations de glucose en réponse à des quantités égales de glucides provenant d'un pain blanc au blé consommé avec soit de l'eau (■), du thé (▲) ou du jus de citron (●). Les points pour lesquels des différences significatives ont été identifiées (ANOVA suivie d'un test-t par paires) sont identifiés par les symboles # et \*. # indique une différence statistiquement significative entre le jus de citron et l'eau ( $P < 0,01$ ;  $P = 0,051$  entre le jus de citron et le thé). \* indique des différences statistiquement significatives entre le jus de citron et les deux autres boissons, eau et thé, à 30 min ( $P < 0,0001$  et  $0,001$ ) et à 45 min ( $P = 0,014$  et  $0,019$ ).  $n = 17$  adultes sains

Les résultats obtenus avec le jus de citron concordent avec de nombreuses études cliniques portant sur l'effet d'autres aliments acides (notamment le vinaigre, le pain au levain et les conserves au vinaigre) sur la réponse glycémique aux aliments riches en amidon. D'après les articles de revue qui se sont intéressés à ce sujet, il semblait clair que le principal mécanisme était lié à l'acidité, bien que l'effet réel de l'acidité n'ait pas été identifié. L'effet sur les enzymes amylolytiques, en particulier l'amylase salivaire, a rarement été mentionné et une autre explication possible, bien que non encore totalement acceptée ni rejetée, a été avancée, à savoir le fait que l'acidité pourrait retarder la vidange de l'estomac.

L'indice glycémique, les concentrations maximales de glucose et le temps pour atteindre le pic sont présentés dans le Table 12, ainsi que les changements correspondants sous forme de pourcentage de l'expérience témoin (repas avec de l'eau). Encore une fois, aucune différence n'a été trouvée pour aucun de ces paramètres entre l'eau et les repas de thé ( $0,66 \geq P \leq 0,96$ ). Malgré l'absence de différences significatives au niveau de la valeur de l'indice glycémique ( $P = 0,76$ ), le jus de citron a permis d'abaisser de 33 % ( $P = 0,03$ ) les concentrations maximales de

glucose sanguin. De plus, il retarde significativement ( $P < 0,01$ ) le temps nécessaire pour atteindre le pic glycémique, de plus de 30 min en moyenne (78 contre 41 min).

Table 1 – Indice glycémique (IG), concentration glycémique maximale et temps pour atteindre le peak de glycémie après les repas tests composés de 100 g de pain blanc au blé et de 250 mL d'eau, de thé ou de jus de citron

		Eau	Thè	Jus de citron
<b>IG</b> <sup>2</sup>		100 ± 9	107 ± 12	77 ± 7
	Variation % <sup>3</sup>	-	8	-23
<b>Pic</b>	mmol/L	2.7 ± 0.3	2.9 ± 0.2	1.8 * ± 0.3
	Variation % <sup>3</sup>	-	7	-33
<b>Temps pour atteindre le pic</b>	min	41 ± 4	43 ± 3	78 * ± 9
	Variation % <sup>3</sup>	-	5	90

<sup>1</sup>Toutes les valeurs sont  $\bar{x} \pm \text{SEM}$ . Les valeurs à l'intérieur d'une même rangée identifiées par \* sont significativement différentes (ANOVA suivie d'un test-t par paires) de celles du repas pain+eau. La concentration maximale de glucose dans le sang ( $P < 0,05$ ) et le temps avant le pic ( $P < 0,01$ ) ont été significativement plus faibles pour le jus de citron que pour l'eau et le thé.  $n = 17$  adultes sains.

<sup>2</sup>Calculé à partir de la surface sous la courbe (0 à 120 min) et en utilisant le repas pain + eau comme référence.

<sup>3</sup>Variation exprimée en pourcentage de la valeur obtenue pour le repas de référence pain + eau.

La consommation d'énergie *ad libitum* (kJ) après les repas d'eau, de thé et de jus de citron a été de  $2051 \pm 193$ ,  $1764 \pm 216$  et  $2168 \pm 182$  ( $\bar{x} \pm \text{SEM}$ ), respectivement. Ces résultats sont également présentés à la Figure 60. Les effets du repas ( $P = 0,34$ ) et du jour de la session d'étude ( $P = 0,70$ ) sur la consommation énergétique ont été statistiquement non significatifs (données non présentées).

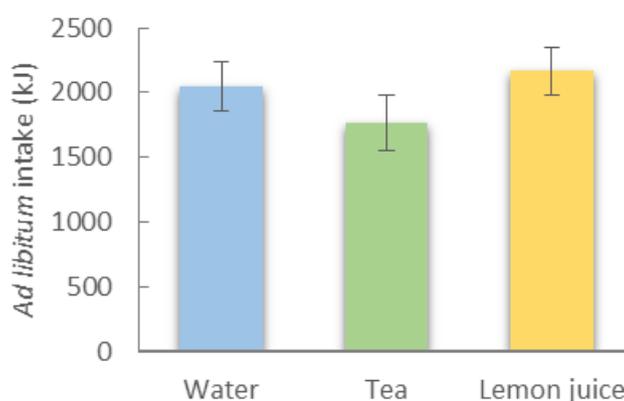


Figure 10 - Consommation *ad libitum*. Consommation énergétique ( $n=16$ ,  $\bar{x} \pm \text{SEM}$ ) 3 heures après un repas test composé de 100 g de pain blanc au blé et de 250 mL d'eau, de thé ou de jus de citron.

## PARTIE 2:

L'objectif principal de la deuxième partie de l'étude chez l'homme était de déterminer si les mécanismes de vidange gastrique sont ou non les mêmes quand un repas de pain est associé à de l'eau, du thé ou du jus de citron. En utilisant les mêmes repas que dans la partie 1, la digestion gastrique a été étudiée par imagerie par résonance magnétique (IRM) lors d'une étude contrôlée et randomisée à laquelle 10 volontaires ont participé. Afin d'assurer la comparabilité avec les résultats précédents, des mesures de la glycémie ont également été incluses. Les réponses glycémiques obtenues ont été similaires à celles obtenues dans la première partie de l'étude. Comme on peut l'observer à la Figure 65, le contenu de l'estomac (orange) présentait suffisamment de contraste pour délimiter ses contours. Comme les sujets étaient allongés sur leur côté droit (en décubitus droite), le chyme était sur leur côté droit et l'air était confiné dans des "poches" au-dessus, sur leur côté gauche. Ainsi, sur les images, ce schéma apparaît inversé et donc le chyme apparaît à gauche et l'air à droite.

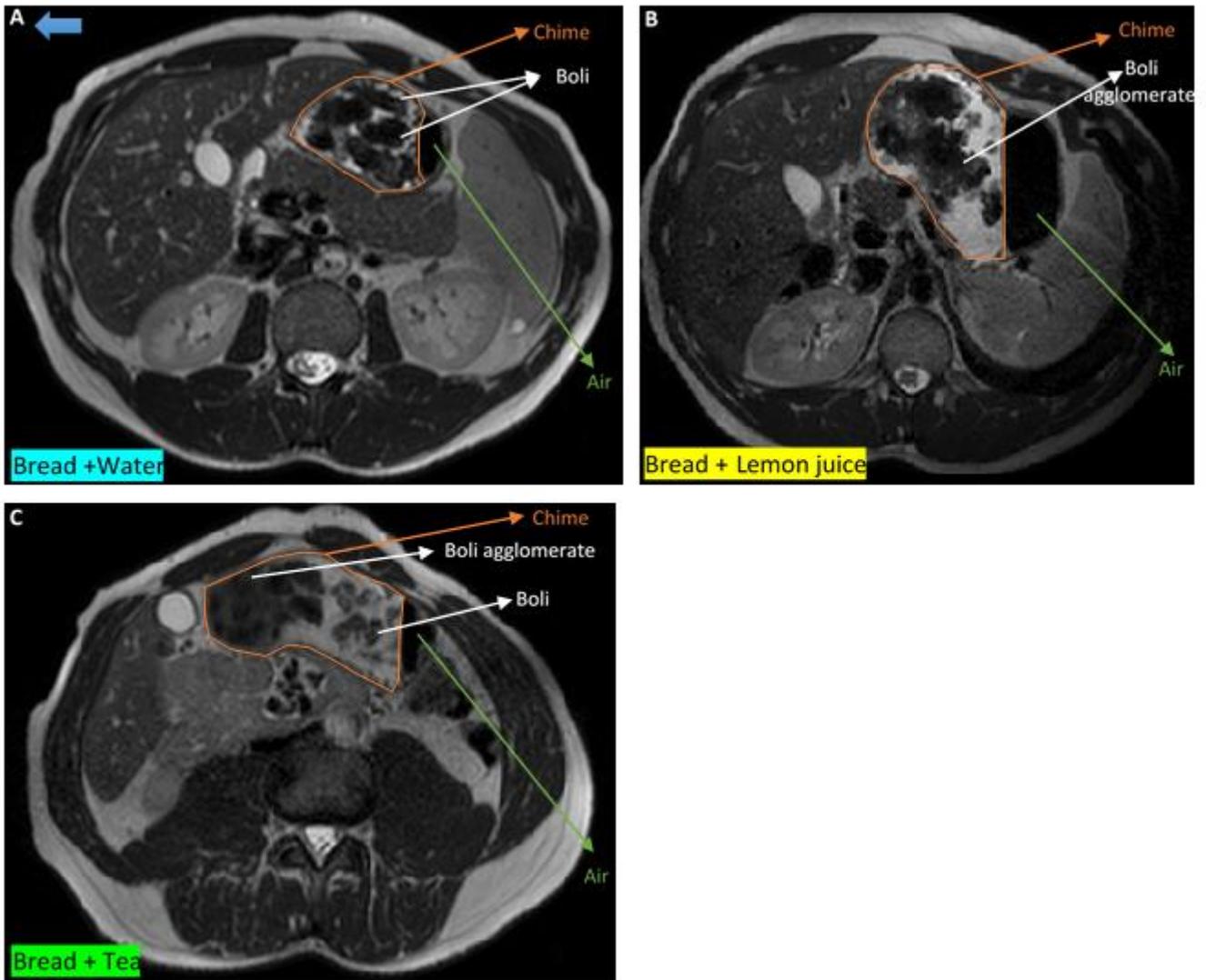


Figure 11 - Exemples des images obtenues par résonance magnétique de la région abdominale d'adultes sains qui ont ingéré des quantités égales de pain blanc au blé, 20 petits pois et 250 mL d'eau (A), de jus de citron (B) ou de thé (C). Les sujets étaient allongés sur le côté droit et les images ont été extraites de scans effectués 30 à 45 minutes après le repas. Le repas / chyme apparaît dans l'estomac à gauche en différents niveaux de gris et l'air apparaît en noir du côté droit. Les boli sont visibles à l'intérieur du chyme, ils apparaissent plus foncés que la phase environnante, dispersés ou en agglomérats. La flèche bleue épaisse en haut à gauche indique la direction de la gravité.

Quel que soit le type de repas consommé (Figure 65) deux catégories de contenu peuvent être distinguées : (1) une phase plus solide composée de boli individuels ou d'agglomérats de boli et (2) une phase plus liquide contenant les boissons et les sécrétions gastriques et probablement des particules de pain. Les agglomérats de boli se sont concentrés près du côté droit de l'estomac en raison du positionnement des sujets, mais les boli individuels sont apparus quelque peu dispersés. Au fur et à mesure que la digestion progressait, les boli individuels semblaient être pour la plupart unifiés en agglomérats plus gros qui semblaient présenter des nuances de gris de plus en plus claires. (Figure 67). A la fin des sessions d'étude, le volume total de l'estomac avait été réduit et contenait surtout des sécrétions (qui apparaissent en blanc).

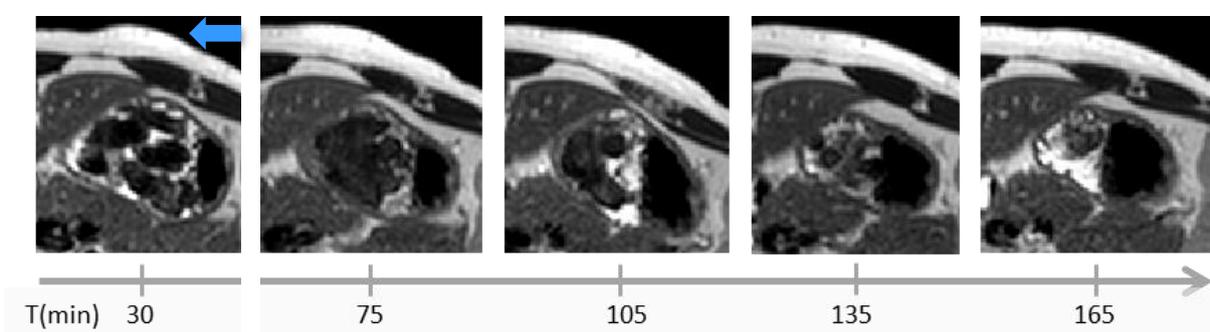


Figure 12 - Exemples des images obtenues par résonance magnétique de la région abdominale d'adultes sains qui ont ingéré 100 g de pain blanc au blé, 20 petits pois et 250 mL d'eau. Les images correspondent à des coupes axiales dans la même région anatomique et ont été extraites des scans anatomiques de volume effectués 30, 45, 75, 105, 135 et 165 minutes après le début du repas. Le chyme apparaît à gauche dans l'estomac contenant des boli individuels ou des agglomérats de boli qui semblent plus foncés que le chyme environnant et l'air apparaît noir sur le côté droit. Le sujet était allongé sur son côté droit. La flèche bleue en haut à gauche indique la direction de la gravité.

En délimitant individuellement le chyme et l'air dans l'estomac, il a également été possible d'obtenir des reconstructions 3D qui illustrent la position relative et la proportion de chaque composant (comme le montre la Figure 66). Comme souhaité, l'air est resté confiné dans les régions gastriques supérieures pendant toute la durée du suivi par IRM. De plus, en suivant la taille des zones bleues et rouges (correspondant au chyme et à l'air), ces reconstructions indiquent également que le rapport air/chyme dans l'estomac augmente au fur et à mesure que la digestion avance.

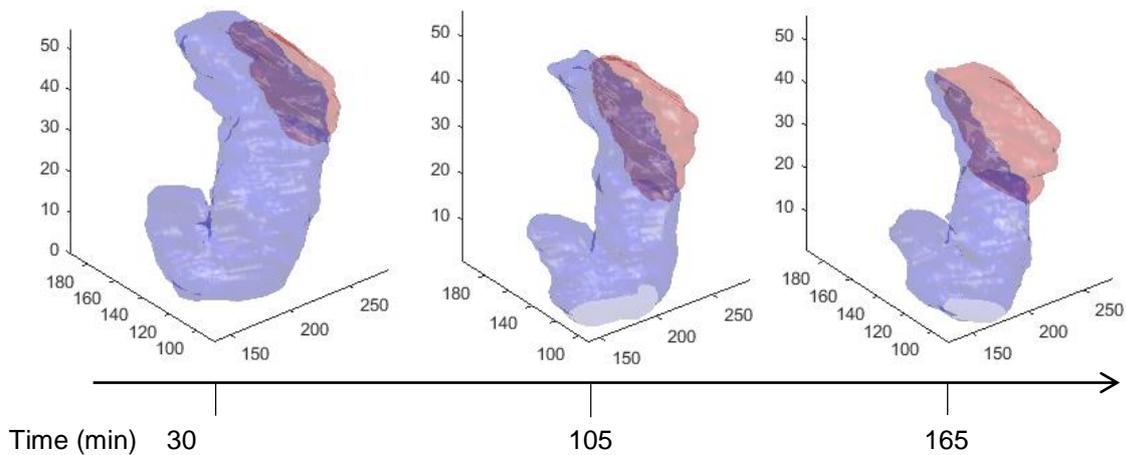


Figure 13 - Exemple d'une reconstruction 3D de l'estomac. De gauche à droite, les images correspondent aux examens effectués 30, 105 et 165 minutes après le début du repas. Les couleurs bleu et rouge illustrent la position et les volumes relatifs du chyme et de l'air, respectivement.

Les volumes postprandiaux du contenu gastrique sont présentés à la Figure 71.

Les résultats obtenus avec les repas contenant de l'eau et du thé ont été similaires pour tous les paramètres étudiés. Le volume postprandial du contenu gastrique 30 min après le début du repas était environ 50% plus élevé lorsque le pain était consommé avec du jus de citron par rapport au repas d'eau ( $454,0 \pm 18,6$  vs  $298,4 \pm 19,5$  mL,  $\bar{x} \pm \text{SEM}$ ). Le taux de vidange gastrique global était également environ 50% plus rapide pour le jus de citron ( $1,8 \pm 0,2$  pour l'eau contre  $2,7 \pm 0,1$  mL/min pour le jus de citron,  $\bar{x} \pm \text{SEM}$ ). Ces deux différences étaient statistiquement significatives avec  $P < 0,00001$  et  $P < 0,01$ , respectivement. En effet, le taux de vidange gastrique (significativement plus élevé avec du jus de citron) semble avoir été ajusté pour assurer un taux d'apport nutritif au duodénum similaire à celui des autres repas.

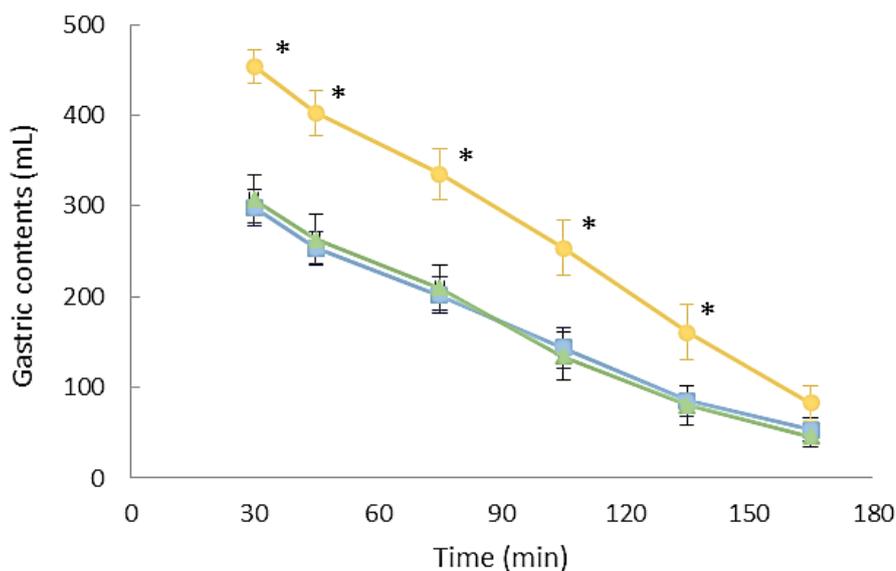


Figure 14- Volume post-prandial du contenu gastrique. Volumes moyens ( $\pm$ SEM) après consommation de trois repas de volume égal contenant des quantités égales d'amidon provenant de pain et d'eau (■), de thè (▲) au de jus de citron (●). Le symbole \* indique des différences significatives (ANOVA suivie d'un test-t par paires) entre le repas contenant du jus de citron et les deux autres repas. n = 10 adultes sains.

Les résultats de l'évaluation préliminaire des volumes des phases solides et liquides dans l'estomac sont présentés à la Figure 72. Les volumes des phases solides ont été très similaires pour les trois repas étudiés tout au long de la digestion. Les volumes de la phase liquide présentaient quelques différences. Pour l'eau et le thé, la phase liquide semble avoir eu un comportement similaire. Pour le repas de jus de citron, le volume de la phase liquide a eu tendance à être beaucoup plus élevé que dans les autres repas pendant la majeure partie de la période étudiée. Cependant, comme on peut l'observer, avec le jus de citron, le volume du contenu liquide a diminué à plus rapidement de sorte que, après 180 minutes, le volume gastrique restant était le même que pour les deux autres repas.

Le volume de liquide plus élevé après le repas de jus de citron peut provenir d'un niveau plus élevé de sécrétions salivaires et/ou gastriques. De plus, une courte phase de latence immédiatement après la consommation du repas pourrait également avoir contribué à l'augmentation initiale du volume de liquide. Dans tous les cas, il est important de souligner qu'après 3 heures, les volumes gastriques étaient les mêmes pour tous les repas. Par conséquent, nos résultats n'appuient pas vraiment l'hypothèse selon laquelle les repas à faible pH atténuent la réponse glycémique aux repas riches en amidon en raison d'un ralentissement de la vidange gastrique. Ils sont plus conformes à l'idée que cet effet sur les concentrations de glucose dans le sang est, au moins en partie, une conséquence de l'état auquel le chyme arrive dans l'intestin grêle. Comme nous l'avons observé dans nos études *in vitro*, l'acidification précoce du chyme gastrique par le jus de citron, et l'inhibition prématurée de la  $\alpha$ -amylase salivaire qui en résulte, auraient grandement réduit l'hydrolyse gastrique de l'amidon. Par conséquent, en contraste avec les autres repas, après le repas contenant du jus de citron, le chyme vidangé dans l'intestin grêle aurait eu une proportion plus élevée d'amidon pas- et/ou peu-hydrolysé, ce qui aurait exigé un travail d'hydrolyse supplémentaire pendant les étapes suivantes et retardé le relargage du produit final de la digestion, le glucose.

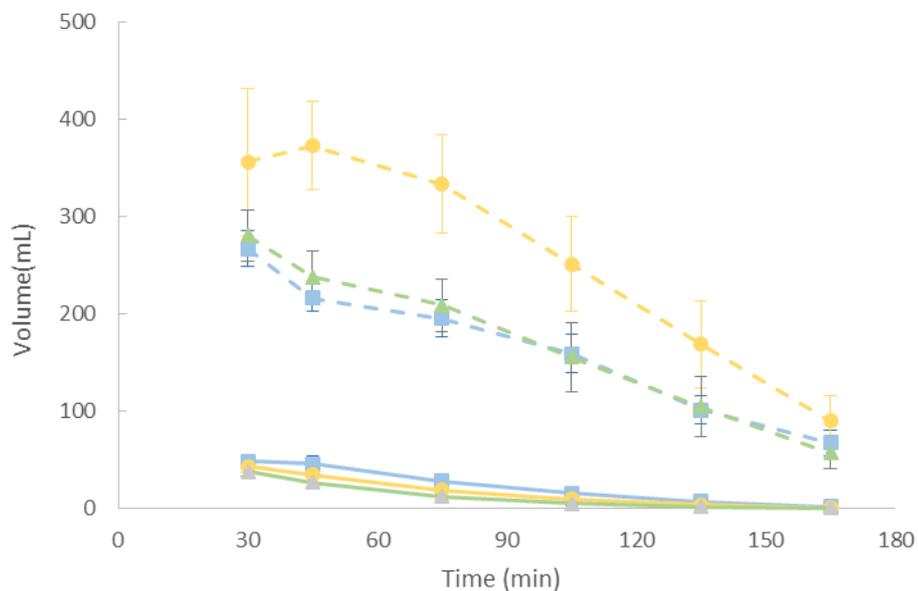


Figure 15 - Estimation du volume postprandial des phases solide (lignes pleines) et liquide (lignes pointillées). Volumes moyens ( $\pm$ SEM, marqueurs SEM plus petits que la taille du symbole pour la phase solide) après consommation de trois repas de volume égal contenant la même quantité de pain et le même volume d'eau (■), de thé (▲) ou de jus de citron (●).

Ce travail a clarifié des aspects importants du processus digestif de l'amidon, un nutriment clé de la nutrition humaine. Il confirme le rôle prépondérant joué par l'HSA lors de la digestion gastrique *in vitro* et la littérature montre que son rôle *in vivo* semble tout aussi important. De plus, en présence d'HSA, des courbes quasi-superposables ont été obtenues quelle que soit la cinétique de réduction du pH gastrique imposée. Ceci, révèle la grande efficacité de cette enzyme et justifie la nécessité de réévaluer sa contribution au processus digestif de l'amidon. Différentes stratégies d'atténuation du taux d'hydrolyse de l'amidon ont aussi été testées *in vitro*, permettant d'élaborer des stratégies diététiques visant à améliorer la réponse glycémique provoquée par ces aliments, qui ont ensuite été testées dans une étude humaine. Les résultats de la première partie de cette étude confirment que l'abaissement du pH d'un repas riche en amidon peut atténuer significativement la réponse glycémique. En raison de la nature semi-contrôlée de cette partie de l'étude, nos résultats suggèrent fortement que cette stratégie simple et efficace pourrait facilement être adoptée dans les repas de la vie quotidienne. De plus, à la suite d'une étude IRM réalisé dans la deuxième partie de cette étude, avec les mêmes repas, la réduction de la réponse glycémique due à l'acidité des repas semble provenir (au moins en partie) de l'inhibition de la  $\alpha$ -amylase salivaire, et non d'un ralentissement de la vidange gastrique.

En conclusion, ce travail a mis en évidence le rôle de l' $\alpha$ -amylase salivaire dans la digestion des aliments riches en amidon, et a fait émerger de nouvelles perspectives pour le développement de stratégies visant à améliorer la réponse glycémique aux aliments riches en amidon. Une stratégie simple basée sur l'abaissement du pH d'un aliment ou d'un repas riche en amidon (en réduisant le pH de l'aliment ou du repas, en combinant des aliments riches en amidon avec des boissons acides par exemple) pourrait constituer une approche simple et efficace pour atténuer la réponse glycémique aux repas riches en amidon.



## LIST OF ABBREVIATIONS

<b>AUC</b>	Area under the curve of glucose concentration versus time
<b>eSGF</b>	Simulated Gastric Fluid electrolyte solution
<b>eSIF</b>	Intestinal Fluid electrolyte solution
<b>GF</b>	Gluten-free
<b>GI</b>	Glycemic Index
<b>GL</b>	Glycemic load
<b>HSA</b>	Human salivary $\alpha$ -amylase
<b>MRI</b>	Magnetic Resonance Imaging
<b>RDS</b>	Rapidly digested starch
<b>RS</b>	Resistant starch
<b>SDS</b>	Slowly digested starch



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## INTRODUCTION

All of us spend over three quarters of our lives in the postprandial state (Vors, Nazare, Michalski, & Laville, 2014). However, if the link between the fasting metabolism and health has been extensively investigated, less attention has been dedicated to the study of the postprandial state. A PubMed search for articles containing the terms “fasting” or “postprandial” in the title reveals the current gap between studies of these two states (Figure 16). Although research on the postprandial state has drawn increasing attention since the 1970’s, at the time of writing this manuscript, there are still twice as much articles addressing fasting-related subjects.

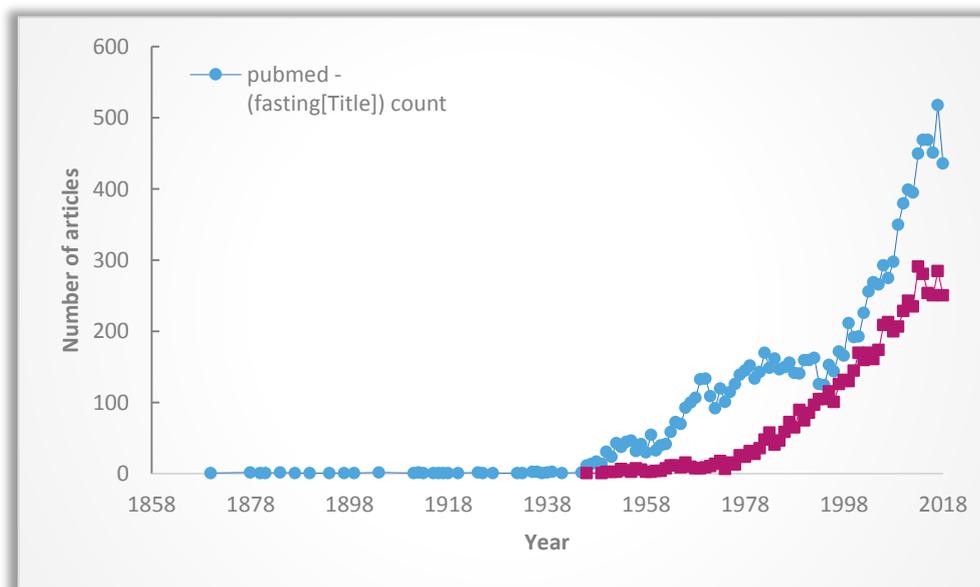


Figure 16 - Published research about the fasting and postprandial states. Results of a PubMed search for articles containing the term "fasting" (●) (total of 12131 articles) and/or "postprandial" (■) in the title (total of 6054 articles). Retrieved on September 10<sup>th</sup> 2018.

The postprandial state is a dynamic state resulting from the ingestion and digestion of a meal, and lasting 4 to 5 hours depending on the type of nutrient. It is distinguished from the post-absorptive state (which corresponds to the 6h period following the postprandial state) and the fasted state (which follows the post-absorptive state, 10-12 hours after the last meal). These states succeed and can overlap each other (Vors, Nazare, Michalski, & Laville, 2014).

If we think about it, our skill of precisely sourcing each nutrient from the miscellanea in our plates is, by itself, a fascinating field of research. In our current society, the consumption of 3 to 4 meals per day means that we rarely leave the postprandial state (Vors, Nazare, Michalski, &

Laville, 2014), and the mismatch between nutrient need and supply has placed diet as the second-leading global risk factor for deaths (Gakidou, et al., 2017). It has also become clear that not only the type and quantity of nutrients ingested are important, but also their rates of digestion and absorption. This is valid for proteins, for instance, as their resistance to digestion and digestion rate can influence the mechanisms that trigger food allergies (Bøgh & Madsen, 2016) and the efficiency of post-prandial protein metabolism (Dangin, Boirie, Guillet, & Beaufrère, 2002; Koopman, et al., 2009; Walrand, et al., 2016). The same holds true for carbohydrates, with a growing body of evidence relating increased postprandial glucose levels with an increased risk of type-2 diabetes (Bhupathiraju, et al., 2014), coronary heart disease in women (Dong, Zhang, Wang, & Qin, 2012; Mirrahimi, et al., 2012), breast cancer (Mullie, Koechlin, Boniol, Autier, & Boyle, 2016), as well as obesity related complications (Schwingshackl & Hoffmann, 2013).

Carbohydrates play a key role in human nutrition, supplying up to 45% of the energy requirements in developed countries, and up to 85% in developing countries. They can be found in the form of sugars (monosaccharides and disaccharides), starch or non-starch polysaccharides (Stylianopoulos, 2012). Starch is the most important, abundant, and digestible polysaccharide. It is exclusively made up of glucose residues, and accounts for 20 % to 50 % of the total energy intake (Stylianopoulos, 2012), therefore playing a major role in the post-prandial glycaemic responses elicited by our diets.

THE OVERALL AIM OF THIS PHD PROJECT WAS:

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**TO STUDY THE DIGESTION OF STARCH-RICH MEALS WITH DIFFERENT DIGESTION KINETICS *IN VITRO* AND IN HUMANS**

More precisely, my work has mostly focused on the following two objectives:

- I. Re-evaluate the contribution of salivary  $\alpha$ -amylase to the hydrolysis of starch-rich meals using semi-dynamic *in vitro* digestions.
- II. Test some of our working assumptions in humans and assess some physiological responses associated to the consumption of starch-rich meals through blood glucose measurements, visual analogue scales, and magnetic resonance imaging (MRI).

THE PHD PROJECT – COLLABORATIONS AND FUNDING

---

This PhD was a multidisciplinary project carried out at the **GMPA** research unit (Génie et Microbiologie des Procédés Alimentaires, UMR782 INRA - AgroParisTech, Thiverval-Grignon, France) in collaboration with the **IR4M** research unit (Imagerie par Résonance Magnétique Médicale et Multi-Modalités, UMR8081 Université Paris Sud - CNRS, Orsay, France), the **SHFJ** (Service Hospitalier Frédéric Joliot, Orsay France) and the **Avicenne Hospital - CEFRED** (Centre d'exploration fonctionnelle et de rééducation digestive, Service de gastro-entérologie, Hôpital Avicenne, Bobigny Cedex, France). My PhD was financed by the 2015 IDEX (Excellence Initiative) of the Paris-Saclay University.

During my master studies in Food Innovation and Product Design I completed my final internship in the GMPA lab, studying the influence of bread formulation and production process on its digestion profile. This work was the starting point of the PhD and part of the results presented in the first article in this manuscript were obtained during my internship and then completed during the PhD.

ORGANIZATION OF THE MANUSCRIPT

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This manuscript is organized into 4 parts:

- I. State of the art – a bibliographic review addressing key fundamental aspects of the subject of study namely: an overview of the properties of starch, its digestion process, the tools that can be employed in the study of digestion (*in vitro* and *in vivo*), with a focus on MRI studies, and an overview of the postprandial impact of starch rich foods. In order to draw the reader's attention to specific topics that were studied within this PhD project, paragraphs that contain important related information were identified with a vertical banner as exemplified below:

|| Paragraph text

- II. *In vitro* studies – this section is divided into 4 chapters, each consisting of a research article reporting the results of *in vitro* experiments that were conducted in preparation of the *in vivo* studies.
- III. *In vivo* studies – this section starts with a brief description of the preparatory steps that preceded the clinical study and of the configuration of this study. The presentation of the results is then divided into two chapters.
- IV. Conclusions and perspectives.



I

# STATE OF THE ART



## 1. THE PROPERTIES OF STARCH AND STAPLE STARCH-RICH FOODS - AN OVERVIEW

### 1.1. COMPOSITION OF STARCH

Starch is made up of two types of polymers, amylose and amylopectin. Both polymers can be found in cereals and other common foods, with amylose comprising 15-20% and amylopectin 80-85% of total starch (as reviewed by Stylianopoulos, 2012). The structural differences between these two polymers (Figure 17) contribute to significant differences in starch properties and functionality. Amylose is considered an essentially linear polymer composed almost completely by  $\alpha$ -1,4-linked glucose residues (D-glucopyranose). It often has a helical structure. The interior of its helix is hydrophobic and therefore has the ability to form complexes with free fatty acids, fatty acid components of glycerides, some alcohols and iodine. Another well-known property of amylose is its capability of forming a gel after the starch granule has been cooked. Amylopectin is much larger and branched. It is composed of  $\alpha$ -1,4-linked glucose residues in linear segments and  $\alpha$ -1,6-linked glucose residues at branch points. (as reviewed by Tomas & Atwell, 1999).

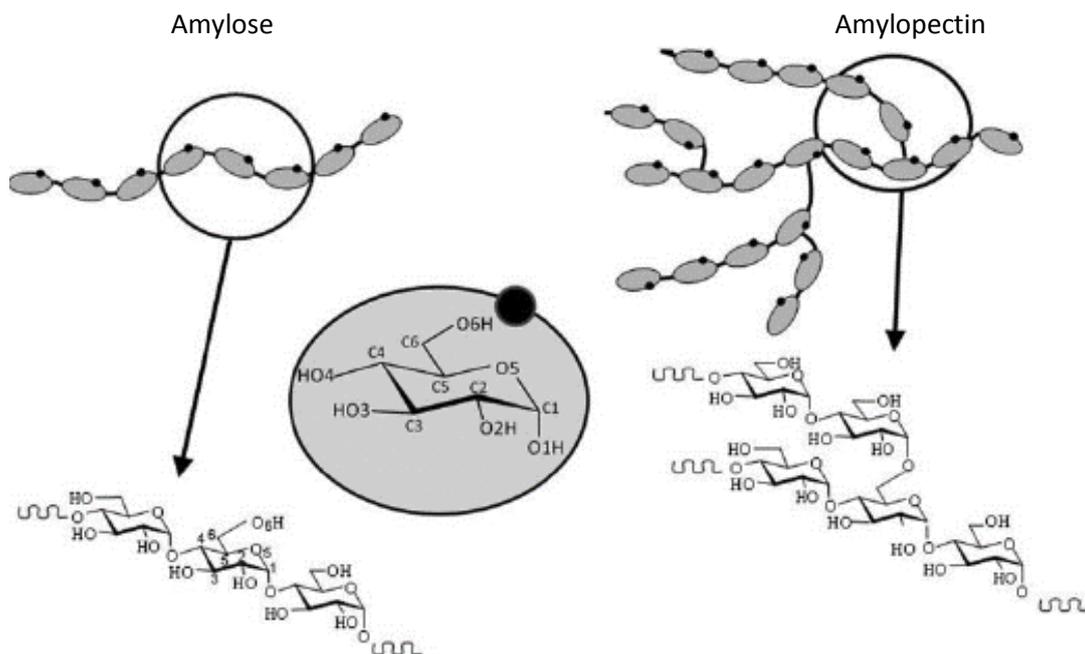


Figure 17 - Basic structural motifs of amylose and amylopectin and extension of the basic motifs to macromolecular structures. Extracted from (Pérez & Bertoft, 2010).

## 1.2. STRUCTURAL CLASSIFICATION OF STARCH

### 1.2.1. NATIVE STARCH AND STARCH GRANULES

Amylose and amylopectin do not exist free in nature, they occur as components of aggregates called starch granules. Even though these molecules are always the main constituents of starch granules, there is great diversity in the structure and characteristics of the native granules from different botanical sources. The arrangement of amylose and amylopectin within the starch granule is not yet fully understood, but, it is known that their configuration in the native granule is not random. (as reviewed by Tomas & Atwell, 1999)

Native starch granules are semi-crystalline. They consist of concentric rings alternating amorphous regions, largely composed of amylose and the branch-sections of amylopectin, and semi-crystalline regions, formed from the linear and more closely packed sections of amylopectin. Granules from cereal starches have surface pores that open onto channels with diameters that range from 5 to 400nm. (as reviewed by Butterworth, Warren, & Ellis, 2011) An illustration of this organization is presented in Figure 18.

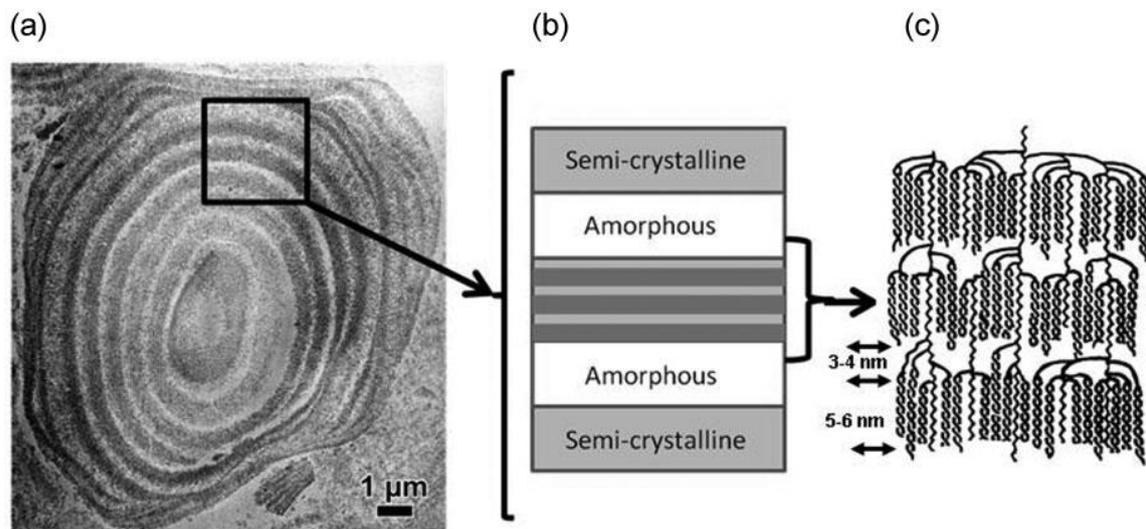


Figure 18 - Schematic representation of the several levels of ultrastructure of starch. (a) Ultrathin section of a waxy maize starch granule treated to exhibit the concentric rings alternating semi-crystalline and amorphous regions. (b) Alternation of semi-crystalline and amorphous rings; (c) clustered model of amylopectin. Extracted from (Pérez & Bertoft, 2010)

### 1.2.2. NON-NATIVE STARCH

---

Most of the starch consumed by humans will have been submitted to a cooking step and/or other processes capable of disrupting, to a greater or lesser extent, the native structure of the starch granule (Butterworth, Warren, & Ellis, 2011).

#### GELATINIZED STARCH

When heated in the presence of water, starch undergoes an irreversible order-disorder transition called gelatinization. Various changes can be observed in the starch granule during this process. Globally, the granules swell, absorb water, lose crystallinity, and leach amylose. (as reviewed by Donald, 2004)

Initially, swelling can be reversible, but as heating proceeds and the native granule structure is progressively altered, this process becomes irreversible. As temperature rises, the starch polymers undergo strong vibratory motion breaking intermolecular bonds and allowing their hydrogen bonding sites to engage additional water molecules further promoting the separation of the starch chains. (as reviewed by Cauvain & Young, 2007). Water uptake is initiated in the amorphous regions, and since amylopectin molecules in the edges of crystalline regions bond with the constituents of the amorphous regions, the swelling of the amorphous areas imposes a stress upon the amylopectin crystallites. Ultimately, this will cause the dissociation of the corresponding amylopectin double helices, further impairing the granule integrity. (as reviewed by Donald, 2004) Continuous heating and water penetration result in an increased randomness, eventually causing complete loss of crystallinity (as reviewed by Cauvain & Young, 2007).

#### RETROGRADED STARCH

Starch retrogradation starts upon cooling of a gelatinized system. It is a process of structural change during which the starch molecules that have been dispersed during gelatinization randomly re-associate to form double helix crystalline structures. Both amylose, which tends to re-crystallize faster, and amylopectin retrograde. (as reviewed by Cauvain & Young, 2007)

### 1.3. STARCH IN FOODS

Starch is the main nutrient in common staple foods. Wheat flour, barley flour, rice, oats and potatoes are composed of 68%, 56%, 53%, 30% and 15% starch, respectively (CIQUAL, 2017). Wheat derived foods such as bread and pasta are major sources of starch in our diets. The study of the dietary habits of over half a million European adults has revealed that these foods can contribute with up to 32.6% of the total carbohydrate intake (Wirfält, et al., 2002). Traditional wheat bread and pasta products share the same basic ingredients (water and wheat flour), and therefore the same main caloric nutrients (starch and protein). However, the different

processing steps that are employed in producing the ready-to-eat final products, result in two types of foods with clear structural differences.

The main constituent of flour is starch. It accounts for approximately 65% of the total materials in traditional wheat flour (14% moisture basis) and plays a crucial role in bread's structure, physical and nutritional properties and quality (as reviewed by Cauvain & Young, 2007). In wheat flour, the size of the starch granules varies from 1-45  $\mu\text{m}$  and two types of shapes, round and lenticular, can be found (as reviewed by Tomas & Atwell, 1999). Wheat starch comprises about 23% amylose and 73% amylopectin (equivalent to 15% and 50% of the flour weight, respectively), and appears predominantly in the intact granular form with a high degree of crystallinity (as reviewed by Cauvain & Young, 2007).

#### 1.3.1. STARCH IN WHEAT BREAD

---

Starch is the main component of bread, a loaf of bread consists of about 50% starch, 40% water and 7% protein. The main ingredients of bread are flour and water, with small quantities of fat, salt, possibly dough improvers and yeast. (Cauvain & Young, 2007)

During the course of bread production, starch undergoes several changes. During the baking step gelatinization occurs but the small quantity of water available in the dough limits the swelling of starch granules, which therefore retain their identities as discrete particles by the end of this step. Nonetheless, upon swelling, the linear amylose fraction becomes more soluble and diffuses into the aqueous phase, forming a concentrated solution. Immediately after baking, when bread starts to cool, the amylose molecules in this solution rapidly retrograde, re-associating by hydrogen bonding, to set up an insoluble gel that contributes to the loaf structure. (as reviewed by Cauvain & Young, 2007)

At a macroscopic level, bread is characterized as a solid foam. At a molecular level, the solid continuous phase is composed in part of an elastic network of cross-linked gluten molecules and, in part leached starch polymers, both non-complexed and complexed with polar lipid molecules. The discontinuous phase is made up of entrapped, gelatinized, swollen, deformed starch granules. These starch granules are partly fused with neighbouring granules. (J. Gray & J. Bemiller, 2003; Hug-Iten, Handschin, Conde-Petit, & Escher, 1999). The microstructure of bread dough and baked bread crumb is presented in Figure 19.

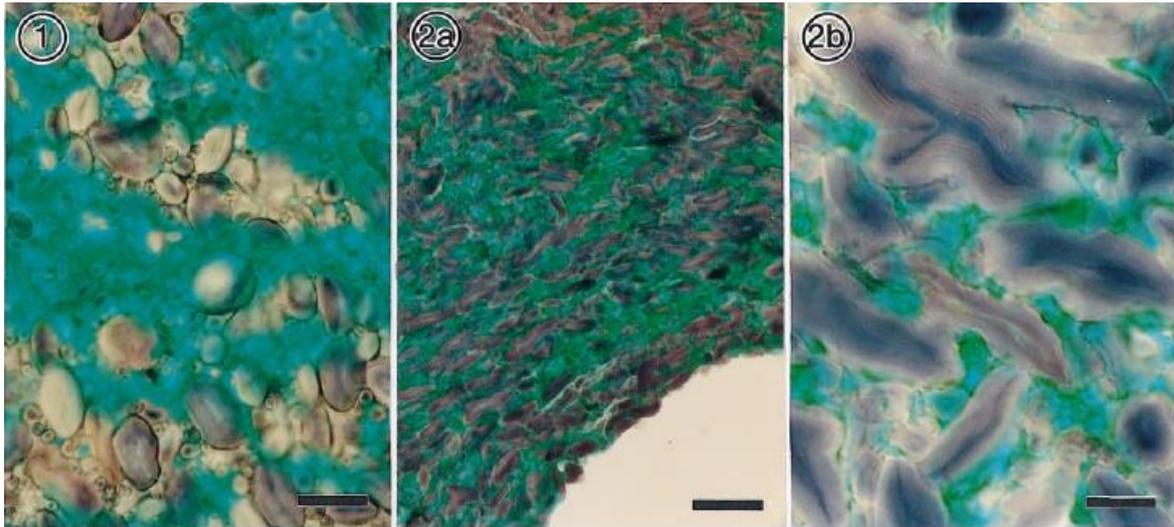


Figure 19 -Light micrograph of a cross-sections of proofed dough and bread crumb stained with Light Green and Lugol's solution. the native starch granules stain slightly violet, amylose and amylopectin stain blue and brown/violet, respectively, with iodine the protein fraction appears green as coloured with Light Green (1) Proofed dough (bar = 25  $\mu\text{m}$ ), the starch granules show the characteristic shape of native wheat starch granules. (2) Bread crumb at two different magnifications (a) (bar = 50  $\mu\text{m}$ ) starch granules appear swollen and elongated, but still retain their granular identity, (b) (bar = 10  $\mu\text{m}$ ) shows that starch granules partly fused with neighbouring granules (Hug-Iten, Handschin, Conde-Petit, & Escher, 1999).

### 1.3.2. STARCH IN WHEAT PASTA

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According to the French table of food composition (2017), cooked wheat pasta is composed of about 68% of water, 22% of starch and 4% of protein. The interior of raw spaghetti strands present a homogeneous somewhat porous structure where starch granules (with a visibly retained shape) embedded in a protein network. Cooked wheat spaghetti exhibits a more heterogeneous microstructure where three concentric zones containing starch that is progressively more gelatinized from the interior to the surface of the strand have been identified (Cunin, Handschin, Walther, & Escher, 1995; Fardet, et al., 1998). The most inner layer contains un- or partially- gelatinized starch granules and a continuous dense protein network still remains (Cunin, Handschin, Walther, & Escher, 1995). The progression of starch gelatinization is prevented in that inner layer because of a limited water absorption in the centre of the strand. The most outer layer consists of a film that contains small openings and cracks. It is made of protein and starch that are no longer discernible from one another with greatly swollen starch granules and leached starch, in close interaction with a diffuse coagulated protein matrix (Cunin, Handschin, Walther, & Escher, 1995; Fardet, et al., 1998). The middle layer consists of an intermediate zone with incompletely swollen starch granules, no leaching and a dense protein

network (Cunin, Handschin, Walther, & Escher, 1995). The microstructure of raw and cooked spaghetti is visible in Figure 20.

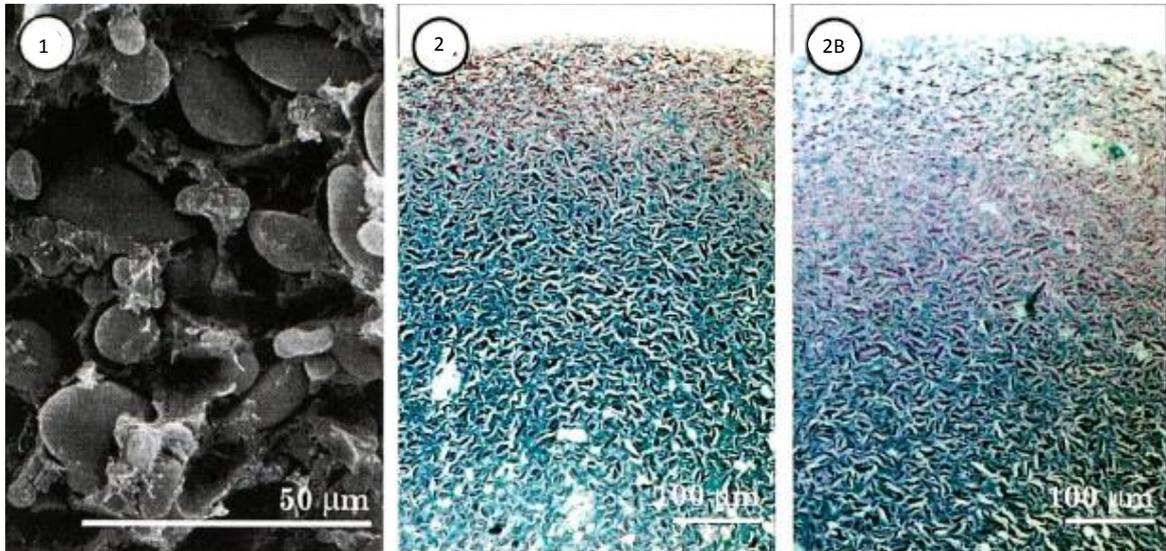


Figure 20 - Micrographs of cross-sections of spaghetti. (1) Scanning electron micrograph, dry spaghetti. Homogeneous and porous structure with starch deeply embedded in a protein matrix is observed (2) Light micrographs, pasta cooked for: (A) 5 min; (B) 13 min (*al dente* time). Cooked pasta was stained with Light Green and Lugol's solution. Three concentric zones are visible. Obtained from (Cunin, Handschin, Walther, & Escher, 1995).

## 2. STARCH IN HUMAN NUTRITION – DIGESTION AND PHYSIOLOGICAL RESPONSE

Because starch is exclusively made up of glucose residues, and accounts for 20 % to 50 % of the total energy intake (Stylianopoulos, 2012), starch-derived glucose is a major determinant of the post-prandial blood glucose concentrations (postprandial glycaemia) elicited by our diets. Maintaining plasma glucose concentration within narrow limits is essential to provide energy and prevent damages to our organs and tissues. Additionally, a growing body of evidence is relating diets that elicit elevated postprandial responses with increased risk of type-2 diabetes, cardiovascular disease and some types of cancer (Bhupathiraju, et al., 2014; Mirrahimi, et al., 2012; Mullie, Koechlin, Boniol, Autier, & Boyle, 2016; O'Keefe & Bell, 2007). Accordingly, there is scientific evidence to support that an optimum diet for health requires consideration of the glycaemic impact of eaten foods in preference to consideration of carbohydrate content alone (Blaak, et al., 2012; Livesey, Taylor, Hulshof, & Howlett, 2008).

Blood glucose concentrations are normally maintained between approximately 3.3 and 8.3 mmol/L throughout the day. This is ensured by a dynamic regulation mechanism. After a meal,

blood glucose concentration increases. In healthy persons, the peak postprandial concentration is usually reached within the first hour and seldom exceeds 8.3 mmol/L. In response to this rise, the pancreas increases its secretion of insulin and suppresses the release of glucagon. As a result, glucose uptake by the muscle and fat tissues is promoted, and glucose release by the liver is limited. Therefore, after consumption of a starch-rich food, blood glucose concentration is the result of (1) the influx of starch-derived glucose, (2) the appearance of endogenously produced glucose, and (3) the rate of glucose removal from the blood stream. The influx of glucose derived from the meal, is generally considered the main factor influencing postprandial glucose response. (Priebe, Eelderink, Wachters-Hagedoorn, & Vonk, 2018). The cause-effect relationship has been well summarized as follows: “the degree to which blood glucose loading exceeds blood glucose clearance determines the acuteness of the net increase in blood glucose concentrations”(Mishra, Hardacre, & Monro, 2012).

Major determinants of the rate of glucose absorption are the gastrointestinal transit kinetics, as well as the rates of starch hydrolysis and starch-derived glucose absorption (Priebe, Eelderink, Wachters-Hagedoorn, & Vonk, 2018). The rate of starch hydrolysis appears to be a predominant factor. Indeed, the positive relationship between the rate at which foods’ starch is being hydrolysed in controlled *in vitro* conditions, and the degree to which they raise blood glucose has been repeatedly acknowledged (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; D. Jenkins, et al., 1982). It is therefore clear that attention should not only be given to the starch content in our diets, but also to its susceptibility to digestive enzymes.

## 2.1. DIGESTION - AN OVERVIEW WITH A FOCUS ON STARCH HYDROLYSIS

The following sections present a brief overview of the digestive process with a focus on aspects that are relevant for the comprehension of the research work that is presented in the subsequent parts of this manuscript. The oral and gastric phases are described in more detail than the later stages of digestion, and more attention is given to the process of starch release and hydrolysis than to other nutrient sources. Some specific features associated to the digestion of breads and pastas, which have been studied during the thesis, are also provided.

### 2.1.1. ORAL PHASE

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Oral food processing can be divided into 4 steps: (1) transportation of the ingested food from the front of the mouth to the teeth; (2) breakdown of food particles through crushing/grinding action of the teeth; (3) as particles are broken down to appropriate size, they are gradually

moved to the back of the oral cavity and manipulated with the tongue to form a bolus; (4) transportation of the bolus to the back of the tongue in preparation for swallowing (Bornhorst & Singh, 2012). During this process, ingested food is not only reduced in size but also lubricated with saliva. Saliva secreted in the oral cavity plays a key role in making chewed food particles adhere to each other as they begin to form a bolus (Bornhorst & Singh, 2013).

Moreover, it is in the oral cavity, as saliva is mixed with the food particles, that the enzymatic degradation of starch is initiated by salivary  $\alpha$ -amylase (HSA), the main enzyme in saliva. This enzyme breaks down amylose and amylopectin into maltose, maltotriose and  $\alpha$ -limit dextrins by cleaving their  $\alpha$ -1-4 glycosidic bonds in (Bornhorst & Singh, 2012).

Researchers who have studied the oral phase of white-wheat bread digestion have observed that chewing times range between 18 and 41 seconds (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000; Jourden, et al., 2016), with an average of 27-28 s for 3 g of bread servings (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000). By the end of this process, salivary impregnation is about 220 g/kg of fresh bread consumed, and chewed bread particles have no clearly defined shape with diameters bi-modally distributed around 30 and 620  $\mu$ m (range: 5-1500  $\mu$ m) (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000; Hoebler, et al., 1998). The smaller particles correspond to starch granules and the larger to fragments of the protein network (Hoebler, et al., 1998).

Investigations of the oral processing of 5.4-g servings of pasta (spaghetti and tortiglioni, with similar dry weight as 3 g of bread) have revealed that mastication times tend to be shorter and saliva impregnation to be lower than for bread: 19-20 s of chewing time and 39 to 70 g of saliva per kg of pasta. The characteristics of pasta boluses appear to be influenced by the shape of pasta and particle size tends to be higher than for bread, with characteristic sizes between approximately 1 to 5-7 mm (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000).

Specific characteristics of saliva before (basal) and after (activated) chewing of bread and pasta are presented in Table 2, showing significant differences in salivary flow and osmolarity (Hoebler, et al., 1998).

Table 2- Characteristics of saliva (mean  $\pm$  SEM) before (basal) and after (activated) chewing starch-rich foods (Hoebler, et al., 1998).

	SALIVA	BREAD	COOKED SPAGHETTI
<b>OUTFLOW (G/MIN)</b>	Basal	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1
	Activated	1.3* $\pm$ 0.1	1.1* $\pm$ 0.1
<b>PH</b>	Basal	7.1 $\pm$ 0.1	7.1 $\pm$ 0.1
	Activated	7.4 $\pm$ 0.1	7.4 $\pm$ 0.1
<b>OSMOLARITY (MOSMOL/KG)</b>	Basal	66.3 $\pm$ 4.5	61.4 $\pm$ 3.0
	Activated	80.8* $\pm$ 5.7	59.3* $\pm$ 2.7
<b>AMYLASE ACTIVITY (MKAT/L)</b>	Basal	3122 $\pm$ 5.14	4587 $\pm$ 737
	Activated	3500 $\pm$ 823	3983 $\pm$ 647

Asterisk (\*) denotes statistically significant different from basal saliva for a given parameter,  $p < 0.05$ .

Despite its short duration, the oral processing of bread results in about 31% of starch granules being released from the gluten network (and therefore more susceptible to enzymatic action), and in the hydrolysis of 13% of bread starch into oligosaccharides. For pasta, the lower salivary impregnation and the higher particle size are less favourable to amylolysis. Therefore, hydrolysis is limited mostly to the surface of particles and results in the hydrolysis of about 9% of the total starch into oligosaccharides. (Hoebler, et al., 1998)

#### 2.1.2. GASTRIC DIGESTION AND EMPTYING

When they are swallowed, the food boli formed in the mouth reach the stomach via the oesophagus. The gastric phase of digestion has been less studied than the oral phase due to the limited accessibility. However, expanding knowledge on this digestive step is essential to assess the bioavailability of nutrients in the gastrointestinal tract. (Kong & Singh, 2008)

Four anatomic regions can be distinguished in the stomach: fundus, body, antrum and pylorus (Figure 21). The term proximal stomach is often used in reference to the fundus and body regions, and distal stomach is used in reference to the antrum and pylorus. (Kong & Singh, 2008)

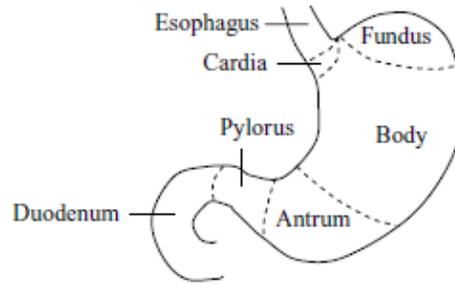


Figure 21 - Diagram of the stomach showing the different regions (Kong & Singh, 2008)

The main function of the stomach is to store the food ingested during a meal and to regulate its release into the duodenum (M. E. Smith & Morton, 2011) but also to churn and mix the food with gastric secretions forming a multiphase slurry called chyme (Kong & Singh, 2008; M. E. Smith & Morton, 2011). Additionally, the stomach has also range of other functions (M. E. Smith & Morton, 2011):

- exocrine: secretions of digestive juices, collectively known as gastric juice, into the stomach lumen,
- paracrine: mainly through the secretion of histamine that stimulates gastric acid secretion,
- endocrine: the major endocrine secretion is gastrin, an hormone which acts both locally on the stomach smooth muscle and mucosa to stimulate gastric motility and acid secretion, and distally on the intestines, pancreas and liver.

In the fasted state, resting gastric volume is close to 20 mL (Grimm, et al., 2018), and the pH is  $\leq 2$  (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Kalantzi, et al., 2006; Malagelada, Go, & Summerskill, 1979; Troost, Steijns, Saris, & Brummer, 2001). During consumption of a meal, the stomach expands to accommodate the boluses that enter the stomach and the gastric juice that is secreted. The volume expansion is adjusted as a function of the gastric contents (Kwiattek, et al., 2009) and it is this flexibility that confers the stomach its reservoir function (Kong & Singh, 2008). Depending on the properties of the meal, postprandial pH increases to the range of 4.5 to 7 (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Kalantzi, et al., 2006; Malagelada, Go, & Summerskill, 1979; Troost, Steijns, Saris, & Brummer, 2001).

Mixing of gastric contents can result in the formation of a homogeneous mass or in the formation of layers (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Marciani, et al., 2013). The occurrence of layering effects depends on the properties of the meal. It is possible to distinguish at least 3 behaviors: non-dissociated boluses that gather in the lower part of the stomach (Figure 22A), separation of high water from high fat phases (Figure 22B), and sedimentation of food particles (Figure 22C) (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Marciani, et al., 2013). Simultaneously, the pH is lowered and food keeps being broken down both physically and chemically, due to the peristaltic movements of the stomach walls and presence of gastric secretions.

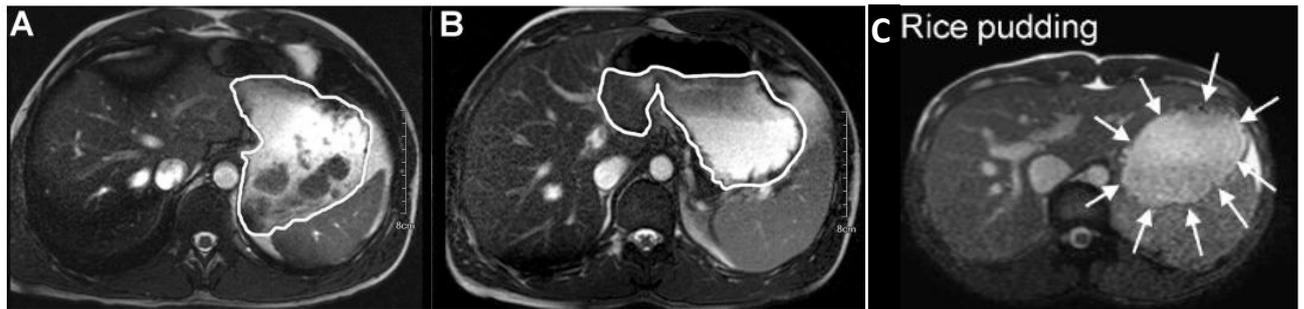


Figure 22 - Examples of MRI scans of the abdominal region showing intra-gastric layering effects. The gastric region is contoured by a white line in (A) and (B) and identified by white arrows in (C). In (A) it is possible to observe individual boluses of cheese that appear as dark regions. In (B) the separation of two liquid phases is visible with aqueous and high fat layers that appear as bright and darker regions, respectively. Figure (C) illustrates the layering of a semi-solid rice pudding meal resulting in the formation of a particulate sediment (darker layer at the bottom) below a more homogeneous, higher water content phase (brighter layer at the top). (A) and (B) were obtained from (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013), (C) was obtained from (Marciani, et al., 2013).

Two key components of gastric juice secreted into the stomach are gastric acid, and digestive enzymes (human gastric lipase and pepsin) (Rumsey, 2005).

The gastric acid (HCl) has a maximum concentration of 160 mmol/L (pH 0.8) (Koziolek, Garbacz, Neumann, & Weitschies, 2013). Its secretion causes acidification of the gastric contents, and the resulting pH reduction kinetics is dependent on the buffering capacity of the meal. It has been observed that it can take anywhere from 60 to 200 min to reach  $\text{pH} \leq 2$  (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Kalantzi, et al., 2006; Malagelada, Go, & Summerskill, 1979; Troost, Steijns, Saris, & Brummer, 2001). Typical examples of gastric pH reduction kinetics are presented in Figure 23A for a liquid and a solid meal.

The gastric lipase has an optimal pH at 5.4 and is therefore more active during the first half of gastric digestion. It initiates the hydrolysis of ingested lipids, with extents up to about 10 to 25% depending on the meal and lipid types (Carriere, Barrowman, Verger, & René, 1993). The gastric

protease, pepsin, is responsible for a limited extent of protein hydrolysis, typically up to 10% of the peptide bonds (Norton, Wallis, Spyropoulos, Lillford, & Norton, 2014). It is secreted into the lumen in an inactive form (pepsinogens) which is then activated in the presence of HCl (Rumsey, 2005). HCl has a double action, it causes proteins to unfold or uncoil due to the breakage of hydrogen and electrostatic bonds, and enables the activation of two pepsin molecules (S. Gropper & J. Smith, 2013; Piper & Fenton, 1965). Human pepsin starts being active below pH 5.5 and exhibits maximum activity at pH 2 (Piper & Fenton, 1965). The products of gastric proteolysis consist of a mixture of large polypeptides, oligopeptides and only few free amino acids (S. Gropper & J. Smith, 2013).

During gastric digestion, starch hydrolysis by salivary amylase can continue until the gastric acid penetrates the food bolus and lowers the pH sufficiently to inactivate the enzyme (S. Gropper & J. Smith, 2013). This occurs at pH values ranging from less than 3.0 and up to 4.0 both *in vivo* and *in vitro*, depending on factors such as the type of meal (M. Fried, S. Abramson, & J. H. Meyer, 1987). Considering the gastric acidification rates reported above, reaching this inactivation threshold might take well more than one hour. Accordingly, data from human studies show that HSA can remain active in the stomach long after the short oral processing phase, and can even contribute to about 14% of the total amylolytic activity at the small intestine stage over a 3h post-prandial period (M. Fried, S. Abramson, & J. H. Meyer, 1987). This is also consistent with another human study conducted with a liquid test meal showing that gastric pH can be as high as 5.0 at half-emptying time (Figure 23B) (Carrière, Renou, Ville, Grandval, & Laugier, 2001). These data clearly indicate that HSA can be responsible for hydrolysing an important fraction of starch before, and even after, the chyme is emptied from the stomach. An interesting work from the early 20<sup>th</sup> century, also conducted in humans, led to the conclusion that up to 76% of the starch in mashed potatoes and 59% of that in bread is hydrolysed into maltose by HSA in the stomach (Bergeim, 1926). Since no other amylase is present at this stage, only that of salivary origin can be responsible for the enzymatic hydrolysis of starch during gastric digestion (Bornhorst & Singh, 2012). Nonetheless, these works seem to have been forgotten, or missed, within the scientific community, considering that the extent of HSA's contribution to starch digestion remains unclear (Butterworth, Warren, & Ellis, 2011) and that the final digestive stage (which occurs in the small intestine) is often considered of higher importance (des Gachons & Breslin, 2016).

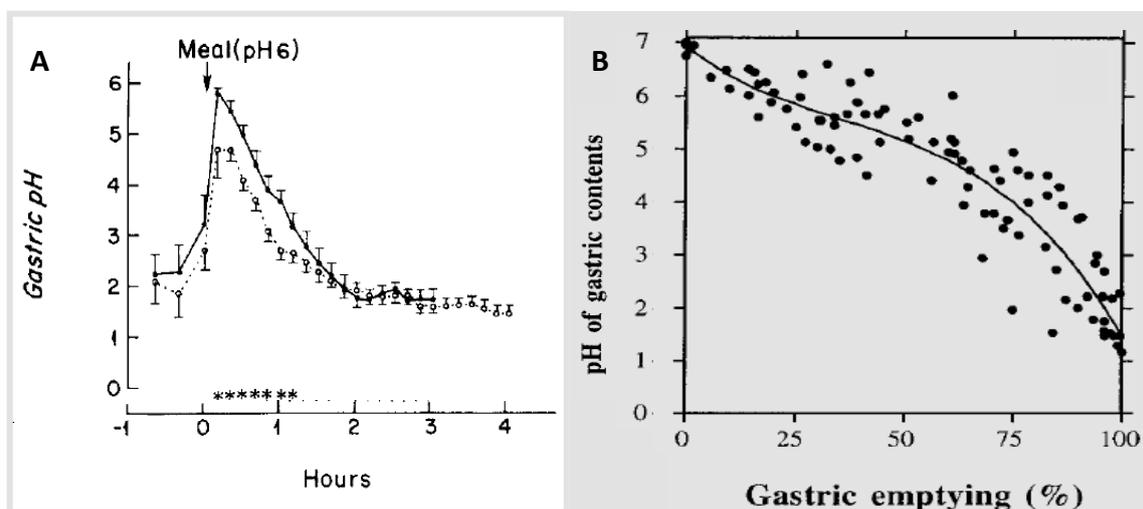


Figure 23 – Postprandial intragastric pH. (A) - Gastric pH after a solid meal (dashed line) and a homogenized meal (full line) of equivalent nutrient content. Mean  $\pm$  SEM, n=6. (Malagelada, Go, & Summerskill, 1979) (B) Variations in the pH of the gastric contents after the ingestion of the liquid test meal as a function of gastric emptying. Experimental values from ten individual experiments (●) and model (full line) (Carrière, Renou, Ville, Grandval, & Laugier, 2001).

Gastric emptying is a gradual process that is influenced by both the physical and chemical properties of the meal. Regarding the physical properties of the meal, key aspects include the state of the meal (liquid vs. solid) (Marciani, et al., 2013; Siegel, et al., 1988) and the size of food particles (Olausson, et al., 2008; Vincent, et al., 1995). Typical examples of gastric emptying patterns obtained with liquid and solid meals are presented in Figure 24.

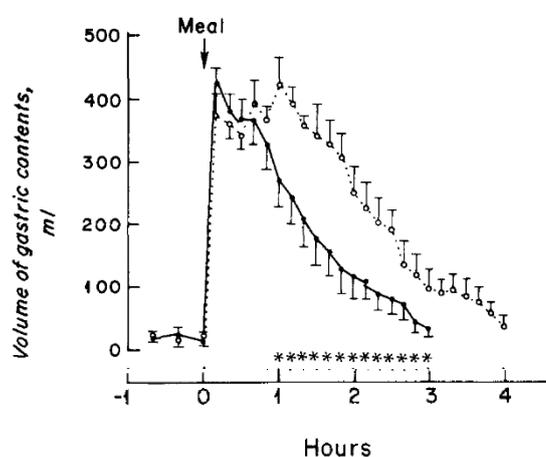


Figure 24 - Postprandial volume of gastric contents after a solid meal (dashed line) and a homogenized meal (full line) of equivalent nutrient content. Mean  $\pm$  SEM, n=6. Obtained from (Malagelada, Go, & Summerskill, 1979).

Generally, it has been observed that the liquid phase is emptied faster than the solid phase (Marciani, et al., 2013; Siegel, et al., 1988), and that particles are usually emptied with fluids when they have been degraded to a diameter of about 2 mm or less (S. Gropper & J. Smith, 2013). Nonetheless, even for particles smaller than 2 mm, variations in particle size appear to influence the rate of gastric emptying (Vincent, et al., 1995). Additionally, for large particles (> 2 mm), increased resistance to fracture (breaking strength > 0.65N) has also been linked with a delayed exit from the stomach (Marciani, Gowland, Fillery-Travis, et al., 2001). Other physical properties such as meal viscosity (Marciani, et al., 2000) and volume (Kwiatek, et al., 2009) are of minor relevance. The chemical properties of meals, however, appear to exert a predominant impact, particularly the nutrient density. Overall, it seems that the stomach adapts to the physical and chemical properties of the meals to maintain a constant rate of nutrient delivery to the small bowel (Calbet & MacLean, 1997; Hunt, Smith, & Jiang, 1985; Kwiatek, et al., 2009). In this context, it is caloric density that appears most relevant, while the nature of calories seems to play a minor role (Calbet & MacLean, 1997).

During my PhD we have gathered gastric emptying data from numerous studies. A plot of gastric emptying, expressed in calories per minute as a function of caloric density of the meal, is presented in Figure 25 to illustrate this effect: regardless of meals properties, gastric emptying rate fall between 1.5 and 3 kcal per min for almost all meals. A total of 15 studies were included in this analysis. They cover a wide range of physical and chemical meal properties as well as methods for gastric volume determination (Cecil, Francis, & Read, 1999; French & Read, 1994; Fruehauf, et al., 2009; Grimm, et al., 2018; Jones, et al., 1997; Kunz, Feinle, Schwizer, Fried, & Boesiger, 1999; Kwiatek, et al., 2009; Kwiatek, et al., 2006; Lavin, French, & Read, 2002; Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Marciani, Gowland, Spiller, et al., 2001; Mourot, et al., 1988; K. Murray, et al., 2014; Toepfer, et al., 1999; Vesa, et al., 1997; Vesa, Marteau, Briet, Boutron-Ruault, & Rambaud, 1997). We did not apply any filter in this selection although we excluded studies that resorted to invasive methods to monitor gastric volumes.

An interesting description of the process of gastric emptying was presented by the team of Kwiatek (Kwiatek, et al., 2009). Their results appeared to suggest that the early phase of gastric emptying provides a meal sample to the small intestine in conditions (amount and speed) determined by the meal volume. This would influence the small intestine's feedback response so that, after a short delay, subsequent nutrient delivery is adapted as a function of the caloric

density of the emptied chyme (i.e. a greater volumic emptying flux for chyme with lower caloric density).

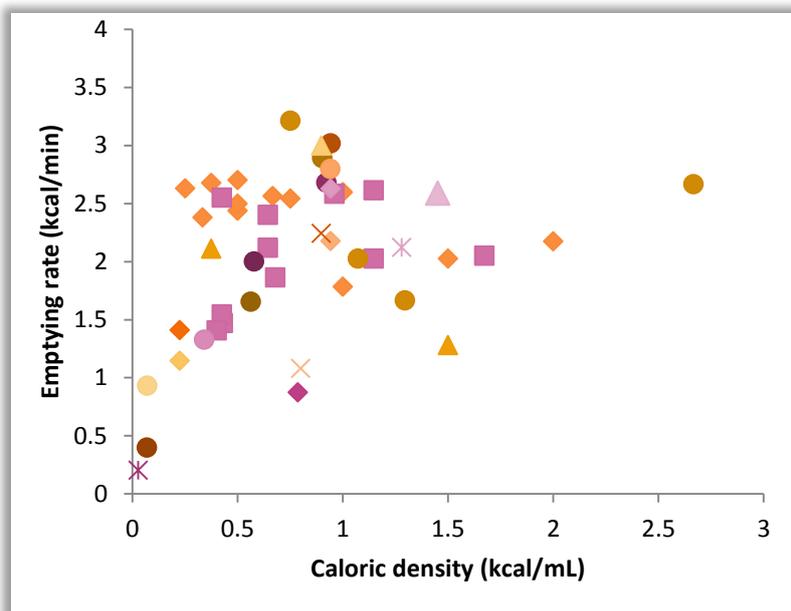


Figure 25 - Emptying rate in kcal/min plotted as a function of caloric density of the meal. Results from 15 studies investigating different meals with distinct physical and chemical properties and using non-invasive methodologies to determine gastric volume. (Cecil, Francis, & Read, 1999; Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999; French & Read, 1994; Fruehauf, et al., 2009; Grimm, et al., 2018; Jones, et al., 1997; Kwiatek, et al., 2009; Kwiatek, et al., 2006; Lavin, French, & Read, 2002; Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Marciani, Gowland, Spiller, et al., 2001; Mourot, et al., 1988; K. Murray, et al., 2014; Toepfer, et al., 1999; Vesa, et al., 1997; Vesa, Marteau, Briet, Boutron-Ruault, & Rambaud, 1997).

A brief overview of some of the findings of researchers who assessed the gastric emptying and the pH during digestion of starch-rich meals can be found in Table 2.

### 2.1.3. INTESTINAL DIGESTION

The key functions of the small intestine are the hydrolysis and absorption of nutrients. This organ is over 6 m in length and has a luminal diameter of approximately 4 cm. Starting from the pyloric region, three regions are distinguished: the duodenum (first 20 cm), the jejunum (approximately 2.5 m), and the ileum. (Rumsey, 2005)

As the chyme reaches the duodenum, the pH of the digested material is neutralized quickly by the secretions coming from the Brunner's glands (located in the duodenum) and the pancreas.

Table 3- Summary of findings on the GER and pH variation after consumption of bread-rich meals

Test meal		Measured parameter and method	Results (Source)
Composition	Macronutrients		
<b>1 cup of tea + 15 g sugar + 70 g white bread</b>	(not available)	Gastric pH using a radiocapsule ingested with the test meal	Gastric pH range after test meal First min.: 4.0 - 5.0; at 30 min.: 1.6 - 2.5 1-1.5h: 1.3 - 1.7; 1.5-3h: 1.0 - 1.5 (Dedlovskaya, 1968)
<b>170 g hamburger + 2 slices of bread + 57 g hash brown potatoes + 1 tbsp each of ketchup and mayonnaise + 28 g each of tomato and lettuce + 227 g milk</b>	1000 kcal (macronutrient profile not available)	Gastric pH monitored with a radiocapsule	Gastric pH after test meal: First min.: 5 28 min.: 4 56 min.: 3 107 min.: 2 (Dressman, et al., 1990)
<b>125 g steak + 200-250 g boiled potatoes + 200-250 g vegetables + 50 g salad; 200 mL dessert; 200 mL water</b>	(not available)	Gastric pH using a pH recording system with electrodes	Gastric pH after test meal: First min.: 4.5 1h: ≈3.5 2h: ≈1.5 (Gardner, Ciociola, & Robinson, 2002)
<b>155 g whole meal barley bread + 8.8 g butter + 18.9 g cheese; 250 ml water; 150 ml coffee or tea</b>	Carbohydrates 50 g Protein 15 g Fat 12 g	Antral cross-sectional area using ultrasonography	Median antral cross sectional area (mm <sup>2</sup> ): 0 min: 257, 15 min: 973, 30 min: 766 ; 105 min: 355 ; 120 min: 340 Half gastric emptying time: 54 min. (Darwiche, et al., 2001)
<b>150 g whole-meal rye bread + ham + 300 ml fun light fruit drink</b>	Carbohydrates 63 g Protein 13 g Fat 5 g	Antral cross-sectional area using ultrasonography	Median antral cross sectional area (mm <sup>2</sup> ): 15 min: 660, 90 min: 416; (Hlebowicz, et al., 2009)
<b>150 g white bread + ham + 300 ml light fruit drink</b>	Carbohydrates 52 g Protein 14 g Fat 0 g	Antral cross-sectional area using ultrasonography	Median antral cross sectional area (mm <sup>2</sup> ): 15 min: 963, 90 min: 523; (Hlebowicz, et al., 2009)
<b>190 g whole meal bread + 34 g raspberry jam; 100 g orange juice</b>	Carbohydrates 98 g Protein 22 g Fat 6 g	Gastric volumes with MRI	Half gastric emptying time: 132 min Baseline gastric volumes at 270 min (Marciani, et al., 2013)

Secretions from Brunner's gland have a pH of approximately 8.2 to 9.3. (S. Gropper & J. Smith, 2013). Pancreatic juice is a solution of sodium bicarbonate containing lipolytic, proteolytic and amylolytic enzymes (Rumsey, 2005). Pancreatic lipase is the main source of lypolytic activity in the small intestine and its action is supported by bile salts, which emulsify dietary lipids and facilitate their absorption at the water-oil interface. Pancreatic proteases (mostly trypsin and chymotrypsin) and  $\alpha$ -amylase are the proteolytic and amylolytic enzymes that act in the

intestinal lumen. The role of these enzymes is to complete, almost entirely, the hydrolysis of nutrients, so that they are ready to be absorbed at the intestinal wall.

Similarly to salivary amylase, pancreatic  $\alpha$ -amylase hydrolyses the  $\alpha$ -1-4 glycosidic bonds in the amylose and amylopectin molecules, but cannot cleave the  $\alpha$ -1-6 bonds at branch points in amylopectin (Lentle & Janssen, 2011). The products of starch digestion in the small intestine lumen are a mixture of maltose, maltotriose and  $\alpha$ -limit dextrins (Lentle & Janssen, 2011).  $\alpha$ -limit dextrins are resistant to  $\alpha$ -amylase action because they are made of 3 to 4 glucose units that contain  $\alpha$ -1,6 glycosidic bonds that were located at branch points in the original starch polysaccharides (McGuire & Beerman, 2013). Therefore, starch digestion is not completed in the lumen, the final step of this process occurs along the intestinal wall.

The other proteolytic and amylolytic enzymes present during intestinal digestion (namely peptidases and disaccharidases) are not free in the intestinal lumen, they are fixed to the enterocytes membranes on the side that faces the interior of the small intestine (Rumsey, 2005). The cell membrane area where these enzymes are located exhibits numerous protrusions, termed microvilli, that extend from the cell into the interior of the small intestine (increasing surface area and consequently promoting efficiency of the digestive process) creating what is called the “brush border”. Thus, enzymes located here are referred to as “brush border enzymes”. It is here that the final step of starch hydrolysis takes place. Maltose and maltotriose are hydrolysed by maltase, and  $\alpha$ -limit dextrins by  $\alpha$ -dextrinase, into the same final product: glucose (S. Gropper & J. Smith, 2013).

Glucose is then absorbed into the blood through the intestinal mucosa due to two important mechanisms: active transport (which requires energy input) and facilitated transport (which is triggered when the glucose concentration in the lumen exceeds the blood concentration) (S. Gropper & J. Smith, 2013). The starch that is not digested in the small intestine (resistant starch) ends in the large intestine where it will be fermented by the intestinal microbiota (Higgins, 2004).

A schematic view of the digestive process of digestible starch is presented in Figure 26.

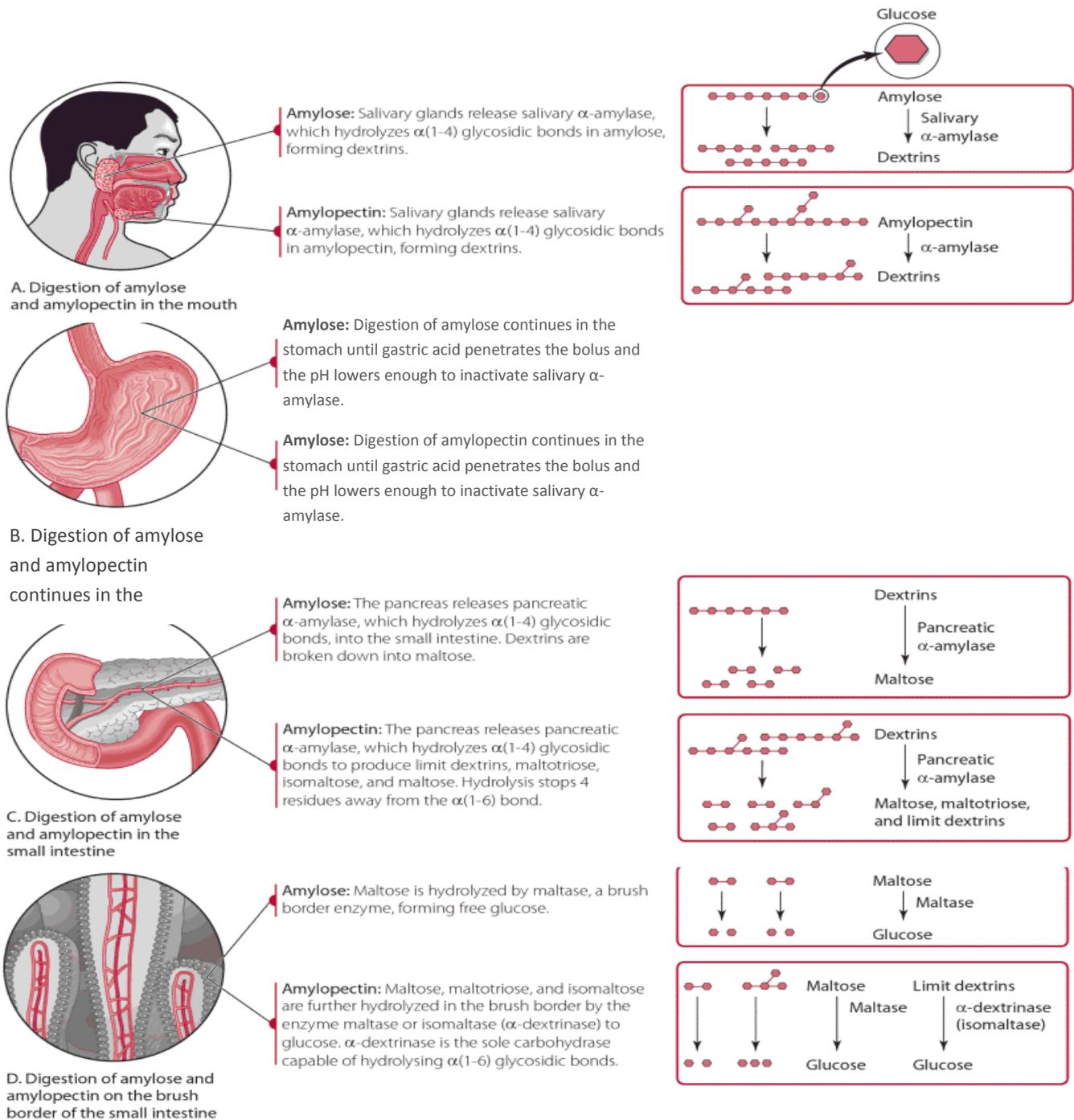


Figure 26- Schematic view of starch digestion. This scheme is originally from (S. Gropper & J. Smith, 2013), the description of the processes that occur in the stomach was modified according to other bibliographic sources.

## 2.2. STARCH DIGESTION RATE – INFLUENCING FACTORS

The rate of starch digestion can vary greatly between different foods (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; D. Jenkins, et al., 1982). Given the relationship between the starch digestion kinetics and the glycaemia response, extensive work has been dedicated to the understanding of the factors that influence starch digestion rate and to the development of strategies to slow it down. The differences between the structural properties of foods can have a major influence because of the potential effects on the accessibility of digestive enzymes. Additionally, there are also factors that can have a direct impact on digestive enzymes (ex.: the presence of inhibitors or the pH).

### 2.2.1. THE STRUCTURAL PROPERTIES OF FOODS

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The structural properties of foods can be examined in numerous ways and at different levels from the molecular scale (ex.: level of starch gelatinization) to the macroscopic scale (ex.: particle size or porosity of foods).

#### 2.2.1.1. EXAMPLES AT A MOLECULAR TO MICROSCOPIC SCALE

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Amorphous areas of the starch granules are generally degraded more easily by  $\alpha$ -amylases than crystalline areas (Tomas & Atwell, 1999). Overall starch digestibility is favoured when (Tester, Karkalas, & Qi, 2004): (a) it is in an amorphous state (especially physically damaged or gelatinized); (b) freely accessible to digestive enzymes rather than entrapped in food particles; (c) not associated with other molecules forming associations or complexes (such as amylose-lipid complexes); (d) not chemically modified in a form that prevents it from acting as a substrate for amylases.

### NATIVE STARCH

The native starch granules are semi-crystalline due to their high degree of organization. This, in association with the presence of a protein and lipid coating at the granule surface, increases the resistance to  $\alpha$ -amylase action; this explains the slow digestion rate of native starch. Different studies have shown that native starch granules appear to be digested from the inside out. This can be explained by the presence of surface pores, which are entrances of channels that reach the near centre (hilum) of the granules, and allow the penetration of water and enzymes into the centre of the granules (Mishra, Hardacre, & Monroe, 2012; Naguleswaran, Li, Vasanthan, Bressler, & Hoover, 2012). An example of the progression of hydrolysis of wheat starch granules is illustrated in Figure 27.

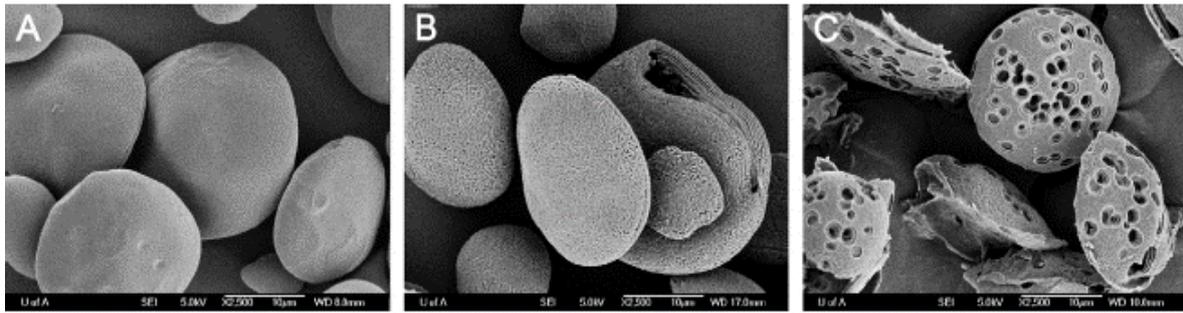


Figure 27 - Scanning electron micrographs of wheat starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A) and 1 h (B), and at 30 °C for 24 h (C). Obtained from (Naguleswaran, Li, Vasanthan, Bressler, & Hoover, 2012)

### GELATINIZED AND RETROGRADED STARCH

As mentioned before, starch gelatinization can be controlled and involves a dramatic loss of structural organization parallel to an increase of amorphous material. As shown in Figure 28, as the swelling and dispersion of starch polysaccharides progresses with the level of gelatinization, the starch therefore becomes increasingly more accessible and less resistant to  $\alpha$ -amylase action. (Mishra, Hardacre, & Monro, 2012)

During retrogradation, amylose chains tend to re-crystallize in an almost irreversible way, hence becoming again very resistant to amylase digestion. Amylopectin's retrogradation is slower and more reversible, so that its digestion by amylase is retarded less than in the case of retrograded amylose (Mishra, Hardacre, & Monro, 2012).

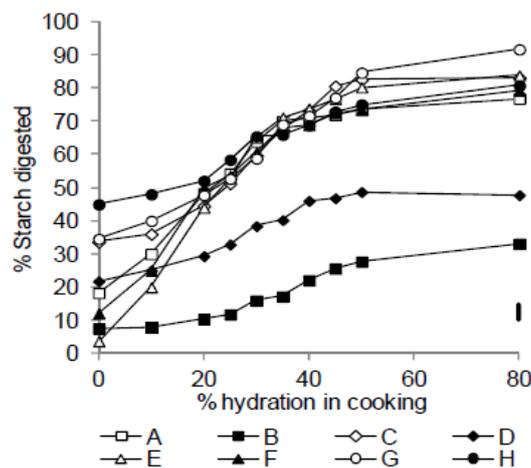


Figure 28 - Effect of structural change in starch granules as a function of hydration during cooking (10 min, 95 °C), on *in vitro* digestion. Digested starch as a % of total starch for seven starches at progressively increasing degrees of hydration (A. Maize; B. High amylose maize; C. Mazaca; D. Pea; E. Potato; F. Rice; G. Tapioca; H. Wheaten corn flour). Obtained from (Mishra, Hardacre, & Monro, 2012)

### OCCLUDED STARCH

In the mature endosperm of most cereals, the cell walls are largely destroyed and the endosperm becomes a protein matrix with implanted starch granules. The density and sealing effect of the protein matrix reduces water uptake during cooking preventing the swelling of the starch granules, which in turn impairs accessibility to digestive enzymes. In species with hard endosperm, the protein matrix is almost continuous, whereas in others, with a soft endosperm, there are discontinuities through which water and enzymes can penetrate into the endosperm. As a result, soft endosperm wheat variants hydrate quicker and present a greater internal surface area of starch for water absorption and enzymatic digestion than hard varieties. (Mishra, Hardacre, & Monro, 2012)

### COMPLEXES

Starch may also form complexes with other molecules that may lead to a structure associated with a reduced digestibility. An example is amylose-lipid complexes, formed when starch is gelatinized in the presence of lipids, which are considered as crystalline and therefore exhibit a lower digestion rate than amylose (but greater than retrograded amylose). (Mishra, Hardacre, & Monro, 2012)

#### 2.2.1.2. EXAMPLES AT A MACROSCOPIC SCALE

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Food processing can modify the structure of the food matrix in numerous ways. This, in turn, can influence the digestibility of foods by, for example, modulating the accessibility of the enzymes to their substrates. Numerous criteria can be used to categorize foods according to their structural properties. An interesting relationship between a global qualitative classification (according to the level of structuration and porosity of foods) and the digestibility of starch has been reported (Mishra, Hardacre, & Monro, 2012). These researchers have quantified the proportions of rapidly digestible starch, slowly digestible starch and resistant starch in a range of foods divided into four categories: (A) little structured porous foods (ex.: extruded and puffed cereal products), (B) somewhat structured, minimally processed foods (ex.: rolled oats), (C) dense structure, low porosity foods (ex.: pasta), and (D) mostly intact, with starch in native tissue structure (ex.: pulses). Their results show that highly processed, little structured and porous foods such as extruded or puffed cereals contain the highest proportion of rapidly digestible starch. This proportion decreases with increased structuration and/or density, and they observed the lowest rapidly digestible starch content for foods in which starch is mostly found in native food structures.

### 2.2.2. DIETARY FACTORS THAT CAN DIRECTLY INFLUENCE ENZYMES INVOLVED IN STARCH DIGESTION

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A simple dietary strategy to achieve a lower glycaemic response is to pair starch-rich foods, or meals, with food products that can somehow exert an inhibitory effect on amylases. Indeed, a similar type of approach has been considered effective in contexts where glycemia management represents an even bigger challenge than in healthy populations. An example is the treatment of type 2 diabetes via the administration of acarbose, an inhibitor of amylolytic enzymes and brush-border glucosidases in the digestive tract (Salvatore & Giugliano, 1996). Some beverages or condiments that are commonly consumed could provide such an opportunity.

Some interesting results have indeed been found *in vitro* for coffee and tea. For coffee, some substances isolated from coffee beans have been reported to exert an inhibitory effect on pancreatic amylase (Funke & Melzig, 2005; Narita & Inouye, 2009). Teas also might be a suitable option as some of them can inhibit salivary (Y. Hara & Honda, 1990; Kashket & Paolino, 1988; Zhang & Kashket, 1998) and pancreatic (Kwon, Apostolidis, & Shetty, 2008; Quesille-Villalobos, Torrico, & Ranilla, 2013; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015)  $\alpha$ -amylases *in vitro*. The same has been observed for tea extracts and purified tea polyphenols (Fei, et al., 2014; Forester, Gu, & Lambert, 2012; K. Hara, et al., 2012; Miao, Jiang, Jiang, Zhang, & Li, 2015; Sun, Gidley, & Warren, 2018; Sun, Warren, Netzel, & Gidley, 2016; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012).

However, results of *in vivo* studies with teas and coffees is unclear. Consumption of caffeinated coffee for example might in fact be linked with impaired blood glucose management (Moisey, Kacker, Bickerton, Robinson, & Graham, 2008). Moreover, investigations on the effect of tea seem to show different effects. In some studies, it has been reported that polyphenols and fibre present in fruits, together with a cup of green tea, have a pronounced lowering effect on postprandial glucose and insulin to bread (Nyambe-Silavwe & Williamson, 2016). In others, as it can be observed in Figure 29, consumption of green tea with a bread-based meal for breakfast, for example, appears to have no glucose lowering effect (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010), and adding green-tea extracts to a starch based confection produced similar results (Sapper, et al., 2016).

Other studies have investigated the effect of condiments. Cinnamon, for example has been reported to attenuate the postprandial glycaemic response to instant cereal (Magistrelli & Chezem, 2012). Another example is cayenne chili, which appears to have no effect on the

glycaemic response but can improve postprandial insulinaemia (Ahuja, Robertson, Geraghty, & Ball, 2006).

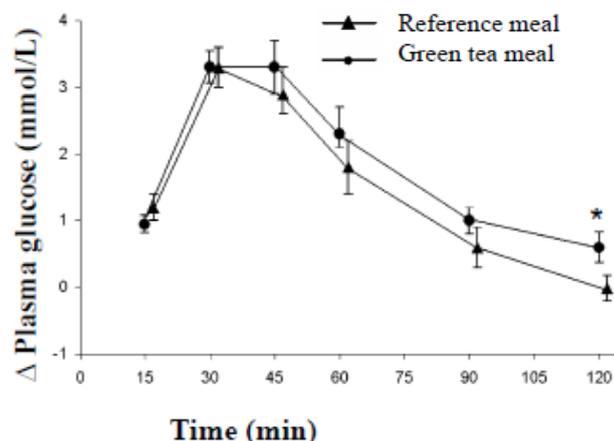


Figure 29 – Effect of green tea on postprandial blood glucose concentrations. Mean ( $\pm$  SEM) incremental postprandial plasma glucose concentrations after ingestion of a meal containing bread and tea ( $\bullet$ ) and a reference meal without tea ( $\blacktriangle$ ). \* Indicates significant difference between the meals ( $n = 14$ ). Obtained from (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010)

One particular strategy that has been gaining increased attention is pairing a starch-rich food/meal with an acidic food (ex.: vinegar). Research in this area has consistently shown that this enables to reduce the glycaemic response in normal subjects by 20% to 50% (Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Leeman, Östman, & Björck, 2005; H. Liljeberg & Björck, 1998; Elin Östman, Granfeldt, Persson, & Björck, 2005; Sugiyama, Tang, Wakaki, & Koyama, 2003). The same holds true for processes that result in the acidification of the food itself (ex: sourdough bread fermentation) (Bo, et al., 2017; Lappi, et al., 2010; H. Liljeberg & Björck, 1996; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009). Additionally, this type of strategy has also proved effective for insulin resistant (Carol S Johnston, Kim, & Buller, 2004), and diabetic subjects (Liatis, et al., 2010; Mitrou, et al., 2010). In Figure 30, two examples are presented to show the attenuation of the glycaemic response in healthy individuals (Figure 30A) and in individuals with type-2 diabetes (Figure 30B).

The consistency of the results obtained in different studies by different research teams has drawn the attention of different research teams who have investigated the possible underlying mechanisms.

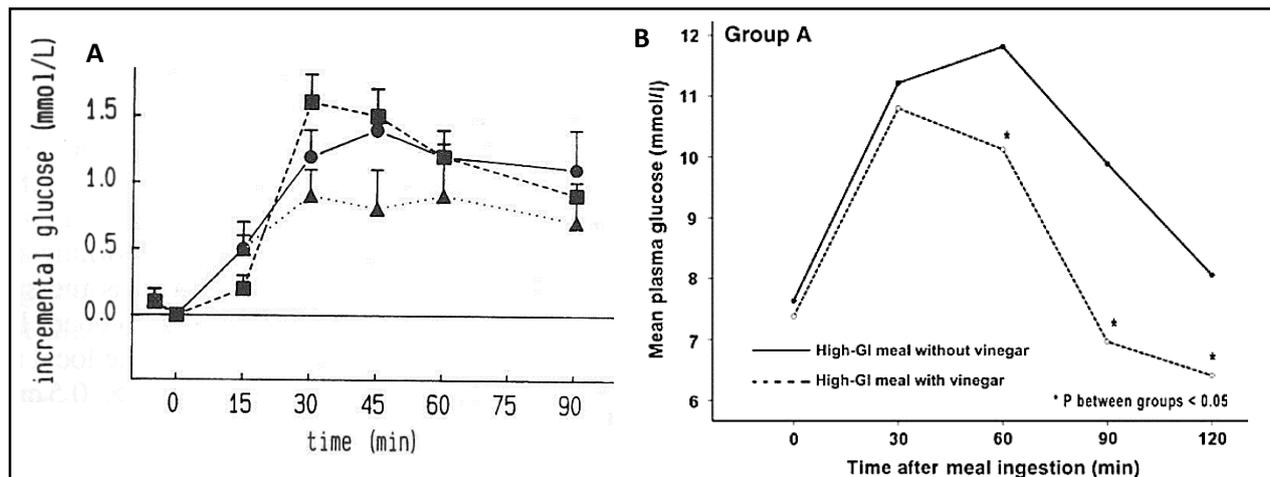


Figure 30 –Effect of meal supplementation with vinegar on plasma glucose concentration. (A) Results obtained with a group of healthy subjects following consumption of a meal without vinegar (●, full line), with vinegar (▲, dotted line) or with sodium acetate (■, dashed line). Mean  $\pm$  SEM, n=5. (B) Results obtained with a group of subjects with type-2 diabetes following consumption of a high glycemic index meal without vinegar (full line) or with vinegar (dashed line). \*Indicates statistically significant differences between treatments. Mean values, n=8.

A number of reviews have been published on this subject and, as illustrated in Figure 31, several mechanisms have been proposed, including a decreased gastric emptying rate or a suppression of the activity of intestinal disaccharides, but no clear conclusions have been reached so far (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014; Poutanen, Flander, & Katina, 2009). There are, however, some factors that have been identified as essential to preserve this effect, particularly the low pH and the presence of starch. Whenever vinegar was compared with a neutral-pH equivalent, the effect on the glycaemic response was lost, as it can be observed in Figure 30A (Brighenti, et al., 1995; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010). The same was observed when starch was replaced with dextrose (Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010). Relating these key factors with some of the possible underlying mechanisms, it could be possible that acidity delays gastric emptying (Hunt & Knox, 1962, 1969, 1972), but research is not conclusive (Brighenti, et al., 1995; H. Liljeberg & Björck, 1996, 1998), and this would not explain the loss of effect when starch was replaced with dextrose. Also, since the pH of the chyme is rapidly neutralised in the duodenum (section 2.1), it seems unlikely that the effect of low pH meals would derive from a limitation of intestinal enzymes.

Interestingly, an impairment of amylolysis by HSA in the early stages of digestion (oral and gastric phase), which are the steps of starch digestion most likely to be altered by meal acidity, is hardly addressed (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016;

Poutanen, Flander, & Katina, 2009), or is not even considered (Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014), as shown in Figure 31. As explained previously (section 2.1), this enzyme initiates starch digestion during mastication and can continue active in the stomach until it is inactivated due to increasing acidity. Because postprandial gastric pH usually rises to 4.5 – 6.7 and is decreased at a steady pace (taking 75-107 min to reach  $\text{pH} \leq 2$ ) (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Malagelada, Go, & Summerskill, 1979), there is a large time window during which HSA can continue active, possibly hydrolysing important starch fractions as previously reported (Bergeim, 1926). It would therefore seem reasonable to hypothesize that a premature acidification by vinegar would influence the performance of HSA, slowing down amylolysis and ultimately attenuating postprandial glycaemia. Overall, further work is therefore needed to pinpoint the mechanism(s) behind the effect of lowering the pH of starch-rich meals. This could indeed open new opportunities to improve the glycaemic impact of starch-rich diets.

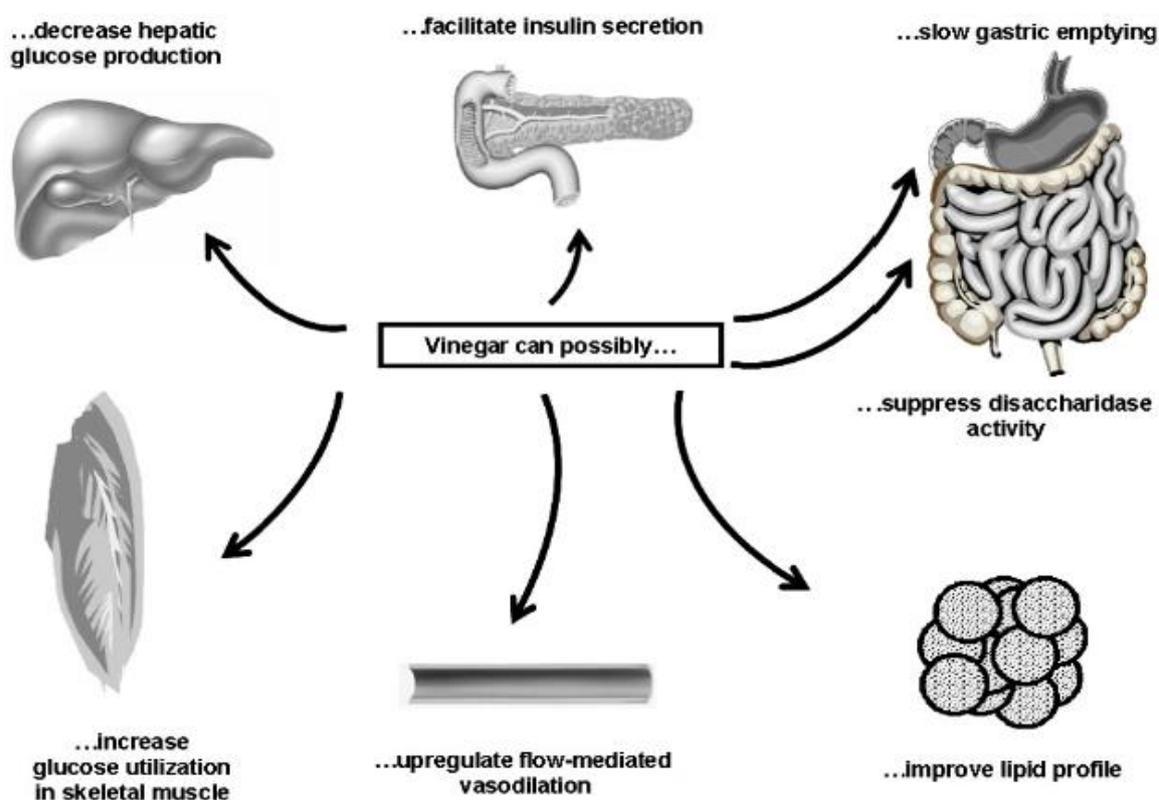


Figure 31 - Proposed underlying mechanisms of the effects of vinegar on glucose metabolism. Extracted from (Petsiou, Mitrou, Raptis, & Dimitriadis, 2014).

### 3. *IN VIVO* APPROACHES TO STUDY DIGESTION

#### 3.1. ANIMAL MODELS

Different animal models have been used to expand our knowledge on food digestion. Using animals as human models has even been considered crucial to study extremes conditions, used for instance for the discovery of essential nutrients, or tolerance levels for excessive nutrient intake (Baker, 2008). They have been, and still are, very useful in the study of nutrient interactions, bioavailability, or fate within the host.

Depending on the objectives of each study, different species can be considered. The rat (Cameron-Smith, Collier, & O'dea, 1994; Sigalet & Martin, 1999; Ton, Chang, Carpentier, & Deckelbaum, 2005) and dog (Graham, Maskell, Rawlings, Nash, & Markwell, 2002; S. Murray, et al., 1999) are examples of some species that have been commonly used. But other than monkeys, it is pigs that are often considered as the best animal model of humans for digestion studies (Miller & Ullrey, 1987; Tumbleson, 1986). Among the different species of pigs, miniature pigs (weighing between 20 and 30kg) are among the most commonly used models because less space is required, and lower amounts of feed and test product are needed (Gaudichon, et al., 1994; Le Feunteun, et al., 2014; Moeller, Sewing, Geisbe, & Schmolke, 1974).

#### 3.2. HUMAN STUDIES

The study of digestion in the human body is essential for an integrated comprehension of all the processes involved, the responses triggered and their consequences. This is, however, a complex task due, in one part, to the difficulty of access to the digestive system, and, in the other, to the miscellany of physical (ex.: rheological properties, liquid vs. solid phase, molecular interactions) and chemical (ex.: molecular and macromolecular composition, water content and minerals) properties of the meal which in turn determine the characteristics of the bolus and chime (Gaudichon, 2013). There are various methods available, each with its specific applications and limitations, advantages and disadvantages. Knowing them is important when selecting the tools for a human study, combining them is key for achieving a thorough and integrated understanding of the digestive system.

A summary of existing methods, as well as their applications, advantages and disadvantages or difficulties is presented in Table 3. It is possible to classify the available methods into two main categories, direct and indirect. Direct methods are those that enable the evaluation of the specific parameter(s) under study. In contrast, indirect methods are based on the analysis of a given tracer for the parameter under study (ex.: accessing gastric emptying through the rate of arrival of a given substance to the bloodstream) (Gaudichon, 2013; Kim, Myung, & Camilleri, 2000). Additionally they can be invasive (requiring intubation for example) or non-invasive

(through medical imaging). Finally, it is important to highlight that in most cases these methods can also be adapted to animal studies even though some of them require anesthetizing the animals.

For the particular purpose of the study of gastric function in the postprandial state, different mechanisms may be investigated, including intragastric meal distribution and gastric accommodation, motility, and emptying. As shown in Table 3, there are at least 6 methods that can be employed for such purposes. Two of them (Paracetamol absorption test, and stable isotope breath test) can be specifically used in gastric emptying studies. They have the important advantages of avoiding exposure to radiation or being of relatively simple application, but they appear to have only been validated for one type of meal (solid or liquid) and the gastric emptying is assessed through an indirect measurement. The four other methods can provide direct measurements of parameters related with gastric function but some present significant limitations. It is the case of intubation techniques, which can provide very precise information about the digestibility of nutrients, but which are also invasive and might thus cause discomfort to the participants and alter the gastric function. The three remaining methods are non-invasive, and mostly based on direct measurements. These are Scintigraphy, Ultrasonography and Magnetic Resonance Imaging (MRI). Scintigraphy is considered as the golden standard for gastric function studies. The fact that this technique has a high temporal resolution and that it allows to monitor gastric emptying of the liquid and solid components of a meal, independently, are among its strongpoints. Interesting results were reported by the team of Siegel and colleagues, who studied the gastric emptying patterns of a meals composed water with either an egg sandwich or chicken liver (Siegel, et al., 1988). The liquid (water) and solid (egg or liver) components of the meals were labelled differently, and this enabled the researchers to study what they have defined as the biphasic nature of gastric emptying. They have observed that emptying of water started immediately and at a fast rate, while the solid phases had initial lag phases, ranging from approximately 30 min for the egg sandwich up to almost 1h with the liver meal. After 30 min of digestion, at least 90% of the solid phase was still in the stomach, while only about 50% of the liquid phase remained. Their results are presented in Figure 32.

Table 4 - Summary of approaches employed in the study of digestion.

<b>Approach Applications</b>	<b>Procedure/Assessed parameters</b>	<b>Advantages</b>	<b>Disadvantages/ Difficulties</b>
<b>Ileostomized patients</b>			
<b>Analysis of intestinal effluents</b>	Sampling of intestinal effluents is possible in patients who have undergone ileostomy (ileum is diverted through an opening in the abdomen)	Direct access to the chime Acceptable correlation with healthy individuals	Patients suffer from digestive pathologies: results not fully representative of healthy individuals
<b>Gastroduodenojejunal intubation</b>			
<b>Characterization of digestion products along the digestive tract</b>	Intubation via the nasogastric passage enables the collection of samples from precise locations (ex.: stomach, ileum, jejunum)	Direct access to the chime Considered the only method to obtain precise digestibility information	Invasive, causes discomfort May impact motor activity and intragastric meal distribution
<b>Motor function</b>	Tubes equipped with a barostat and polyethylene balloon can be used to monitor reflex motor function in the proximal stomach		Intubation required Difficulty to estimate dilution by secretions (some methods require successive removal and reintroduction of the chime)
<b>Scintigraphy</b>			
<b>Gastric emptying and contraction patterns</b> <b>Intragastric meal distribution</b> <b>Gastrointestinal transit</b>	Based on the ingestion of a meal containing a radio-isotope and subsequent detection of radioactivity using a gamma-camera	Gold standard in the evaluation of gastric motor function Independent monitoring of liquid and solid transit	Exposure to radioactivity – not eligible for studies without a direct benefit for the individual (France)
<b>Ultrasonography</b>			
<b>Gastric emptying</b> <b>Antral wall motion</b> <b>Transpyloric flow</b> <b>Gastric accommodation</b>	Changes in the diameter of specific stomach regions (relative to anatomic landmarks) and volume determinations, advanced techniques enable 3D analyses	No exposure to radiation Safety Availability Cost	May be technically suboptimal in the presence of air Impractical for prolonged observations Needs a skilled observer
<b>Magnetic Resonance Imaging (MRI)</b>			
<b>Intragastric meal accommodation, dilution by secretions and layering, detection of boluses and food particles</b> <b>Gastric emptying, and motility</b> <b>Small intestine water content</b>	After ingestion of a meal (containing a tracer element or not) the study region is scanned as a succession of individual planes, called “slices”, each of which constitutes an independent image in the final scan. Various image processing techniques can then be employed according to the aim of the study.	Currently the imaging technique with the most potential applications No exposure to radiation Safety	High cost and low availability Requires skilled operators Image analysis is complex and time-consuming Positioning may alter intragastric meal distribution

Information obtained from (Caride, et al., 1984; Gaudichon, 2013; Kim, Myung, & Camilleri, 2000; Marciani, Gowland, Fillery-Travis, et al., 2001; Marciani, Gowland, Spiller, et al., 2001; Marciani, et al., 2013).

Table 3 - Summary of approaches employed in the study of digestion (continued).

Approach Applications	Procedure/Assessed parameters	Advantages	Disadvantages/ Difficulties
<b>Postprandial blood concentration of a given nutrient</b>			
<b>Digestion kinetics Physiological response</b>	Monitoring the incremental concentration of a given nutrient (aminoacids, tryglicerides, glucose) following a meal is a commonly used technique providing an indirect measure of its digestion kinetics	The only method to understand the metabolism and physiologic response to certain nutrients	Requires repeated sampling Results can be influenced by certain drugs
<b>Paracetamol absorption test</b>			
<b>Gastric emptying</b>	Based on the principle that gastric emptying is the rate-limiting step in the absorption of paracetamol because its absorption is fast and complete in the small intestine. Plasma paracetamol concentrations provide a measure of the gastric emptying rate	Simple test Well tolerated by participants Validated against scintigraphic and duodenal intubation studies for liquid foods Relatively easily available because many laboratories perform the paracetamol assay	May be less sensitive than other approaches to assess emptying of solids Requires repeated blood sampling Interactions with commonly used drugs (ex.: oral contraceptives) may alter the metabolism of paracetamol leading to erroneous results
<b>Stable isotope breath test</b>			
<b>Gastric emptying</b>	The solid component of a meal can be labelled using a substrate containing the stable, nonradioactive isotope $^{13}\text{C}$ . $^{13}\text{C}$ -labeled substrate is absorbed in the duodenum, metabolized in the liver and excreted as $^{13}\text{CO}_2$ to the breath. Which can then be measured by isotope ratio mass spectrometry or laser infrared spectroscopy.	No exposure to radiation Mathematical modelling can enable to simulate the kinetics of gastric emptying	Very indirect because it is based on a measurement made at the final stage of the product's metabolism
<b>Water / Nutrient drink test</b>			
<b>Noninvasive alternative to intra gastric barostat</b>	Subjects ingest water or a liquid nutrient meal at a defined rate, and the maximal tolerated volume is determined. Postingestion symptoms can be measured for up to 30 min	Simple Inexpensive	Possible impact of psychological factors Satiating or reduced tolerated volume may reflect impaired accommodation or hypersensitivity of the stomach

Information obtained from (Caride, et al., 1984; Gaudichon, 2013; Kim, Myung, & Camilleri, 2000; Marciani, Gowland, Fillery-Travis, et al., 2001; Marciani, Gowland, Spiller, et al., 2001; Marciani, et al., 2013).

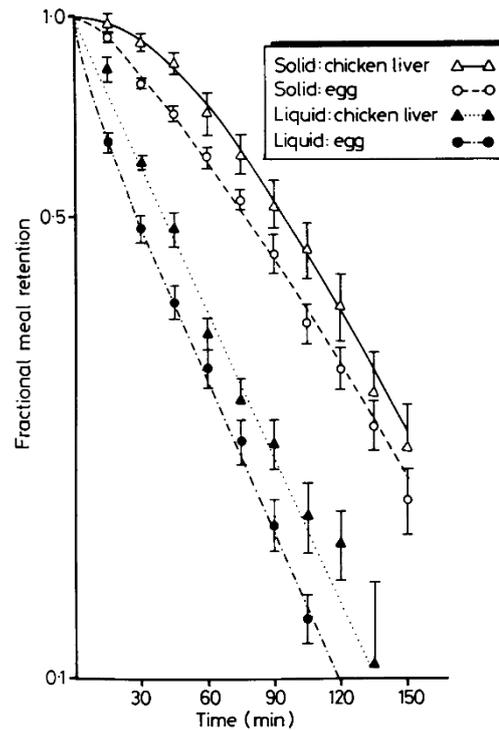


Figure 32 - The mean  $\pm$  SEM fractional meal retention values for 24 subjects receiving either Tc-99m-egg sandwich (n = 14) or Tc-99m *in vivo* labelled chicken liver (n = 10) and In-111 DTPA water on a semilogarithmic plot. (Siegel, et al., 1988)

However, the use of scintigraphy exposes the examined person to radioactivity and therefore alternative tools are needed. Ultrasound and MRI are safer alternatives which do not involve exposure to potentially harmful radiation. Ultrasound imaging has the advantages of being more available and cheaper than MRI. However, the higher resolutions of MR images (Figure 33) can enable a more accurate and detailed study of gastric digestion parameters. This methodology will be discussed in more detail in the following section.

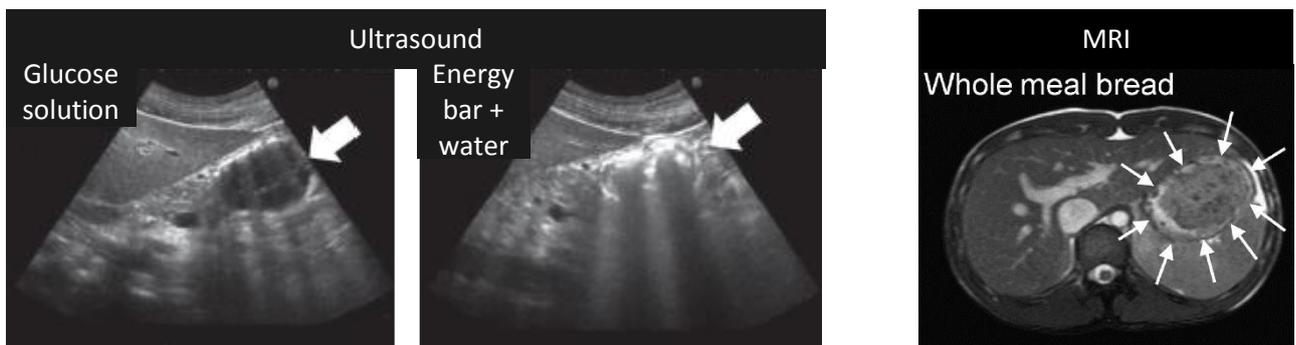


Figure 33 – Images of the abdominal region. Examples of images obtained through ultrasound (left) and MRI (right). The gastric regions are identified by white arrows. Ultrasound and MRI images obtained from (Okabe, Terashima, & Sakamoto, 2017) and (Marciani, et al., 2013), respectively.

## 3.2.1. ASSESSMENT OF THE PHYSIOLOGICAL IMPACT OF STARCH

Regarding the study of starch, and carbohydrates in general, one of the parameters that is regarded is the glycaemic response. Recently, an international consensus has led to a harmonized definition of the glycaemic response as the post-prandial change in blood glucose concentration following consumption of a food or meal that contains carbohydrate (Augustin, et al., 2015). The glycaemic response elicited by different foods is highly variable, therefore, it is useful to have a simple tool to distinguish foods according to the glycaemic response they elicit. A commonly used classification system is the Glycaemic Index (GI), a concept initially proposed by Jenkins and his team in the 1980s (DJ Jenkins, et al., 1981). It is defined as the total incremental area under the blood glucose response curve (AUC - area under the curve of glucose concentration versus time) in the 2 hour period immediately subsequent to the consumption of a portion of food containing 50g of available carbohydrates relative to that of a standard food (glucose or white bread) also containing 50g of available carbohydrates (DJ Jenkins, et al., 1981; Thomas M Wolever, et al., 1985). Although there has been some controversy associated with this tool, scientific evidence from the analysis of over 1000 foods clearly demonstrates that it constitutes a good summary of postprandial glycaemia, predicting important attributes of the glucose response curve, namely the peak or near peak response and the maximum glucose fluctuation (J. C. Brand-Miller, Stockmann, Atkinson, Petocz, & Denyer, 2009). An international standard for the determination of the GI has been proposed, and according to it, it is also possible to classify the GI of foods as low, medium or high, Table 5 (ISO, 2010).

**Table 5 - Categories of GI**

Level	Glycaemic Index
<b>Low</b>	GI ≤ 55
<b>Medium</b>	55 < GI ≤ 70
<b>High</b>	GI > 70

**(ISO, 2010)**

To account for the glycaemic response to different amounts of foods, the glycaemic load (GL) can be used. Still according to the recent international scientific consensus establishing harmonized descriptions of these terms, the GL is defined as the product of the GI and the total available carbohydrate content in a given amount of food. Because available carbohydrates can be expressed in different ways (for ex.: g/serving or g/ 100g of food), the units of the GL will have corresponding units (for example g/serving or g/100g of food). (Augustin, et al., 2015)

## 4. DIGESTION STUDIES USING MAGNETIC RESONANCE IMAGING (MRI)

How fascinating is it to think that we can actually see beyond sight? The first chapter of a book dedicated to this topic (McRobbie, Moore, Graves, & Prince, 2006), starts as follows:

*“HOW WOULD YOU IMPRESS A STRANGER YOU MEET AT A PARTY WITH YOUR INTELLIGENCE? YOU MIGHT CLAIM TO BE A BRAIN SURGEON OR A ROCKET SCIENTIST.*

*WELL MAGNETIC RESONANCE IS NOT ROCKET SCIENCE, IT’S BETTER!”*

Impossible to disagree! It involves an amazing combination of advanced sciences, engineering and technologies, as the authors put it: “a lifetime is not enough to become an expert in all aspects of it” (McRobbie, Moore, Graves, & Prince, 2006). By now the reader might have realized that, with my primary training in Nutrition and Food Sciences, I am not, nor will I pretend to be any sort of MRI expert, neither at any party, nor here. A basic knowledge of the underlying principles is however important in order to be capable of interpreting the results of MRI studies.

### 4.1. MRI PRINCIPLES

#### 4.1.1. BASIC NOTIONS

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The nuclei of atoms are made up of protons and neutrons, both of which have spins. In odd-numbered nuclei, that is nuclei that contain an odd number of protons or neutrons, their spins do not cancel each other out, conferring a net spin to the nuclei. In a simplified fashion, the “spin” property of odd-numbered atomic nuclei can be conceived as the nucleus rotating around its own axis. This motion induces a magnetic field with north and south poles, and so these atoms behave like bar magnets or dipoles. Such atoms, can therefore interact with other magnetic fields and it is this property that enables magnetic resonance techniques to work. (Bitar, et al., 2006; Grover, et al., 2015; Sands & Levitin, 2004)

In clinical MRI, hydrogen ( $^1\text{H}$ ) atoms are the most commonly used nuclei because of their spin properties and their abundance in the body. Although other atoms such as carbon-13, fluorine-19 and phosphorous-31 also possess net spins, usual MRI can be basically defined as the imaging of hydrogen atoms, from now on simply referred to as protons, in free water and in association with macromolecules such as proteins, lipids, etc. (Sands & Levitin, 2004)

When protons are exposed to a dominant magnetic field ( $B_0$ ), such as what is obtained with a powerful superconducting electromagnet built into the bore of an MR scanner, their spins tend to align with the magnetic axis of  $B_0$ . In contrast with bar magnets, which would orientate perfectly parallel or antiparallel to the external field, protons rotate in a cone-shaped fashion, termed precession. The frequency of precession, called the Larmor frequency, is in the radiofrequency domain (RF, in MHz) and depends on the strength of the external magnetic field

(measured in teslas [T]) and a constant (the gyromagnetic ratio) unique to each atom. The cumulative effect of all the magnetic moments of the nuclei is referred to as the net magnetization vector, which is the source of signal for image generation. (Bitar, et al., 2006; Grover, et al., 2015; Sands & Levitin, 2004)

#### 4.1.2. MAGNETIC RESONANCE - PROPERTIES AND SIGNAL CREATION

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The application of a second magnetic field ( $B_1$ ) lasting microseconds and oscillating at the Larmor frequency (RF domain) causes the net magnetization vector to flip by a certain angle, thereby temporarily displacing the net magnetization from the axis of the dominant magnetic field. At this point, two magnetization vector components can be defined: longitudinal and transverse magnetization. In the absence of an RF energy source, the system returns to its thermodynamic equilibrium in the magnet. This process, called relaxation, occurs at different rates along (longitudinal magnetization) and perpendicularly (transversal magnetization) to the static magnetic field. (Bitar, et al., 2006; Grover, et al., 2015; Sands & Levitin, 2004)

This recovery of longitudinal magnetization and decay of transversal magnetization can be described with relaxation times:

- The longitudinal relaxation time (or spin-lattice relaxation time) is denoted  $T_1$ , and corresponds to the time it takes to recover 63% of the initial longitudinal magnetization after an RF pulse. This phenomenon is basically caused by the transfer of the energy initially transmitted to the protons by the RF pulse back to the surroundings (lattice).
- The transverse relaxation time (or spin-spin relaxation time) is denoted  $T_2$ , and corresponds to the time it takes to dissipate 63% of the transverse magnetization after an RF pulse. This phenomenon is basically caused by the dephasing of nuclei orientations as energy is transferred between them, resulting in a more random overall alignment and a reduction of the net transverse magnetization. (Bitar, et al., 2006; Grover, et al., 2015; Sands & Levitin, 2004)

An additional phenomenon generally contributes to the transverse relaxation: inhomogeneities of the dominant magnetic field (which is not exactly the same everywhere due to the presence of air, impurities, or limitations of the magnet construction for example). This is why in practice, the effective (observed) transverse relaxation, denoted  $T_2^*$ , is generally shorter than the theoretical  $T_2$  (Bitar, et al., 2006)

The transverse component of the net magnetization vector can be measured because it induces electromotive forces (emf) in receiver antenna coils (built into separate pieces of hardware). As

transverse magnetization decays, the signal induced in the coil decreases. This signal, called the free induction decay, corresponds to the measurements performed in all MR techniques. (Bitar, et al., 2006; Sands & Levitin, 2004).

#### 4.1.3. MANIPULATION OF THE SIGNAL TO DISCERN TISSUES WITH DIFFERENT PROPERTIES

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For a useful image to be formed it is necessary to somehow differentiate each tissue in the scanned area. According to the above description of MR principles, the amplitude of the signal is a function of the density of protons, and since water has a very high proton density compared to other molecules, proton-density-weighted MRI is one obvious tool to distinguish between tissues with different water contents. Additionally, the local proton environment is also important. Small molecules in solutions, and their protons, are in rapid thermal motions, which contrast with what happens in macromolecules, gels or solids. This is why different biological tissues, or types of material (ex: water vs. fat) have different T1 and T2 values. These differences in relaxation times can thus be used to generate different contrasts in MR images. (Sands & Levitin, 2004)

There are a vast number of ways to manipulate the longitudinal and transversal magnetization, using different RF-pulse sequences, with the purpose of emphasizing different properties of the tissues. A simple option may be only to vary the amount of time between the start of two consecutive acquisitions (repetition time, or TR). Another option is to vary the time between the start of the sequence and the formation of signal (echo) (echo time or TE). Figure 34 illustrates the evolution of the longitudinal and transversal magnetizations for fat and water with time, and the consequences of the duration of TR and TE, respectively. As schematically represented, short TR will enable to distinguish products/tissues with different T1, whereas long TE are useful to distinguish products/tissues with different T2. It is said that MR images are either T1 weighted and/or T2 weighted.

In summary, and although other types of contrast can be used, three basic types of image contrast can be obtained by varying the duration of the TR and TE in an RF-pulse sequence, these are presented in Table 6.

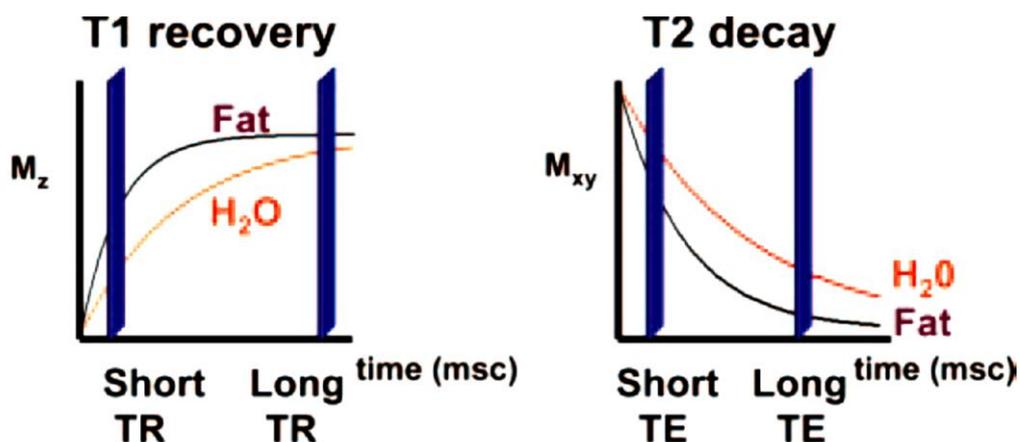


Figure 34 - Graphs show T1 recovery (recovery of longitudinal magnetization [ $M_z$ ]) and the T2 decay (the decrease of transverse magnetization [ $M_{xy}$ ]), as a function of time (in milliseconds [msc]) for fat and water. The effects of short and long TR (left) and short and long TE (right) correspondingly on T1 recovery and T2 decay in fat and water are illustrated by the blue bars: TR relates to T1 and affects T1 weighting, whereas TE relates to T2 and affects T2 weighting. (Bitar, et al., 2006)

**TABLE 6 - EFFECT OF TR AND TE ON MR IMAGE CONTRAST**

IMAGING TECHNIQUE	TR	TE
T1 WEIGHING	Short	Short
T2 WEIGHING	Long	Long
PROTON-DENSITY WEIGHING	Long	Short

FROM (BITAR, ET AL., 2006)

#### 4.1.4. SPATIAL LOCALIZATION OF THE SIGNAL AND IMAGE FORMATION

A basic explanation of the relationships between tissue properties, sequence parameters and magnitude of the signal and contrast between tissues in an MR image was explained above. To obtain images, *i.e.* a spatially encoded signal, one needs to apply three magnetic field gradients, which consist of linear variations of the magnetic field in the three-dimensional space ( $x$ ,  $y$ ,  $z$ ) (Bitar, et al., 2006). These gradients create ordered variations in a specific property of the protons' spins (spin frequency or phase), so that the frequency and phase of a spin is indicative of its spatial location (Bitar, et al., 2006; Sands & Levitin, 2004).

The three steps in the localization of a signal are: (1) the selection of a slice, (2) phase encoding and (3) frequency encoding. These are explained below to obtain the most common type of image (a cross sectional slice).

**(1) SELECTION OF A SLICE** – A first magnetic field gradient is applied, causing the protons' precession frequencies to increase gradually along the gradient axis (*e.g.* the  $z$ -axis).

Then, a slice is selected by applying an RF-pulse, with known band-width, at the frequency of precession of the protons in a particular location (Ridgway, 2010; Sands & Levitin, 2004).

**(2) PHASE ENCODING** – By applying another gradient field along the y-axis, protons will acquire a gradually higher frequency of precession along this axis, and as a result, will also constantly change their relative phase (according to their precession frequency) along the gradient. When this gradient is switched off, the precession frequency becomes again the same across the y-axis, but the relative phases of the protons will have changed according to their location along this axis. (Ridgway, 2010; Sands & Levitin, 2004)

**(3) FREQUENCY ENCODING** – Finally, a third gradient is applied along the x-axis. Similarly to the phase-encoding gradient, it causes the protons to precess at different frequencies, along this axis. But now, the signal is measured at the same time and so the position along the x-axis is discernable according to the different frequencies at the time of measurement. (Ridgway, 2010)

This is the process used while imaging a cross section perpendicular to the position of a person on the bed of an MRI (a transverse or axial slice) (Figure 35). Other slice orientations are obtained by re-assigning each of the gradients (slice, phase and frequency encoding) to a different axis. Additionally, by manipulating the characteristics of the gradients and the RF pulses, it is possible to modify other properties of the slice that is imaged such as its thickness, and number of points with distinct phases/frequencies (which will influence the level of detail or resolution of the final image). (Ridgway, 2010) In summary, at the end of an MR imaging sequence, a complex cluster of data containing information about the intensity of signals (related with proton density, T1 and T2), and the frequencies and phases of these signals is produced (Ridgway, 2010). What emerges is an analysis of how much signal (amplitude, obtained by the emf induced in the receiver coil following excitation RF-pulse) is present in a specific location (assigned by the frequency and phase) of the slice selected. The amplitude of each signal is finally displayed as a shade of gray (Figure 35) at its relative spatial position on the imaged portion of a person. (Sands & Levitin, 2004)

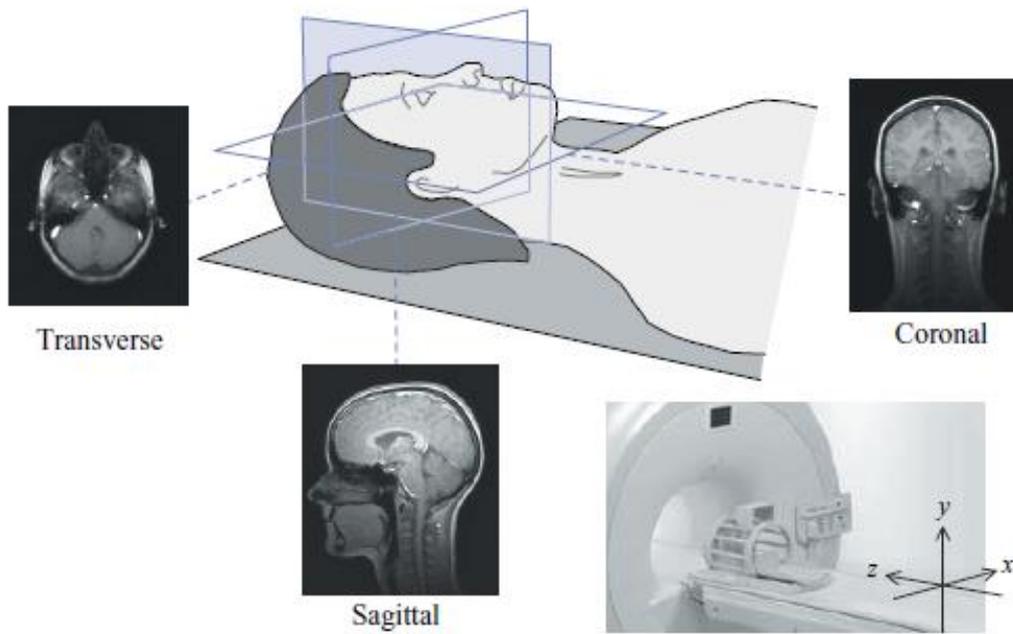


Figure 35 – Anatomical planes and image projection in shades of gray. The 3 main types of slice orientations which are selected by the magnetic gradient coils are illustrated by the blue planes. The resulting anatomical slices are presented and are also illustrative of the projection of signal intensity as shades of gray. Image obtained from (McRobbie, Moore, Graves, & Prince, 2006).

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#### 4.2.5. HIGH SPATIAL OR HIGH TEMPORAL RESOLUTION

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Finally, it is important to note that imaging an organ such as the stomach requires a compromise between the desired contrasts within the images, as well as the spatial and temporal (speed of acquisition) resolutions. According to the objectives of the study, different compromises may thus be chosen. If the aim is to study anatomic or structural features, then spatial resolution is favoured to allow the abdominal area to be scanned once in a given amount of time (often within one single breath hold, to avoid artefacts due to respiratory motion). If, on the other hand, the aim is to study motion or motility features, then it will be necessary to scan the studied area several times in a short time, meaning that the spatial resolution needs to be decreased.

#### 4.2. THE STUDY OF DIGESTION WITH MRI

The first published study of digestive function by MRI was conducted about 30 years ago (Stehling, et al., 1989). When studying the functions of the digestive system, spatial resolution (image quality) must be high so that small and thin walled structures can be delineated and distinguished. Additionally, high spatially resolved images should be acquired fast because the organs are in constant movement, so temporal resolution needs to be adapted to the physiological range of the digestive events under study. Valuable progress was made in this context in the past 30 years. Important innovations to the MR system and external components, as well as the development of acquisition techniques (new sequences, parallel imaging of individual slices...) resulted in significant improvements of imaging speed and/or image quality. Moreover, since then the use of this technique was extended to other organs involved in digestive processes such as pancreas and the small and large intestine. (Schwizer, Fox, & Steingötter, 2003)

The focus of this brief review of the literature is gastric digestion and the knowledge inputs from MRI studies. Assessments are usually based on standard measurements of gastric activity to assess its global function (ex. gastric emptying rate) or individual components (ex. layering effects, particle size) and their influence on the global function (ex. the effect of layering effect or particle size on gastric emptying). The most commonly measured variable in studies of gastric digestion is the gastric emptying kinetics, generally summarized by a half time ( $t_{1/2}$  or  $t_{50\%}$ ) when the gastric volume reaches 50% of the initial volume, and a lag time ( $t_{lag}$ ) which refers to the initial postprandial period before significant emptying is initiated (Schwizer, Fox, & Steingötter, 2003). A key advantage of MRI is that multiple parameters can be assessed simultaneously (Schwizer, Fox, & Steingötter, 2003). The main disadvantages of MRI studies are the high cost and the low availability of MR equipment, the concerns about the impact of the position of the subject on normal digestive function, and the complexity of sequence adjustment and image analysis, both of which are time-consuming task (Table 3). Regarding the potential impact of the positioning in the MR scanner on gastric function, the measurement of gastric emptying of solid and liquid meals with MRI has been validated against scintigraphy (Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999; Schwizer, et al., 1994), and potential biases inducing the formation of air pockets in unusual parts of the stomach can be limited by adopting the right decubitus position (Treier, et al., 2006).

Overall, MRI has provided the grounds for some unique findings about the digestion of foods in the stomach. The work of Marciani and his team on the patterns of dilution by gastric secretions and emptying rate for nutritive and non-nutritive meals with different viscosities is one example

(Marciani, Gowland, Spiller, et al., 2001). They have observed that although a 500 Pa.s increase in meal viscosity produced an increased half gastric emptying time, by 9-14 minutes, the presence of nutrients exerted a predominant effect by almost doubling the half-gastric emptying time (30-35 min longer) for a given viscosity. Inversely, meal dilution by gastric secretions has been shown to be more influenced by viscosity than the presence of nutrients. Furthermore, they were able to map gastric meal dilution by gastric secretions, revealing that a high viscosity meal (though less viscous than many real meals) remains heterogeneous long after ingestion, with gastric secretions only poorly penetrating the center of the food bolus (Figure 36). This challenges the traditional model of rapid and complete meal homogenization upon ingestion, showing that for some meals, gastric contents can be rather poorly mixed.

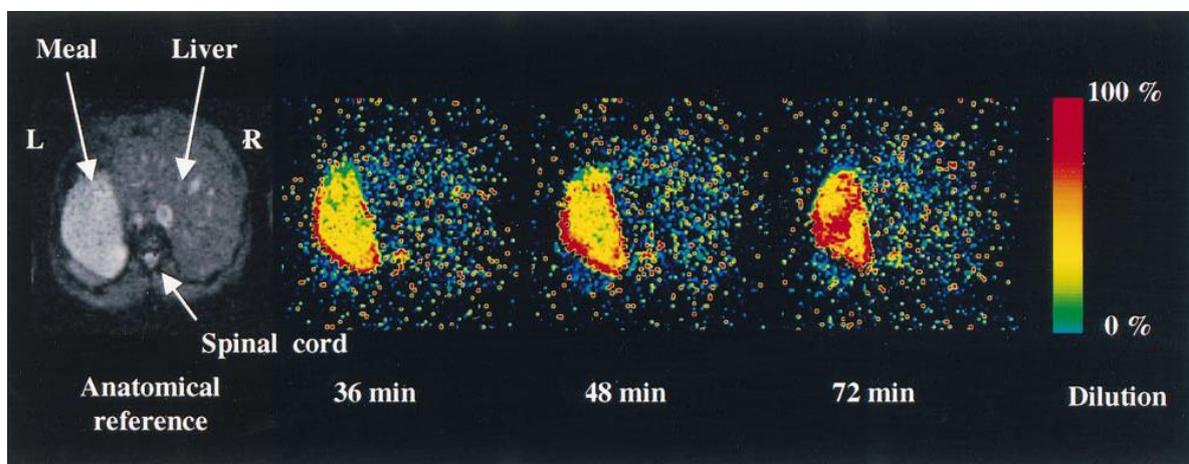


Figure 36 - Color-coded dilution maps acquired at different times after consumption of 500 mL of a viscous locust bean gum meal. The transverse relaxation time T2 of the meal was calibrated against dilution *in vitro*. T2 maps of the stomach contents were then acquired *in vivo*, translated to dilution values using the calibration curve, and color coded. A transverse EPI image is also shown as an anatomic road map (L, left; R, right). Gastric secretion made the outer boundaries of the viscous meal more diluted (in red) at an early time, whereas the inner bolus remained viscous (in green) for longer. As time progressed, the viscous meals appeared more diluted and mixed. Obtained from (Marciani, Gowland, Spiller, et al., 2001).

Another interesting finding was published by Kunz and colleagues who studied the effect of the order of ingestion of the fat component of a solid meal on the kinetics of gastric emptying of fat. By mapping intragastric fat distribution when the fat component was consumed first (Figure 37, F-NF) and last (Figure 37, NF-F), they have concluded that the order of ingestion strongly influences intragastric distribution of fat as well as layering behaviour, and thus the emptying from the stomach. In this study fat emptied significantly faster when consumed first. (Kunz, et al., 2005)

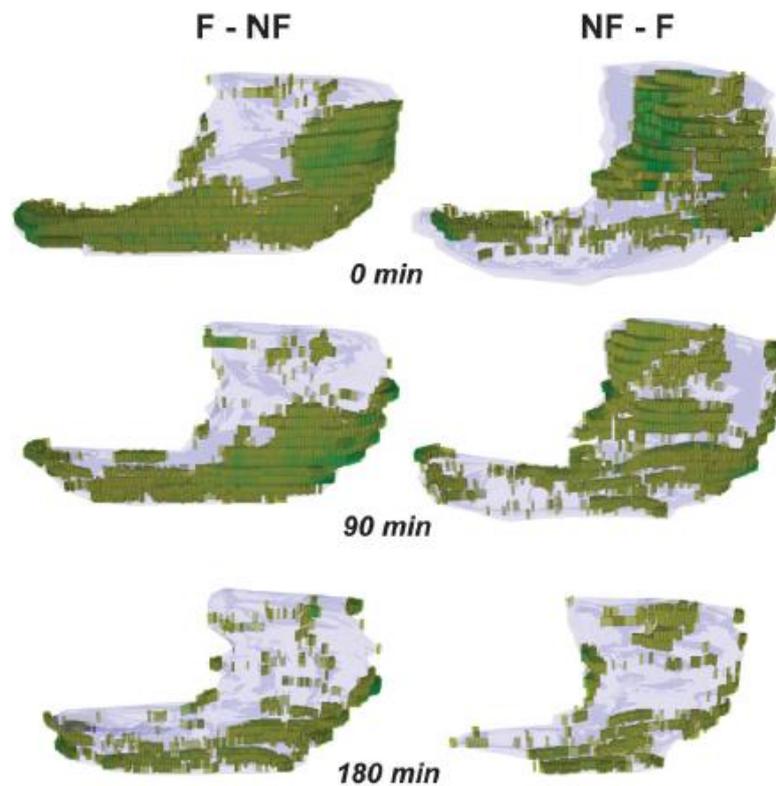


Figure 37 - Frontal view of the three-dimensional reconstruction of the stomach illustrating intragastric fat distribution (in green) immediately (top), 90 minutes (middle) and 180 minutes (bottom) after meal ingestion, when the fat component preceded the nonfat component (F-NF, left) and when the fat component followed the nonfat component (NF-F, right). Obtained from (Kunz, et al., 2005).

Several other interesting results could be referenced. One example is the apparent adaptation of the frequency of contraction in the first 15 min after ingestion of a solid meal in comparison with a liquid meal (Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999). Another is the estimation of the maximum force exerted by the gastric antrum (0.65 N) from the observation that the half gastric emptying time of test meals containing beads with different forces to fracture (from 0.15 N to 0.9 N) was significantly increased for bead strengths > 0.65N (Marciani, Gowland, Fillery-Travis, et al., 2001).

The parallel assessment of gastric emptying by MRI and of blood glucose concentrations would be particularly interesting in the context of starch-rich foods. Two studies combining these measurements to compare different porridge breakfasts have been published so far, demonstrating the feasibility and interest of such approaches (Alyami, Ladd, Pritchard, et al., 2017; Alyami, Whitehouse, Pritchard, 2018).

## 5. IN VITRO APPROACHES TO STUDY DIGESTION

Although *in vivo* studies produce more accurate results than *in vitro* approaches (Hur, Lim, Decker, & McClements, 2011), they can be time consuming, expensive (Hur, Lim, Decker, & McClements, 2011; Minekus, et al., 2014b), they require many subjects with specific attributes (Dona, Pages, Gilbert, & Kuchel, 2010) and are sometimes considered ethically disputable (Minekus, et al., 2014b). Comparatively, *in vitro* studies have the strongpoints of reproducibility, choice of controlled conditions, and ease of sampling at the site of interest (Minekus, et al., 2014b).

Ideally, *in vitro* digestion models should provide accurate results in a short time and could thus serve as a tool for rapid screening of foods or delivery systems with different compositions and structures. In practice, the complexity of the digestion process dictates an inevitable inability to perfectly replicate it *in vitro* (Hur, Lim, Decker, & McClements, 2011). Among the most important limitations, the impossibility to take into account mechanisms related with hormonal responses, neuronal reflexes and cellular activity is clear. This reflects the importance of acquiring a deep understanding of the digestive process before designing an *in vitro* experiment but also during the analysis of the results.

Categorize *in vitro* methods can be a complex task because the protocols are often adapted to the purpose of the studies. One recurring point of divergence is the number of digestive stages included in each protocol. In general, the gastric and intestinal phases are always reproduced (K. N. Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Y Granfeldt, Bjorck, Drews, & Tovar, 1992; Minekus, et al., 2014b; Woolnough, Bird, Monro, & Brennan, 2010), but the oral phase is sometimes omitted (K. N. Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Overall, *in vitro* approaches can nevertheless be divided into two main categories: static and dynamic methods

Because the oral phase is an important step of starch digestion but is not common to all protocols, a first subsection (5.1) is included with information about the study of this digestive phase *in vitro*. Then, because gastro-intestinal phases generate the bulk of the data from *in vitro* experiments, and they are commonly included both in static and dynamic protocols, the two succeeding subsections (5.2.1 and 5.2.2) will focus on each of these approaches individually.

### 5.1. ORAL DIGESTION

Oral digestion is not always considered in *in vitro* studies. Some current *in vitro* methods include the actual chewing of sample foods by volunteers (Y Granfeldt, Bjorck, Drews, & Tovar, 1992; Woolnough, Bird, Monro, & Brennan, 2010). However, because this procedure involved a human

fluid, it may need to be approved by an ethics committee. Moreover, because chewing and salivary amylase activity are highly variable between individuals, some researchers consider this as an irregular method, not suitable for routine *in vitro* digestion studies (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000). As a result, alternative *in vitro* approaches have been proposed. There are three major components that should be considered when mimicking oral digestion: mastication time, level of saliva impregnation and particle size.

Mastication times and levels of saliva impregnation for certain foods can be found in the literature (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000; Jourdren, et al., 2016) and can be considered easy to replicate. It is however important to note that, for practical reasons, the duration of oral phases almost always vary from 2 to 15 minutes (Woolnough, Monro, Brennan, & Bird, 2008), which differs largely from the average 27-28 s chewing times observed *in vivo* for bread samples for example (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000).

There is also detailed information about the bolus particle size for a wide range of foods such as bread, pasta, carrots, cake, cheese, olives among others (Flynn, et al., 2011; Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000; Jalabert-Malbos, Mishellany-Dutour, Woda, & Peyron, 2007; Jourdren, et al., 2016). However, reproduction of the particle size distribution found in an *in vivo* formed bolus can be more difficult as it implies the simulation of the mechanical disruption that occurs during chewing. Literature shows that this might not be a limitation to the study of certain foods since comparisons of the digestive patterns of *in vivo* and artificially (chopping, sieving and mincing) chewed foods have shown that foods with lack a robust structure, such as bread, does not display sensitivity to the method of chewing (Woolnough, Monro, Brennan, & Bird, 2008). A number of different methods, such as mincing, grinding, milling, homogenizing, the use of sieves, food processors or choppers have been proposed (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000; Minekus, et al., 2014b; Woolnough, Monro, Brennan, & Bird, 2008). A meat mincer equipped with a plate pierced with 6-mm diameter holes was found to satisfactory reproduce the distribution of the particle size of bread obtained after *in vivo* mastication when it was used to process freshly cooked pasta or bread crumb moistened with water (100g dry matter/79ml of water) (Hoebler, Devaux, Karinithi, Belleville, & Barry, 2000).

## 5.2. GASTRIC AND INTESTINAL DIGESTION

Different methods have been described to study gastro-intestinal digestion. Major differences between these methods are the number of steps and the way these steps are interrelated. Considering the number of steps, three models have been proposed: single, two-, or three-steps respectively simulating digestion at gastric, gastric/small intestinal, gastric/small intestinal/large stages. Regarding the articulation of each step, two approaches have been used, static and

dynamic. At least four key criteria should be taken into consideration for the design a realistic gastro-intestinal tract model: (1) sequential use of digestive enzymes; (2) appropriate pH, co-factors, co-enzymes, bile salts, etc.; (3) appropriate mixing in each step of the digestive process; (4) respect for the physiological residence times for each digestive step.

#### 5.2.1. STATIC MODELS

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This type of model is the most often described in literature and has been employed in a wide variety of studies focusing on questions such as the digestibility and bio-accessibility of pharmaceuticals, mycotoxins, and macronutrients, including carbohydrates, as well as the matrix release of minerals, trace elements and secondary plant compounds. In the static approach, the steps of digestion are simulated using a batch system in which the test material is sequentially exposed to a particular simulated digestive fluid at 37°C and at a fixed pH (Venema, Havenaar, & Minekus, 2009).

The problem of the persistence of significant variations between the different parameters of the static models described in literature has raised the need for a standardized method (Minekus, et al., 2014b; Woolnough, Monro, Brennan, & Bird, 2008). Recently, an international consensus has been reached in the framework of the European INFOGEST COST action, which gathered about 200 scientists in the field of digestion. A step-by-step static protocol, including a frameset of parameters for oral, gastric and small intestinal digestion was proposed, and an extensive list of recommendations, justifications and possible limitations was also presented (Minekus, et al., 2014b). Even though this protocol is still being subjected to further testing and validation by different research groups, it certainly seems advisable to take it as guidance when designing a research work to study food digestion *in vitro* with a static model.

Although static models are simple and easy to use, they are not always considered as sufficiently physiologically relevant. As a result, these models are considered essentially as screening techniques to identify key parameters that may affect the hydrolysis of macronutrients, and as valuable means to study mechanisms at the molecular level (Ménard, et al., 2014).

#### 5.2.2. DYNAMIC MODELS

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A more accurate simulation of *in vivo* conditions can be obtained using dynamic digestion models, which allow the simulation of dynamic aspects of digestion such as transport of digested meals, variable enzyme concentration and pH changes over time (Ménard, et al., 2014; Minekus, et al., 2014b). As an example, gastric juice can be secreted into a vessel while the gastric contents

are gradually removed from that vessel to simulate gastric emptying. Similarly, gastric pH is not kept at a fixed value, but can be manipulated to replicate the changes occurring during *in vivo* digestion by the addition of hydrochloric acid (Venema, Havenaar, & Minekus, 2009). Examples of three of these models are presented below:

(1) **TIM-1<sup>®</sup>** gastrointestinal tract model consisting of four compartments simulating the stomach, duodenum, jejunum and ileum developed by the Netherlands Organisation for Applied Scientific Research (TNO) (Venema, Havenaar, & Minekus, 2009).

(2) **DYNAMIC GASTRIC MODEL (DGM<sup>®</sup>)** consisting of two stages simulating the main body of the stomach and the lower part of the stomach (antrum) developed by the English Institute of Food Research (IFR) (Wickham, Faulks, & Mills, 2009).

(3) **DiDGI<sup>®</sup>** - gastrointestinal model composed of two compartments, stomach and small intestine developed by the French Institute of Agronomic Research (INRA) (Ménard, et al., 2014). The DiDGI<sup>®</sup> model is common to several INRA labs and was used in *in vitro* digestion conducted during my PhD. For this reason, it is the only model for which a more detailed description is provided.

DiDGI<sup>®</sup> (Figure 38) was developed in order to monitor food deconstruction and hydrolysis kinetics occurring during simulated digestion. This is a computer-controlled system in which gastric and intestinal transit times, kinetics of gastric and intestinal pH, sequential addition of digestive secretions, and gastric and intestinal stirring can all be defined. As previously mentioned, DiDGI<sup>®</sup> consists of two consecutive compartments simulating the stomach and small intestine. Between the gastric and intestinal compartments, there is a Teflon membrane with 2 mm holes that mimics the sieving effect of the pylorus in Humans. Each compartment is

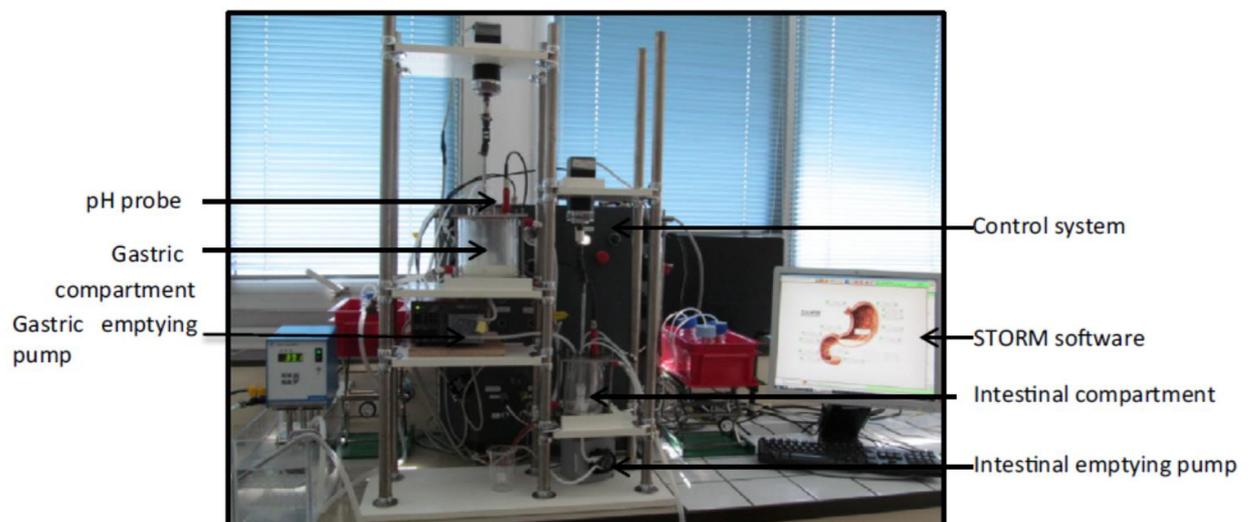


Figure 38- DiDGI<sup>®</sup> dynamic digestion system (Ménard, et al., 2014)

surrounded by a glass jacket filled with water pumped using a temperature controlled water bath. Temperature, pH and redox sensors are connected to the system and the flow rate of meal, HCl, Na<sub>2</sub>CO<sub>3</sub>, bile and enzymes is regulated by computer-controlled peristaltic pumps. It is also possible to simulate anaerobic conditions by purging the air with nitrogen. The system is controlled by the software “StoRM” (Stomach Regulation and Monitoring). This software was designed to accept parameters and data obtained from *in vivo* studies, such as the quantity and duration of a meal, the pH curves for the stomach and small intestine, the secretion rates into the different compartments and the gastric and small intestine emptying. (Ménard, et al., 2014)

### 5.3. ANALYSIS OF DIGESTION PRODUCTS AND STARCH FRACTIONS IN FOODS

#### 5.3.1. PRODUCTS OF *IN VITRO* DIGESTION OF STARCH-RICH FOODS

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Procedures to monitor starch digestion can be direct or indirect, measuring the amount of digested or undigested starch respectively. When undigested starch is measured, digested starch can be estimated from the difference between total and undigested starch. An approach that is common to a number of *in vitro* starch digestion procedures is the complete hydrolysis of all the products of starch digestion into glucose (via an additional incubation with amyloglucosidase, for example), which is then quantified and from this, the digested starch can be estimated glucose x 0.9 (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Additionally it is also possible to separate different fraction of digestion products, and then quantify them separately. An example is the quantification of oligosaccharides with degrees of polymerization up to 10, after selective precipitation of larger fractions via an incubation with 80% aqueous ethanol (v/v) (Hoebler, et al., 1998). The most widely used methodology to determine glucose concentration is based on spectrophotometry. More recently studied for in lab-use, glucometry is another technique that has been proposed as a quick, inexpensive, readily-available and easy to use alternative to screen samples for starch digestibility; it is suitable for semi-continuous measurement of glucose concentration and, as such useful for the understanding of time-course changes of digested starch. (Sopade & Gidley, 2009)

#### 5.3.2. QUANTIFICATION OF DIFFERENT STARCH FRACTIONS IN FOODS

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For nutritional purposes, starch fractions can be classified into three categories depending on their rate and extent of digestion; three main categories have been defined: rapidly digested starch (RDS), slowly digested starch (SDS) and resistant starch (RS). RDS is the starch fraction responsible for the rapid increase in blood glucose concentration following ingestion of carbohydrates, however, it is not to be confused with the total sugar content of a meal which also contributes to the rate of increase in blood glucose concentration. SDS is the fraction of starch that is digested slowly but completely in the human small intestine. RS is the fraction of

starch that escapes digestion in the small intestine and may be subject to bacterial fermentation in the large intestine. Under standard *in vitro* conditions of substrate and enzyme concentration, RDS can be defined as the amount of starch digested in the first 20 minutes, SDS as the fraction of starch digested after the RDS but in no longer than 120 minutes and RS as the remaining fraction of undigested starch. (H. N. Englyst, Kingman, & Cummings, 1992)

#### 5.4. *IN VITRO* PROTOCOLS – COMPLEMENTARITY TO *IN VIVO* STUDIES

*In vitro* digestion models can be an important complement to *in vivo* studies by allowing investigations on processes that are otherwise not accessible, and enabling to pre-screen products and conduct preliminary tests on a given hypothesis (Minekus, et al., 2014b). This type of studies can be of particular importance for the study of specific nutrients or nutrient fractions that trigger allergic responses (Petitot, Abecassis, & Micard, 2009; F. Smith, et al., 2015). Additionally, *in vitro* protocols have also been used to predict some parameters of the physiological response to foods. An example is the prediction of the GI of foods (K. N. Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Gibson, Schönfeldt, & Pretorius, 2011; Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Depending on the protocols used, some discrepancies have been reported between *in vivo* and *in vitro* studies of the digestion of starch-rich foods (Berti, Riso, Monti, & Porrini, 2004; J. Brand-Miller & Holt, 2004; Casiraghi, Brighenti, & Testolin, 1992). However, both static (Araya, Contreras, Alvina, Vera, & Pak, 2002; Ells, Seal, Kettlitz, Bal, & Mathers, 2005; H. N. Englyst, Veenstra, & Hudson, 1996; Ferrer-Mairal, et al., 2012; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; D. Jenkins, et al., 1982; John A. Monro & Mishra, 2010; J. A. Monro, Mishra, & Venn, 2010; Seal, et al., 2003) and dynamic (Ballance, et al., 2013; Bellmann, et al., 2010) *in vitro* protocols are generally considered as reliable indicators of the digestive profiles of these foods *in vivo* (specifically the glycaemic response). As it has recently been concluded, considering the practicality of *in vitro* assays, and their highly significant correlations with *in vivo* data, it certainly seems wise to continue optimizing these techniques and take advantage of them in research and early stages of product development (Bohn, et al., 2017).

## OBJECTIVES

In the previous section, we have discussed the relevance of starch in human nutrition, and seen that beyond the amount of starch within our diet or consumed foods, it is also very important to consider their digestion rates to better understand their overall glycaemic impact. Moreover, it is of common knowledge that the two main enzymes that participate in starch digestion are: salivary amylase, which is active in the mouth and stomach, and pancreatic amylase, which is active in the small intestine. However, their respective contribution to the digestive process of starch seems to remain unclear. The potential contribution of the oral and gastric phases of digestion is frequently overlooked, and it is often considered that most starch is digested in the small intestine by pancreatic amylase. However, we have found hints in the literature that this might not always be the case. In fact, because postprandial acidification of gastric contents can take anywhere from 60 to 200 min to reach  $\text{pH} \leq 2$  (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Kalantzi, et al., 2006; Malagelada, Go, & Summerskill, 1979; Troost, Steijns, Saris, & Brummer, 2001), it seems that there is a large time window during which amylase of salivary origin can remain active during gastric digestion. A first objective of my PhD work was, thus, to re-evaluate the role of salivary  $\alpha$ -amylase in starch digestion. We were also interested in understanding what the extent of its contribution was: does it only release large starch fractions, or does it also hydrolyse them more extensively, into smaller fractions with low degrees of polymerization?

We have also highlighted that a simple strategy to reduce the glycaemic response, based on pairing starch-rich foods with a beverage or condiment that can inhibit amylolytic enzymes, could be easy to adopt by the general population. We have analysed the reports of clinical studies showing that lowering the pH of starch-rich meals, with vinegar for example, consistently attenuated the glycaemic response to these meals (Figure 30). The mechanism is clearly related to acidity, but its exact nature remains a controversial topic (Figure 31). Taking into account the long kinetics of gastric pH acidification observed *in vivo*, it seems that any means of accelerating pH reduction could limit the contribution of salivary  $\alpha$ -amylase to starch hydrolysis, possibly influencing its overall digestion rate and the glycaemic response. Could a premature acidification, and thus inhibition, of salivary amylase during gastric digestion explain the results of these clinical studies? Would similar results be achievable with other acidic foods or beverages? Could other non-acidic food items produce similar effects? Are such strategies effective *in vivo*?

Finally, related with the possible attenuation of the glycaemic response to a meal by lowering its pH, two other aspects can be debated. The first is the possible impact of the attenuation of the glycaemic response *per se* (that is, without modifying the nutritional content of the meal) on satiety/appetite-related sensations? This has been difficult to ascertain because lowering the glycaemic response of a meal often implies changing its nutritional content which adds confounding factors that are difficult to account for. The hypothesis that the attenuation of the glycaemic response to acidic starch-rich meals is explained by a delayed gastric emptying has also been put forward. This has been investigated by some research teams, but different results were obtained (Brighenti, et al., 1995; H. Liljeberg & Björck, 1996, 1998). It is known that the rate of gastric emptying can be influenced by numerous factors, including the pH (Hunt & Knox, 1962, 1969, 1972), but literature shows that caloric density appears to be a major determinant of gastric emptying (Figure 25). So, which factor is predominant, the pH or caloric density?

The research work was designed to provide some answers to the main questions summarized above, which can be translated into the following main objectives:

6. To evaluate the role of salivary  $\alpha$ -amylase during oro-gastric digestion of starch and to assess the extent of its contribution to the hydrolysis of starch during the digestion of high- and low-GI foods *in vitro*.
7. To screen commonly consumed beverages (tea, coffee, wine, lemon juice) and condiments (vinegar), for their capacity to inhibit salivary and pancreatic amylases.
8. To test if the effect of consuming starch-rich foods with either water, an acidic drink (lemon juice), or a polyphenol-rich drink (tea) could reduce the digestion rate of starch *in vitro*.
9. To determine whether lemon juice and tea could effectively attenuate the glycaemic response to bread in a real-life setting.
10. To investigate if lowering the glycaemic response of a meal without modifying its nutrient composition could influence satiety/appetite related perceptions and/or energy intake at the next meal.
11. To study the gastric digestion patterns (mostly gastric emptying) of a bread meal paired with either water, lemon juice, or tea by magnetic resonance imaging.

II

*IN VITRO* STUDIES



IN THIS PART OF THE MANUSCRIPT THE RESULTS OF THE *IN VITRO* STUDIES WILL BE PRESENTED.

THE MAIN GOAL WAS TO ANSWER THE FOLLOWING QUESTIONS:

**WHAT IS THE CONTRIBUTION OF EACH DIGESTIVE STAGE TO THE DIGESTION OF STARCH-RICH FOODS?**

**CAN THIS KNOWLEDGE BE USED TO DESIGN STRATEGIES TO SLOW DOWN STARCH DIGESTION?**

This work is presented in the form of four articles:

- Chapter I - The important role of salivary  $\alpha$ -amylase in gastric digestion of wheat bread starch
- Chapter II -Oro-gastro-intestinal digestion of starch in white bread, wheat-based and gluten-free pasta: unveiling the contribution of human salivary  $\alpha$ -amylase
- Chapter III - Acid induced reduction of the glycaemic response to starch-rich foods: The salivary  $\alpha$ -amylase inhibition hypothesis
- Chapter IV - Inhibitory effect of black tea, lemon juice, and other beverages on salivary and pancreatic amylases: What impact on bread starch digestion? A dynamic *in vitro* study.

An informal text summarizing the reasoning behind the work presented as well as a scientific abstract were included at the start of each chapter. At the end of each chapter the key results are recapitulated in written and schematic form.



II. *IN VITRO* STUDIES

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# CHAPTER 1

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## THE IMPORTANT ROLE OF SALIVARY AMYLASE IN GASTRIC DIGESTION OF WHEAT BREAD STARCH

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### Informal presentation

*As explained in the introduction, part of the results presented here were obtained during my internship in the GMPA lab in the fulfilment of the requirements of my master studies. The laboratory work was then completed during the PhD.*

Nutrition is a complex process in which not only the type and quantity of nutrients ingested are important, but also their rates of digestion and absorption. Because of the positive correlation between the rate of starch hydrolysis for a certain food and the corresponding glycaemic response extensive work has been dedicated to identify ways of slowing down starch digestion.

However, some aspects of its digestive process remain unclear. It is clear that two key enzymes participate in this process, salivary amylase (present during the oral and gastric phases) and pancreatic amylases (present during the intestinal phase). The role of salivary amylase is often considered of little significance, and it is often thought that most starch is digested in the small intestine by pancreatic amylase and brush border enzymes but some hints in the literature point to the opposite. Research addressing this matter is scarce, but essential: how can one define strategies to improve the digestive profile of starch-rich foods if there are pieces missing in the puzzle?

**THE FIRST QUESTION WAS: WHAT IS THE CONTRIBUTION OF THE SALIVARY AMYLASE TO THE ORAL AND GASTRIC PHASES OF STARCH DIGESTION?**

## ABSTRACT

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The role of salivary  $\alpha$ -amylase (HSA) in starch digestion is often overlooked in favour of that of pancreatic  $\alpha$ -amylase due to the short duration of the oral phase. Although it is generally accepted that amylase of salivary origin can continue to be active in the stomach, studies ascertaining its contribution are lacking. This study aimed to address this issue by coupling *in vitro* oral processing with an *in vitro* dynamic system that mimicked different postprandial gastric pH reduction kinetics observed *in vivo* following a snack- or lunch-type meal. Digestion of both starch and protein from wheat bread, as well as the interplay between the two processes were studied. We have observed that the amylolytic activity of saliva plays a preponderant role hydrolysing up to 80 % of bread starch in the first 30 min of gastric digestion. Amylolysis evolved exponentially and nearly superimposing curves were obtained regardless of the acidification profiles, revealing its high efficiency.

## 1. INTRODUCTION

Nutrition is a complex process in which not only the type and quantity of nutrients ingested are important, but also their rates of digestion and absorption, particularly for carbohydrates and proteins. The most frequently used reference tool to classify the rate of carbohydrates digestion and absorption is the Glycaemic Index (GI) (Dona, Pages, Gilbert, & Kuchel, 2010). Different meta-analysis studies have found evidence of an association between high GI diets and an increased risk of type-2 diabetes (Bhupathiraju, et al., 2014), coronary heart disease in women (Dong, Zhang, Wang, & Qin, 2012; Mirrahimi, et al., 2012), breast cancer (Mullie, Koechlin, Boniol, Autier, & Boyle, 2016), as well as obesity related complications (Schwingshackl & Hoffmann, 2013). Likewise, protein resistance to digestion and the rate at which it is digested, can influence the mechanisms that trigger food allergies (Bøgh & Madsen, 2016) and the efficiency of post-prandial protein metabolism (Dangin, Boirie, Guillet, & Beaufrère, 2002; Koopman, et al., 2009; Walrand, et al., 2016), respectively.

The association between carbohydrates and proteins forms the elementary structure of many cereal-based staple foods such as wheat bread, one of the main sources of carbohydrates in the human diet (Wirfält, et al., 2002). One loaf of bread consists of about 50 % starch, 40 % water and 7 % proteins by weight (Pateras, 2007). Wheat starch consists of around 23 % amylose (essentially linear) and 73 % amylopectin (larger and branched) (Stauffer, 2007). These polymers are made up of  $\alpha$ -1,4-linked glucose residues in linear segments and  $\alpha$ -1,6-linked glucose residues at branch points (Stauffer, 2007).

Given that starch is generally the main source of digestible carbohydrate in the human diet (Butterworth, Warren, & Ellis, 2011), the physiological impact of its digestion kinetics has been widely studied, and slowly digestible starch has been linked to beneficial effects on common diet-related chronic diseases such as diabetes and pre-diabetes, cardiovascular diseases and obesity (Miao, Jiang, Cui, Zhang, & Jin, 2015).

Starch digestion starts in the oral cavity (when saliva is mixed with food particles) due to the action of human salivary  $\alpha$ -amylase (HSA) which breaks down amylose and amylopectin by cleaving their  $\alpha$ -1-4 glycosidic bonds (Bornhorst & Singh, 2012). Its role is often considered of little significance due to the short duration of the oral phase, but this line of thought can be challenged after analysing some bibliographical data. Indeed, bread boli are typically formed within 16 to 50 seconds, depending on the type of bread and the characteristics of the individual (Jourden, et al., 2016; Panouillé, Saint-Eve, Délérís, Le Bleis, & Souchon, 2014). However, once boli are swollen, HSA can continue hydrolysing starch in the stomach (Bornhorst & Singh, 2012;

S. S. Gropper & J. L. Smith, 2013) until the pH lowers below 4.0 and the enzyme is inactivated (M. Fried, S. Abramson, & J. H. Meyer, 1987).

As postprandial gastric acidification is a gradual process, it can take over 45 min to reach this pH level (Malagelada, Go, & Summerskill, 1979). Data from human studies showing that HSA can remain active in the stomach long after the short oral processing phase, and might even reach the small intestine without becoming inactive, indicate that this enzyme can be responsible for hydrolysing an important fraction of starch (M. Fried, S. Abramson, & J. H. Meyer, 1987). Additionally, according to an interesting article from the early 20<sup>th</sup> century, also reporting results of an *in vivo* human study, up to 76 % of the starch in mashed potatoes and 59 % of that in bread is hydrolysed into maltose by HSA in the stomach (Bergeim, 1926). Since no other amylase is present, only that of salivary origin can be responsible for the enzymatic hydrolysis of starch during gastric digestion (Bornhorst & Singh, 2012). Nonetheless, this work seems to have been forgotten within the scientific community, considering that the extent of HSA's contribution to starch digestion remains unclear (Butterworth, Warren, & Ellis, 2011) and the final digestive stage is often considered of higher importance. The last stage of starch digestion occurs in the small intestine, where pancreatic  $\alpha$ -amylase and brush border enzymes complete amylolysis, and the ultimate product of this process, glucose, is finally absorbed into the blood stream (S. S. Gropper & J. L. Smith, 2013).

Proteins in wheat bread consist of about 25 % of soluble proteins and 75 % of insoluble gluten proteins (Belderok, Mesdag, & Donner, 2000). Contrarily to the digestion of starch, proteolysis only begins in the stomach with the combined action of hydrochloric acid (HCl) and pepsin, the gastric protease (S. S. Gropper & J. L. Smith, 2013) HCl has a double action, it causes proteins to unfold or uncoil due to the breakage of hydrogen and electrostatic bonds (S. S. Gropper & J. L. Smith, 2013) and enables the activation of pepsin (S. S. Gropper & J. L. Smith, 2013; Piper & Fenton, 1965). Human pepsin starts being active below pH 5.5 and exhibits maximum activity at pH 2 (Piper & Fenton, 1965). The products of gastric proteolysis consist of a mixture of large polypeptides, oligopeptides and free amino acids (S. S. Gropper & J. L. Smith, 2013). Protein digestion is completed in the small intestine by the action of different proteases and peptidases before the absorption of small peptides and free amino acids (S. S. Gropper & J. L. Smith, 2013).

At a macroscopic level, bread is characterized as a solid foam (J. A. Gray & J. N. Bemiller, 2003). At a molecular level, the bread foam contains a continuous and a discontinuous phase. The continuous phase is formed by an elastic network of cross-linked gluten molecules and leached starch polymers that may integrate starch-gluten associations (starch-gluten fibrils) (J. A. Gray

& J. N. Bemiller, 2003). The discontinuous phase consists of gelatinized, swollen, deformed starch granules entrapped in the continuous phase (J. A. Gray & J. N. Bemiller, 2003). Given the intricate association of starch and gluten in bread, an interdependence of their digestive processes can be expected. This was recently shown using an *in vitro* static digestion model where the pH during each digestive stage was kept stable (F. Smith, et al., 2015). Expanding our understanding of the digestive process of starch, and how this influences the digestion of proteins, is an important step towards the development of food products with potential health-supporting digestion rates. However, to preserve the biological relevance of the results, it is important that the digestive conditions reproduce as closely as possible those found *in vivo*. Therefore, our purpose was to re-evaluate the role of HSA in the form in which it is found in the human digestive tract (i.e. in saliva, non-purified), and to study the digestion kinetics of bread starch and proteins during oral and gastric processing using DiDGI<sup>®</sup>, an *in vitro* model capable of mimicking key dynamic aspects of our digestive environment. Bread was selected as a model food due to its macronutrient profile and the importance it has in our diet.

## 2. MATERIALS AND METHODS

### 2.1. MATERIALS

The bread used in this study was a French wheat baguette supplied by Lesaffre (Marcq-en-Baroeul, France) that has been characterized during previous studies carried out in our lab (Jourden, et al., 2016). Only the crumb of the baguette was used in all the experimental work.

Human saliva from one non-smoker volunteer was used for the determination of HSA activity as a function of pH and for the preparation of saliva-based boli after informed consent was obtained. The subject was asked to abstain from tooth brushing, food and drinks for at least two hours prior to saliva collection. On each trial day, mechanically stimulated saliva was collected at 9 AM, aliquoted and conserved at 4 °C until usage (for a maximum period of 6 hours). Saliva was used in all experiments requiring HSA. Given that HSA's activity can be influenced by the salts naturally present in saliva, (P Bernfeld, Staub, & Fischer, 1948) no pre-treatments (excluding freezing and thawing) were employed in order to reinforce the physiological relevance of the results. Immediately before each digestion experiment, saliva aliquots were placed in a water-bath for 5 min and brought to 37 °C. The commercial enzymes used in this study were: amyloglucosidase from *Aspergillus Niger* (ref 10 102 857 001, from Roche, Switzerland), pepsin from gastric mucosa (P-6887) and pancreatic  $\alpha$ -amylase (A-3176), both of porcine origin (Sigma-Aldrich, France). The substrate used for the determination of HSA activity was potato starch (ref. 101 253, from Merck, Germany).

### 2.2. BREAD CHARACTERIZATION

**PROTEIN:** Total protein content was determined by an independent laboratory (ABIOPOLE - Groupe Qualtech, France) using the Kjeldahl method.

**STARCH:** Total and resistant starch determination assays were carried out in our laboratory in triplicate.

**TOTAL STARCH** was determined according to a modified version of Goñi's method (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Bread crumb samples ( $50 \pm 5$  mg) were dispersed into 10 mL of 2 M KOH solution and mixed (150 rpm) at room temperature for 30 min before 5.7 mL of 2 M HCl and 3 mL of sodium acetate buffer were added and the pH adjusted to  $4.75 \pm 0.1$ . This resulted in solutions of solubilized starch which were subsequently hydrolysed into glucose by adding 60  $\mu$ L of undiluted amyloglucosidase and incubating in a shaking water-bath (60 °C, 150 rpm, 45 min). After centrifugation (4 °C, 3000 g, 10 min) glucose concentration in the supernatant was determined using an enzymatic kit (D-Glucose kit, ref. 103.21, BioSenTec;

Toulouse, France) followed by absorbance measurements (340 nm) in 1 cm plastic cuvettes with a UV-Vis spectrometer (Evolution 201, Thermo Scientific). Total starch content was then calculated from the measured glucose as mg of glucose  $\times$  0.9.

**RESISTANT STARCH** was determined according to a modified version of the method of Goñi (Goñi, Garcia-Diz, Mañas, & Saura-Calixto, 1996). Bread crumb samples ( $250 \pm 5$  mg) were incubated with 10 mL of HCl-KCl buffer (pH 1.5) and 0.2 mL of a  $2.5 \times 10^5$  U/mL pepsin solution in a shaking water bath (40 °C, 150 rpm) for 60 min. Then, 9 mL of tris-maleate buffer (pH 6.9) and 5 mL of a 0.52 U/mL pancreatic  $\alpha$ -amylase solution were added. A 16 h incubation at 37 °C under agitation (150 rpm) followed. After centrifugation (4 °C, 3000 g, 15 min) and supernatant removal, the starch remaining in the pellet (resistant starch) was quantified using the procedure described above for total starch.

**DIGESTIBLE STARCH** was calculated as the difference between total and resistant starch.

### 2.3. HSA ACTIVITY AS A FUNCTION OF PH

An adapted version of Bernfeld's method (Peter Bernfeld, 1955) was used to estimate the activity of HSA as a function of pH. Human saliva, collected as described in section 2.1, was diluted 10 folds (w/w) with deionized water. 13 solutions of 1% (w/v) starch and 5.8 mM NaCl were prepared in the range of pH 2 to 7.5 using HCl or NaOH for pH adjustments. For each pH level, four glass tubes containing 0.5 mL of the corresponding starch solution were incubated at 20 °C for 3 min with different volumes of diluted saliva (0, 50, 70 or 100  $\mu$ L). Immediately after, solutions with a pH < 6.5 were neutralized to 7 with pre-determined volumes of NaOH solutions, and the amyolytic activity was interrupted by adding 0.5 mL of a DNS solution (containing 96 mM 3, 5-Dinitrosalicylic Acid, 0.5 M NaOH and 0.16 M of potassium tartrate). The saliva content of each tube was then standardized to 100  $\mu$ L. All tubes were placed in a boiling water bath for 15 min and cooled to room temperature in ice, before adding 4.95 mL of a  $\text{Na}_3\text{PO}_4$  buffer solution and adjusting at pH 6.9. Absorbance was then read at 540 nm, and the maltose concentration was determined from a previously established calibration curve obtained using a series of maltose solutions (0.005 % to 0.2 % w/v). Enzymatic activity was defined as follows: 1 unit liberated 1.0 mg of maltose from potato starch per mL of saliva in 3 min, at pH 6.9 and 20°C.

### 2.4. *IN VITRO* DIGESTION: ORAL AND GASTRIC PHASES

All digestion experiments were carried out with bread crumb only. The crumb was separated from the crust with the help of a round, 3 cm diameter cookie cutter, used to cut crumb cylinders from bread slices 1.5 – 2 cm thick.

**ORAL PHASE:** After cutting each crumb cylinder into four (lengthwise), a standard volume of baguette crumb was artificially chewed using a domestic kitchen food chopper (Multi-moulinette 400W, Moulinex, France) during 4 segments of 5 s to simulate food comminution through human mastication (no liquid was added at this stage). Individual portions of 3 g were kept in an air-tight container at 20 °C until usage (maximum of 4 hours). Each saliva-based bolus was formed by transferring one artificially chewed bread portion into an individual plastic syringe with a cut end, and mixing in 1.5 g of saliva for 30 s with a spatula. Mixing time was set in accordance with *in vivo* data from a previous work with the same bread (Jourden, et al., 2016) and other results from the literature (Hoebler, Devaux, Karinthe, Belleville, & Barry, 2000), both reporting chewing times between 18 and 41 s for white wheat bread. Particular attention was paid to prevent an overestimation of HSA's role by defining a bolus hydration level at 0.5 mL of saliva per gram of crumb, just below the 0.6 – 1.0 mL/g range previously reported as the optimal saliva level for wheat bread bolus (Bornhorst & Singh, 2013).

The suitability of our *in vitro* boli to appropriately mimic the textural and digestive properties of *in vivo* boli was preliminarily assessed. *In vivo* boli were produced by the volunteer who donated the saliva by chewing 3 g crumb portions for 30 s. Texture profile analyses (TPA) were conducted using a TA-XT2 texture analyser (Stable Micro Systems Ltd., Godalming, UK) equipped with an ebonite 60-mm-diameter cylindrical probe. Each sample was compressed twice at 4°C and a speed of 0.83 mm.s<sup>-1</sup>, up to 65 % of deformation (rest period of 1 s between each cycle) to determine its hardness, adhesiveness and cohesiveness (3 replicates). Figure 39 shows that only hardness was significantly different (higher for *in vitro* formed boli). As presented in Figure 40, the digestion profiles of both types of bolus were also highly comparable, with a plateau being reached in about 15 min in all cases.

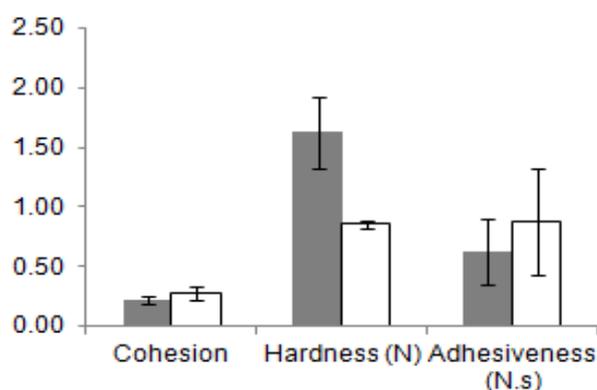


Figure 39 - Texture Profile Analysis of *in-vitro* (grey columns) and *in-vivo* (white columns) formed boli. Different letters (a-b) denote statistically significant differences ( $p < 0.05$ ) between the two types of bolus for each parameter (cohesion, hardness and adhesiveness) according to one-way ANOVA.

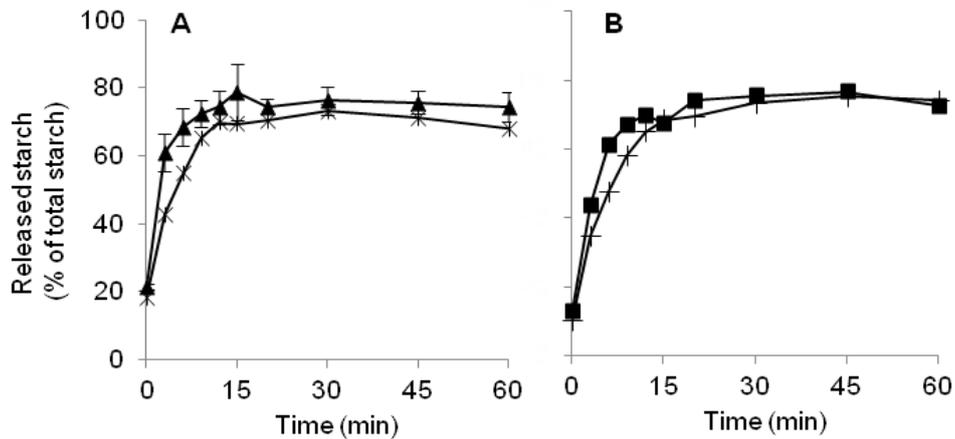


Figure 40 - Assessment of the *in vitro* boli's ability to reproduce *in vivo* conditions. Proportion of starch released during digestion of *in vivo* and *in vitro* formed bread boli. (A) —▲— SSB - Snack-type digestion of an *in vitro* Saliva-based bolus (average  $\pm$  SD, 3 rpt.), —∗— Snack-type digestion of an *in vivo* Saliva-based bolus (1rpt). (B) —■— CSB - Constant pH Digestion of an *in vitro* Saliva-based-bolus (1rpt), —⊕— Constant pH Digestion of an *in vivo* Saliva-based-bolus (1rpt).

**GASTRIC PHASE:** Bread boli were submitted to individual dynamic gastric digestions using DiDGI®, a dynamic digestion system. This system is controlled by a software (STORM®) that allows one to continuously add pepsin and HCl solutions to an *in vitro* chime (henceforth simply called chime) and also to monitor and control its pH, temperature and stirring conditions (Ménard, et al., 2014). Immediately after being prepared, each bolus was introduced in the gastric compartment of DiDGI® (capacity  $\sim$  600 mL), previously set-up with an operating temperature of 37 °C, and a pre-meal water volume of 50 mL to ensure that the pH probe was properly submerged and facilitate sample collection. After a 30 s stirring period followed by a 30 s sedimentation step (no stirring), the first sample was collected from the supernatant and the *in vitro* gastric digestion was initiated by activating the influx of pepsin and HCl using the software. Due to practical experimental limitations (ex. need to stir for 30 s before the first sample), a total of 2.5 min passed between the start of bolus formation and the beginning of gastric digestion. This was the shortest possible time to complete all the preparatory tasks and initiate the gastric phase. Temperature (37 °C), stirring conditions with an anchor blade (10 rpm, to obtain homogeneous mixing) and pepsin flux (5000 U/min) were equal in all experiments. Other parameters were selectively modified to investigate the respective influences of HSA, pepsin and gastric pH reduction kinetics, leading to 4 different types of trials.

(1) “Snack-type digestion of a Water-based Bolus” (SWB): saliva was replaced by water during the bolus preparation (*i.e.* a condition with no HSA), and gastric digestion lasted 60 min. The pH was gradually reduced from 6.0 to 2.0 by continuously adding 0.5 M HCl during the first 30 min

of gastric digestion, and kept constant (pH 2) during the last 30 min. This pH reduction kinetics was intended to reproduce the evolution of human gastric pH following ingestion of a bread-based snack-type meal (Dedlovskaya, 1968), and could be classified as a physiologically fast pH reduction kinetics. SWB served as a control trial for starch digestion.

**(2)** “Snack-type digestion of a Saliva-based Bolus” (SSB): SSB trials only differed from SWB in the type of bolus used as in SSB saliva-based boli were used.

**(3)** “Lunch-type digestion of a Saliva-based Bolus” (LSB): these trials only differed from SSB in the duration of the gastric phase and the pH reduction kinetics. This digestive stage lasted 90 min and the pH reduction from 6.0 to 2.0 was performed in a 60 min period (vs. 30 min in SSB). This was done to simulate human gastric pH conditions following ingestion of a lunch bread-based meal (Malagelada, Go, & Summerskill, 1979).

**(4)** “Constant pH (pH 6) digestion of a Saliva-based Bolus” (CSB): CSB was carried out to assess the maximum amyolytic capacity of HSA under favourable activity conditions (pH  $\approx$  6.0) upon digestion of bread. This trial only differed from SSB trials in the pH conditions used, and served as a control for protein digestion as pepsin activation was not enabled.

In addition to the first sample collected right before initiating gastric digestions, during the gastric phase samples were also collected at 3, 9, 12, 15, 20 and 30 min, and every 15 min after this. The sampling process consisted in interrupting stirring for 30 s (to allow for particle setting) immediately before collecting a 1 mL sample from the supernatant and placing it in a dry bath (ThermoMixer C, Eppendorf) (99 °C, 5 min) for enzyme inactivation. Samples were conserved at -20 °C until required for further analysis. Assays SSB and LSB were performed in triplicate, while the others were conducted once.

## 2.5. SAMPLE ANALYSIS

After thawing (12-15 hours, 4 °C) and centrifugation (10 min, 3000 g, 4 °C), the supernatant of *in vitro* chime samples was analysed for partially hydrolysed starch and solubilized protein fractions.

**RELEASED STARCH:** samples were treated as solutions of solubilized starch as described in section 2.2 for the determination of total starch. This method accounts for both solubilized starch fractions and products of starch hydrolysis. The rate of starch digestion was expressed as the percentage of total starch released as a function of time for each type of trial.

**RELEASED PROTEIN:** the percentage of total protein in the chime was estimated by direct UV spectrophotometry ( $\lambda=280$  nm) using quartz cuvettes (Evolution 201, Thermo Scientific spectrophotometer). The mass attenuation coefficient of wheat flour proteins at 280 nm ( $\epsilon_{280}$ ,  $L.g^{-1}.cm^{-1}$ ) was estimated according to their amino acid composition (Juhász, Békés, & Wrigley, 2014) and the following equation adapted from Pace and colleagues (Pace, Vajdos, Fee, Grimsley, & Gray, 1995):

$$\epsilon_{280} = 5,500 \times n_{Trp} + 1,490 \times n_{Tyr} + 125 \times n_{cystine}$$

where  $n_{Trp}$ ,  $n_{Tyr}$ , and  $n_{cystine}$  are the quantity (mol) of tryptophan, tyrosine, and cystine residues per gram of protein. The mass of solubilized wheat proteins was estimated using the absorbance at 280 nm corrected from pepsin absorbance, and the volume content in the gastric compartment of DiDGI®.

### 3. RESULTS AND DISCUSSION

#### 3.1. BREAD CHARACTERIZATION

The studied baguette contained  $43.4 \pm 1.3$  g of starch per 100 g of crumb as eaten, of which  $95.9 \pm 0.6$  % of was digestible and  $4.1 \pm 0.6$  % was resistant to digestion. Protein content amounted to 5.4 g per 100 g of crumb as eaten. As mentioned above, a loaf of bread generally consists of about 50% starch, 40% water and 7% protein (Pateras, 2007). The higher water content of the bread used in our study ( $49.1 \pm 0.2$ ) (Jourden, et al., 2016) explains the slightly inferior starch and protein in comparison with values reported in the literature.

#### 3.2. AMYLOLYTIC ACTIVITY OF SALIVA AS A FUNCTION OF PH

To understand the contribution of HSA to starch digestion, it is important to first determine its behaviour within the range of pH values it encounters throughout digestion. The evolution of human HSA's activity as a function of pH is presented in Figure 41. The highest amylolytic activity corresponded to  $352 \pm 41$  U/mL, and was observed between pH 6 and 7. At around pH 4, 50 % of the maximum activity was still observed, and complete inhibition occurred between pH 3.0 and 3.5.

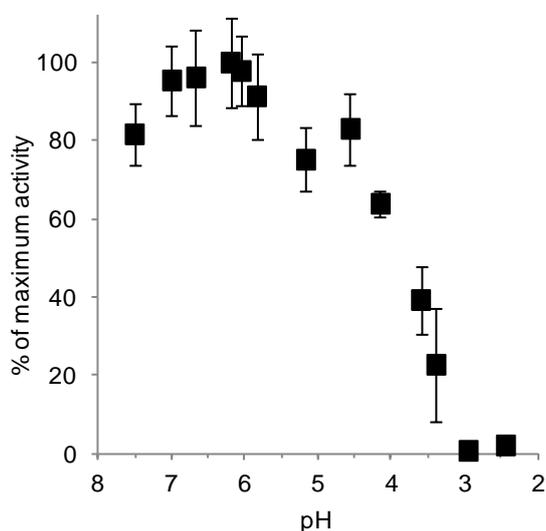


Figure 41 - Amylolytic activity of saliva as a function of pH. Maximum activity, found at pH 6.2, was  $352 \pm 41$  U/mL of saliva (1 unit liberated 1.0 mg of maltose from potato starch in 3 min at pH 6.9 at 20°C).

These findings are well in accordance with the literature on saliva and HSA's properties. The optimum pH of HSA (both in saliva and in purified form) has been reported to be between 6.5

and 7.0 (Peter Bernfeld, 1955; P Bernfeld, Staub, & Fischer, 1948; Walker & Whelan, 1960) and the maximal amylolytic activity of 352 U/mL obtained for saliva is very close to the 410 U/mL previously reported for centrifuged saliva (Peter Bernfeld, 1955) using the same assay as in the present study. Both HSA in saliva and in purified form have also been reported to be inactivated at around pH 3.5, although it seems that the amylolytic activity of saliva decreases slower when approaching this inactivation threshold than that of pure HSA (P Bernfeld, Staub, & Fischer, 1948; Walker & Whelan, 1960). Indeed, it was demonstrated that saliva's amylolytic activity was preserved in human gastric juice at pH 5, and that there was a steady decrease of activity to 50 % of the initial levels after 120 min exposure to gastric juice at pH 4.3 (M. Fried, S. Abramson, & J. H. Meyer, 1987). This somehow contrasts with the activity of pure HSA which was shown to decrease by about 80-90 % when approaching pH 4 (P Bernfeld, Staub, & Fischer, 1948; Walker & Whelan, 1960).

### 3.3. DYNAMIC *IN VITRO* DIGESTION

The role of HSA on starch digestion and the possible interplay between its action and proteolysis were studied by performing a series of *in vitro* digestions varying in oral and gastric conditions. A total of three distinct trials were carried out with saliva-based boli to investigate protein and starch hydrolysis when simulating snack- (SSB) and lunch-type (LSB) gastric acidification kinetics, and when keeping pH constant at 6 (favourable to HSA and unfavourable to pepsin) (CSB). CSB also served as a protein digestion control. For the starch digestion control study, a water-based bolus (free from HSA) was used and *in vitro* gastric digestion (SWB) was carried out under the same conditions as in SSB trials. The results obtained are presented in Figure 42, B, C and D for trials SWB, SSB, LSB and CSB, accordingly. These graphs show the proportion of starch (black symbols) and total protein (white symbols) released over time, as well as the pH conditions (dotted line) during each trial. The first point in each graph corresponds, simultaneously, to the end of the oral phase ( $t = 2.5$  min) and the beginning of gastric digestion.

#### 3.3.1. DIGESTION OF STARCH (FIGURE 42, BLACK SYMBOLS)

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In the absence of amylase (SWB trial, Figure 42A), it can be observed that at the start of the gastric phase (first point in the graph), about 5 % of the total starch was solubilized in the chime. This fraction of solubilized starch increased to 10 % in about 5-6 min, when a plateau was reached. The plateau lasted around 15 min and was followed by another rise starting at around  $t = 20$  min. This second rise lasted approximately 5 min, until a second plateau, corresponding

to around 20 % of the total starch, was reached. Because there was no amylase in this experiment, the first plateau can be attributed to the solubilisation of starch. The second increase started immediately after pepsin activation (at  $\text{pH} \approx 4$ ) suggesting that more starch was released during the breakdown of the protein network.

HSA was present in all other trials (SSB, LSB and CSB). Matching its activity at different pH levels (Figure 41) with the pH conditions imposed, inhibition of its activity by 50 % was anticipated at around  $t = 15$  min and  $t = 30$  min during snack-type (SSB, Figure 42B) and lunch-type (LSB, Figure 42C) digestions, accordingly. In the last type of trial (CSB, Figure 42D) amylolytic activity was expected to be preserved at all times. Surprisingly, the starch digestion profiles in all types of digestions were equivalent, showing an exponential evolution up to a plateau just under 80 % of the total starch released, reached within the first 20 min of digestion. Amylolysis did not seem to be impaired through early HSA inhibition (SSB trials), nor promoted through the extension of its activity period (LSB, and CSB trials). In fact, no matter the pH conditions, and therefore the time HSA remained active, starch hydrolysis evolved exponentially into nearly superimposable curves with similar plateaus always being reached before HSA inactivation (Figure 42). This suggests that the process had already been completed within the first 20 min of digestion, demonstrating the effectiveness and relevance of this enzyme.

By considering the first data points in Fig. 3B, C and D (dark symbols, SSB and LSB digestions), we found that  $18.5 \pm 3.6$  % (mean  $\pm$  std, 9 replicates) of starch were released at the start of the gastric phase. By comparing these results with those obtained in the absence of HSA (SWB trial) (Figure 42A), it is possible to deduce that this enzyme was responsible for hydrolysing at least 13 % of bread starch by the end of the oral phase and an additional 50 % within the first 30 min of gastric digestion.

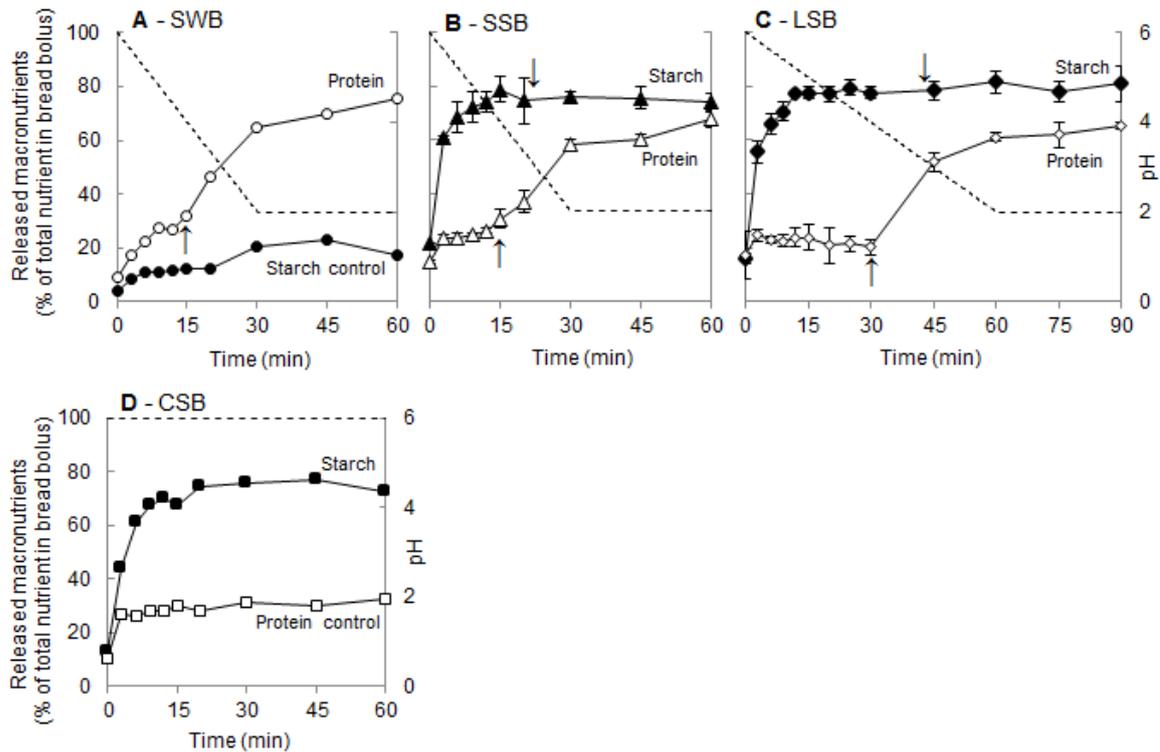


Figure 42 - Starch and protein digestion during *in vitro* oral-gastric digestion of bread. Proportion of total starch (black symbols) and protein (white symbols) released into the chime throughout digestion. **(A)** SWB - Snack-type digestion of a Water-based Bolus (1 rpt.). **(B)** SSB - Snack-type digestion of a Saliva-based Bolus (average  $\pm$  SD, 3 rpts.). **(C)** LSB - Lunch-type digestion of a Saliva-based Bolus (average  $\pm$  SD, 3 rpts.). **(D)** CSB - Constant pH (pH 6) Digestion of a Saliva-based Bolus (1 rpt.). In all graphs, dotted lines (- -) correspond to gastric pH conditions. In graphs A, B and C, up arrows ( $\uparrow$ ) denote the moment when pH conditions start enabling the activation of pepsin (porcine origin) (pH  $\approx$  4). In graphs B and C, down arrows ( $\downarrow$ ) denote the moment of complete HSA inactivation (pH  $\approx$  3).

Starch hydrolysis during the oral phase has been investigated before. The team of Woolnough and colleagues, for example, found that 13 % of bread starch was hydrolysed (either completely to glucose, or partially to dextrins) by HSA during a typical chewing cycle *in vivo* (Woolnough, Bird, Monro, & Brennan, 2010) which is close to the results obtained in our study. Furthermore, despite possible intra-individual variations (Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012) the similarity between the oral phases containing HSA (first point in SSB, LSB and CSB graphs) indicates that any possible variations in saliva's amylolytic profile were minimal. The same team also concluded that the presence of this enzyme did not influence the starch digestion profiles during *in vitro* intestinal digestions, questioning its relevance to the digestive process (Woolnough, Bird, Monro, & Brennan, 2010); However, they used a method that simulated an instantly acidified gastric phase (Woolnough, Bird, Monro, & Brennan, 2010) leading to immediate inhibition of amylolytic activity after the oral phase, which, as discussed above, does

not represent human biological reality (Dedlovskaya, 1968; M. Fried, S. Abramson, & J. H. Meyer, 1987; Malagelada, Go, & Summerskill, 1979).

The similarity between our results and those from Bergeim's *in vivo* studies (Bergeim, 1926), both showing hydrolysis levels of at least 50 % at the end of gastric digestion of bread, strengthens the biological significance of our study as well as the usefulness and importance of using dynamic digestion models in such studies. To the best of the authors' knowledge, this was the first time that the contribution of HSA to starch digestion during the gastric phase was quantitatively assessed with a dynamic digestion model. Our results support not only its relevance in the process, but also highlight its efficiency as, overall, the process was not affected by the pH reduction kinetics.

Around 20 % of the starch remained intact by the end of all trials containing HSA. If the limiting factor here were enzymatic inactivation, the degradation of this starch fraction would have proceeded when amylolytic activity was preserved (CSB), which was not the case (Figure 42D). Our findings appear to reveal the existence of some form of starch resistance to gastric digestion. The concepts of resistant and digestible starch used to characterize the bread studied here are well defined in the literature and refer to the whole process of digestion (Dona, Pages, Gilbert, & Kuchel, 2010). The initial bread characterization revealed that only around 4 % of the total starch in bread was resistant to digestion. Our present results thus suggest that there is a larger fraction of starch, of about 20%, that resists digestion up to the gastric phase. As further discussed in section 3.3.3., this is possibly due to the *in vivo* chronology of salivary amylase and pepsin actions, and the fact that starch is embedded in the gluten network of bread.

### 3.3.2. DIGESTION OF PROTEIN (Figure 42, WHITE SYMBOLS)

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In the protein digestion control trial (CSB, Figure 42D), no proteolysis was expected to occur according to the constant pH of 6. Similarly to the starch digestion control trial (Figure 42A), we have observed that by the end of the oral phase there was already a fraction of protein solubilized in the chime and, after the start of gastric digestion, protein concentration increased rapidly, reaching a plateau in less than 3 min. Around 20-25 % of the total protein was solubilized in the chime. Because no protein fractions from the insoluble gluten network could have been hydrolysed nor solubilized, the increase in protein concentration in the chime is likely to correspond to the dispersion of the soluble protein fraction from wheat flour and was followed by a plateau that lasted until the end of the trial. Indeed, soluble proteins represent around 25 % of the total wheat protein (Belderok, Mesdag, & Donner, 2000), which is consistent with our findings.

In the other *in vitro* digestions, proteolysis was enabled through an acidification of the chime to levels that supported pepsin activation (SSB, SWB and LSB trials). Porcine pepsin, the type of pepsin used in our experiments, starts being active at pH below 4 and exhibits maximum activity at around pH 2 (Kondjoyan, Daudin, & Santé-Lhoutellier, 2015; Pletschke, Naudé, & Oelofsen, 1995). In SSB, SWB and LSB digestions, and similarly to the CSB control, during the first minutes of gastric digestion, while the pH was above 5, there was an increase of the protein concentration followed by a plateau at around 25 % of the total protein solubilized. This plateau lasted only until the pH lowered to about 4, enabling proteolysis. At this transition point, a second rise was initiated. In LSB trials (Figure 42C) the pH reduction was twice as slow as in SSB and SWB, explaining the twice as long duration of the initial plateau. By the end of all the trials, 65 % to 75 % of the total protein in bread had been released.

Considering it appears safe to presume that the initial plateau in the SSB, LSB and SWB trials corresponds to the soluble protein fraction, the transition from this plateau to the second rise, representing about 45 % of the total protein, resulted from the release of protein fractions from the insoluble gluten network (equivalent to about 50 % thereof). Peptic digestion was relatively slow and incomplete. This result is in accordance with findings reported by other researchers indicating that gluten proteins can be quite resistant to *in vitro* gastric digestion (F. Smith, et al., 2015); Additionally, our protein quantification method accounted for all solubilized protein fractions indiscriminately, therefore, it is possible that large fragments resisted proteolysis as demonstrated in the work of Smith and colleagues (F. Smith, et al., 2015).

### 3.3.3. INTERPLAY BETWEEN PROTEIN AND STARCH DIGESTION

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Provided that some of the starch in bread is entrapped within the gluten network, and possibly participates in the formation of starch-gluten interactions (J. A. Gray & J. N. Bemiller, 2003), there is a possible interdependence between amylolysis and proteolysis. In the absence of amylolytic activity, starch-gluten interactions could certainly cause the delay in the initial protein solubilisation (Figure 42A) but they did not seem to impair peptic hydrolysis of the insoluble gluten fraction. Other researchers have observed that bread gluten proteins were digested rapidly only in the presence of pancreatic amylase during an *in vitro* duodenal digestion (F. Smith, et al., 2015), which confirms the intricate nature of starch-gluten interactions and their capacity to influence the digestive process. In their work, an immediate gastric acidification restricted starch hydrolysis to the intestinal phase mainly. However, physiological reality determines that, under normal conditions, HSA is active before any protease and our results show that starch is already extensively hydrolysed when proteolysis is initiated.

By comparing the CSB results (Figure 42D) with those from SSB and LSB, we can observe that starch hydrolysis did not seem to be impaired in the absence of proteolytic activity. However, as previously stated, the second rise in the starch release profile in the absence of HSA (Figure 42A) can be attributed to the effect of pepsin action, thereby reflecting starch-gluten interactions. Indeed, this increase coincided with pepsin activation and degradation of the gluten network, what probably could have led to the release of starch fractions initially entrapped within it. Moreover, these molecular arrangements can also explain, at least in part, the fact that the proportion of starch remaining intact by the end of the gastric digestion trials was about 5 times higher than the fraction of resistant starch found in this bread. This explanation is supported by the distinct protocols used in the resistant starch and digestion experiments. To determine the resistant starch fraction (section 2.2.3) foods are first incubated with a pepsin solution for one hour and amylolysis is initiated only after this process is complete. Due to this inversion of the proteolytic and amylolytic processes, by the time amylolysis starts, protein was already digested and the gluten network disrupted, facilitating the hydrolysis of starch which may explain finding a smaller fraction of resistant starch.

## CONCLUSION

We have demonstrated that HSA plays a preponderant role in the digestion of bread starch during gastric digestion *in vitro* and literature shows that its role *in vivo* appears equally important. Moreover, in the presence of HSA, nearly superimposing exponential curves were obtained regardless of the pH reduction kinetics imposed, which reveals its high efficacy and justifies the need to re-evaluate the relevance of this enzyme in the digestive process of starch. Approximately 20 % of the starch was resistant to gastric digestion, and the initial stages of protein digestion appeared to be impaired in the absence of HSA. Both of these observations could be related with starch-gluten interactions. A specific analysis of the starch and/or protein fractions produced during digestion can provide further insight into the extent of hydrolysis, and it is recommended for future studies.

Finally, our results offer key insight into the digestive process of starchy foods and show the need for accurately replicating the dynamic character of digestive conditions in *in vitro* studies in order to correctly determine the contribution of each mechanism.

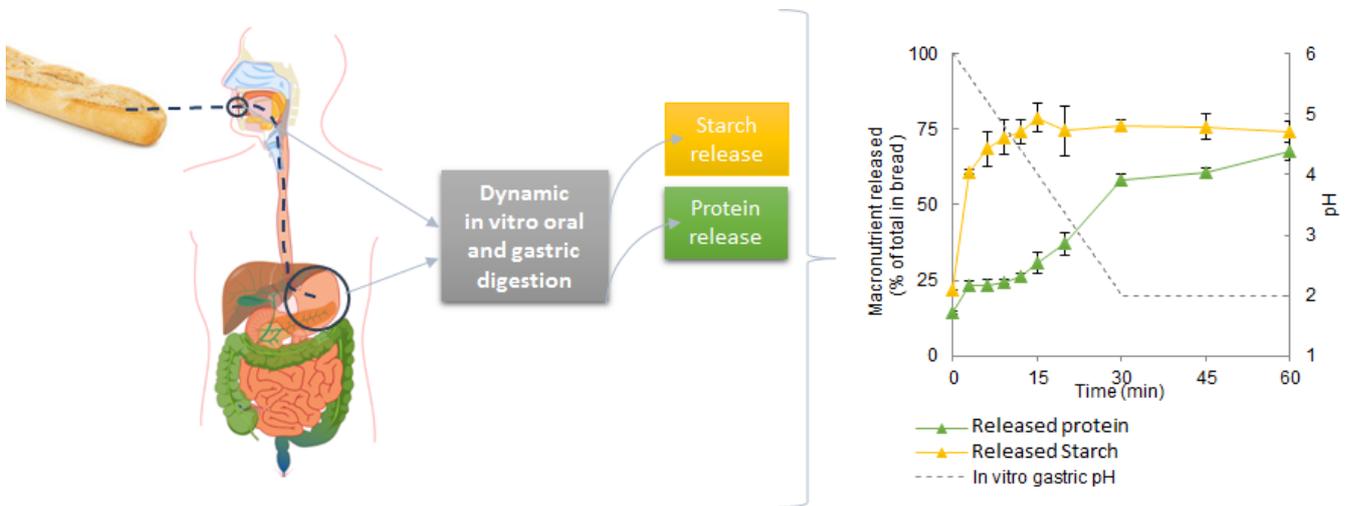
## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## KEY-MESSAGES



- We studied oral-gastric bread digestion mimicking dynamic postprandial conditions
- Salivary  $\alpha$ -amylase hydrolysed up to 80 % of starch in the first 30 min of digestion
- 20% of starch resisted gastric digestion, possibly due to starch-gluten interactions
- Up to 75 % of protein was hydrolysed to some extent during gastric digestion
- A close relationship between starch and protein digestion was observed and could be due to molecular interactions.



## CHAPTER 2

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### ORO-GASTRO-INTESTINAL DIGESTION OF STARCH IN WHITE BREAD, WHEAT-BASED AND GLUTEN- FREE PASTA: UNVEILING THE CONTRIBUTION OF HUMAN SALIVARY $\alpha$ -AMYLASE

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### Informal presentation

The preceding chapter provided first results regarding the role of each digestive stage on the digestion of starch from bread. Can these results be confirmed with other breads? And what about other starch-rich foods? Even when they have similar compositions, starch-rich foods often exhibit different patterns of starch digestion. It is the particular case of wheat-based bread and pasta which are important sources of starch our diets. Their basic ingredients are the same, but the differences in the processing steps lead to two distinct products that generally impact glycaemia differently. Additionally, alternative formulations are often developed to suit particular dietary needs. An example is gluten-free pasta, which can sometimes have a higher glycaemic impact.

**WE HAD MORE QUESTIONS:**

**CAN THE PREVIOUS RESULTS BE TRANSPOSED TO OTHER PRODUCTS?**

**WHAT IS THE CONTRIBUTION OF THE ORAL, GASTRIC AND INTESTINAL PHASES TO THE DIGESTION OF STARCH FROM FOODS THAT ELICIT DIFFERENT GLYCAEMIC RESPONSES?**

## ABSTRACT

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Starch is a major determinant of the glycaemic responses elicited by our diets, but the exact contribution of the two main amylolytic enzymes (salivary and pancreatic  $\alpha$ -amylases) remains a matter of debate. Our aim was to investigate the contribution of the oral, gastric and intestinal phases to the hydrolysis of starch in bread and pasta during dynamic *in vitro* digestions using DiDGI®. Before its inactivation by the low gastric pH, salivary  $\alpha$ -amylase released about 80 % of the starch in bread and 30 % of that in pasta, hydrolysing over half of it into oligosaccharides. Accordingly, the contribution of pancreatic  $\alpha$ -amylase during the intestinal phase was lower for bread than pasta. Our results are well correlated with *in vivo* data, and demonstrate the importance of salivary  $\alpha$ -amylase during oro-gastric processing of starchy foods. This finding is discussed in relation with observations regarding salivary  $\alpha$ -amylase from other fields of knowledge.

## 1. INTRODUCTION

Starch is the most important, abundant and digestible polysaccharide in human nutrition. Being exclusively made up of glucose residues, and accounting for 20 % to 50 % of the total energy intake (Stylianopoulos, 2012), it plays a major role in the post-prandial glycemc responses elicited by our diets. The positive relationship between the rate at which foods are digested and the degree to which they raise blood glucose has been repeatedly acknowledged (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; Jenkins, et al., 1982). It is therefore clear that attention should not only be given to the starch content in our diets, but also to its digestive process, which remains to be fully understood.

There are two key enzymes that participate in the digestion of starch: human salivary  $\alpha$ -amylase (HSA) and pancreatic  $\alpha$ -amylase. HSA initiates the amylolytic process in the oral cavity (Bornhorst & Singh, 2012) and can remain active in the stomach (Bornhorst & Singh, 2012; S. S. Gropper & J. L. Smith, 2013) until it is inactivated due to the increasing acidity, between pH 3.0 and 3.8 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. H. Meyer, 1987). Pancreatic  $\alpha$ -amylase and brush border enzymes then complete starch digestion in the small intestine, where glucose is finally absorbed (S. S. Gropper & J. L. Smith, 2013). The role of HSA during oral digestion of starch is somewhat well established, but little attention is given to its impact during the gastric phase of digestion. Extensive work has been conducted *in vitro* with the aim of better understanding the digestive process of starch (Berti, Riso, Monti, & Porrini, 2004; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; Jenkins, et al., 1982; Woolnough, Bird, Monro, & Brennan, 2010), but the oral phase is either ignored (Berti, Riso, Monti, & Porrini, 2004; Goñi, Garcia-Alonso, & Saura-Calixto, 1997) or followed by a gastric phase at an acidic pH, usually between 1.5 and 3 (Yvonne Granfeldt, Hagander, & Björck, 1995; Jenkins, et al., 1982; Minekus, et al., 2014a; Woolnough, Bird, Monro, & Brennan, 2010). Consequently, even when taken into account in these protocols, HSA is immediately inactivated at the beginning of the gastric phase, and therefore most starch is hydrolysed during the intestinal phase. Moreover, because such studies are generally in very good agreement with *in vivo* data (Bohn, et al., 2017) an analogy between intestinal hydrolysis kinetics and glycemc response is repeatedly established, thereby reinforcing the idea that the great majority of starch is digested by pancreatic amylase (des Gachons & Breslin, 2016).

However, simulating gastric digestion at a stable acidic pH is not representative of the *in vivo* dynamics, as it has been observed that it takes 75-107 min to reach  $\text{pH} \leq 2$  after a meal (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Malagelada, Go, & Summerskill, 1979). This is in line with the reports that the action of HSA can continue long after the oral

phase, and even reach the intestinal lumen in its active form (M. Fried, S. Abramson, & J. H. Meyer, 1987; Skude & Ihse, 1976). These observations suggest that HSA may play a more significant role in starch digestion than often considered. The scientific literature on this topic is scarce, but evidences that HSA can hydrolyse as much as 76 % of mashed potatoes' starch and 59 % of that in bread into maltose, within the stomach, date back to the beginning of the 20<sup>th</sup> century (Bergeim, 1926). More recently, we obtained similar results with an *in vitro* protocol that closely mimicked *in vivo* gastric pH reduction kinetics after a snack- or lunch-type meal and showed that HSA could hydrolyse up to 80 % of bread starch within the first 30 min of gastric digestion (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). In the present study, we intended to confirm that the amylolytic activity of saliva plays a preponderant role in the digestion of starch in bread and to widen our investigations to the intestinal phase of digestion, to the kinetics of starch hydrolysis into oligosaccharides, and to other highly consumed starchy foods: wheat and gluten free (GF) spaghetti. Our hypothesis is that HSA can significantly contribute to the digestion of starch before it is inactivated during the gastric phase, and therefore plays a more important role than generally considered.

## 2. MATERIALS AND METHODS

### 2.1. MATERIALS

The three test foods were purchased from a supermarket: freshly baked white wheat French baguette (common baguette, Auchan, Plaisir, France), no. 5 wheat spaghetti (Barilla Alimentari, Parma, Italy), and no. 5 gluten-free (GF) spaghetti (Barilla Alimentari, Parma, Italy) in which the wheat flour was replaced by a mixture of 95 % maize and 5 % rice flours. Whole saliva pool from 10 human donors (Normal saliva in 5 mL aliquots, ref. 991-05-P-5) was acquired from Lee Biosolutions (Maryland Heights, United States) and conserved at -80 °C. Pepsin from gastric mucosa (P-6887), pancreatin (P-1750) and pancreatic  $\alpha$ -amylase (A-3176), all of porcine origin, were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Amyloglucosidase from *Aspergillus Niger* (ref. 10 102 857 001) was obtained from Roche (Rotkreuz, Switzerland) and was always used undiluted. The amyloglucosidase activity provided by the manufacturer (14 U/mg of protein) was used as reference, whereas the activities of saliva ( $345 \pm 37$  U/mL), pepsin ( $3319 \pm 70$  U/mg) and pancreatin (Trypsin activity of  $3.42 \pm 0.04$  U/mg) were all measured in our lab according to previously described methods (Minekus, et al., 2014b). D-Glucose kits (ref. 103.21) from BioSenTec (Auzeville Tolosane, France) were also used.

### 2.2. FOOD PREPARATION AND CHARACTERIZATION

#### 2.2.1. FOOD PREPARATION

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Only the crumb of the bread, separated from the crust using a 3-cm diameter cookie cutter cylinder, was used for the experiments. Both types of pasta were prepared in a Thermomix food processor (Thermomix TM 31, Vorwerk, Nantes, France) following the same procedure: pasta was cooked in pre-heated salted (7 g/L of NaCl) mineral water (Evian, Évian-les-bains, France) for 11 min at 90 °C under the lowest speed (spoon function). Immediately after, the cooking water was drained and the pasta rinsed 3 times with mineral water. The ratio of pasta to water was kept at 17/100 (w/w) both during cooking and rinsing.

#### 2.2.2. WATER CONTENT

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The water content of food samples ( $5 \text{ g} \pm 0.5 \text{ g}$ ) was determined in triplicate by measuring the weight loss after drying at 110 °C for 24 hours in an oven (UF160, Memmert, Büchenbach, Germany).

#### 2.2.3. STARCH

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Total and resistant starch contents were determined in triplicate and used to estimate digestible starch.

**TOTAL STARCH** was quantified using a modified version of a previously described method (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Food samples ( $50 \pm 5$  mg of bread crumb or cooked

pasta) were dispersed in 10 mL of a 2 M KOH solution at 4°C for 16 h to allow for complete solubilisation. 5.7 mL of 2 M HCl and 3 mL of 0.4 M sodium acetate buffer were then added and the pH was adjusted to  $4.75 \pm 0.1$ . Their solubilised starch was subsequently hydrolysed into glucose by adding 60  $\mu$ L of amyloglucosidase and incubating for 45 min in a shaking water-bath (SW22, Julabo, Seelbach, Germany) (60 °C, 150 rpm). After centrifugation (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) (4 °C, 3000 g, 10 min), glucose concentration in the supernatant was determined using a D-Glucose kit followed by absorbance measurements (340 nm) in 1 cm plastic cuvettes using a UV-Vis spectrometer (Evolution 201, Thermo Fisher Scientific, Massachusetts, United States). The total starch content was calculated as mg of glucose  $\times$  0.9.

**RESISTANT STARCH** was determined using a modified version of Goñi's method (Goñi, Garcia-Diz, Mañas, & Saura-Calixto, 1996). Bread crumb ( $250 \pm 5$  mg) and cooked pasta ( $350 \pm 5$  mg) samples were incubated with 10 mL of HCl-KCl buffer (pH 1.5) and 0.4 mL of a  $1.25 \times 10^5$  U/mL pepsin solution in a shaking water bath (40 °C, 150 rpm, 60 min). Then, 9.5 mL of tris-maleate buffer (pH 6.9) and 1 mL of a 0.52 U/mL pancreatic  $\alpha$ -amylase solution were added and samples were incubated for 16 h at 37 °C under agitation (150 rpm). After centrifugation (4 °C, 3000 g, 15 min) and supernatant removal, the pellet was washed with 10 mL of distilled water and re-suspended in 6 mL of 2M KOH where they were left for 30 min under constant shaking (150 rpm) at 20 °C. 5.5 mL of 2M HCl and 3 mL of 0.4 M sodium buffer acetate (pH 4.75) were added. These samples were then incubated with amyloglucosidase, centrifuged and the starch content of the pellet suspension (i.e. resistant starch) was quantified as glucose using the same conditions as previously provided for the total starch determination.

**DIGESTIBLE STARCH** was calculated as the difference between total and resistant starch.

#### 2.2.4. PROTEIN

The protein content of the spaghetti was determined by the Kjeldahl method in an independent laboratory (ABIOPOLE - Groupe Qualtech, Vandœuvre, France) with a conversion factor of 5.7 between nitrogen and protein contents. The protein content of bread was estimated from the French Table of Food Composition corrected for the nitrogen conversion factor (5.7 instead of 6.25) and adjustment for the measured starch content (Hawkins & Johnson, 2005).

#### 2.3. DYNAMIC *IN VITRO* DIGESTION

Dynamic *in vitro* digestions were conducted in triplicate for each food. Each digestion comprised an oral phase followed by gastrointestinal digestion in DiDGI® (INRA, Grignon, France), a computer controlled dynamic system (Ménard, et al., 2014). The STORM® (INRA, Grignon, France) software was used to continuously monitor digestive conditions and control the fluxes of secretions according to pre-set parameters. The composition of the simulated secretions used

during the gastric and intestinal phases of digestion are described below and were defined in order to remain close to the recommendations provided in the standardized static *in vitro* digestion method (Minekus, et al., 2014b). Whenever possible, data from human studies were used as reference to define the experimental conditions.

#### 2.3.1. ORAL PHASE

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For practical reasons, food comminution and saliva incorporation were dissociated.

**COMMINUTION:** Different procedures were followed for bread and pasta. Bread crumb was fragmented using a domestic kitchen food chopper (Multi-moulinette 400 W, Moulinex, Ecully, France) using 4 segments of 5 s separated by intervals of 5 s. We have previously shown that this enables to accurately replicate the starch digestion kinetics obtained using bread boli formed *in vivo* (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). This procedure was unsuitable for pasta. As most masticated spaghetti pieces have been reported to be between 5 to 30 mm long (Hoebler, Devaux, Karinthe, Belleville, & Barry, 2000), pasta was comminuted by manually chopping spaghetti strands into 10 mm long pieces with a knife. Individual portions of comminuted bread ( $6.4 \pm 0.5$  g) and wheat ( $11.0 \pm 0.5$  g) and GF ( $10.8 \pm 0.5$  g) pasta, equivalent to 3 g of total starch, were kept in an air-tight container at 20 °C until saliva incorporation (maximum of 1 hour).

**SALIVA INCORPORATION:** Saliva was heated to 37 °C in a water-bath for 10 min immediately before each digestion experiment. One portion of comminuted food was mixed with saliva for 30 s using a spatula at a saliva/food mass ratio of 0.2 and 0.05 for bread and pasta, respectively. Saliva incorporation levels and mixing times were based on the results of Human studies with the same types of foods (Hoebler, Devaux, Karinthe, Belleville, & Barry, 2000; Jourden, et al., 2016).

#### 2.3.2. GASTRIC PHASE

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The gastric compartment of DiDGI® was prepared with an operating temperature of 37 °C and a stirring speed of 10 rpm. The fasted gastric content consisted of 20 mL of a Simulated Gastric Fluid electrolyte solution (eSGF), 10 µL of 0.3 M CaCl<sub>2</sub>, and a predetermined volume of water. The composition of all electrolyte solutions was based on the literature (Minekus, et al., 2014b), the only difference being that eSGF was prepared at a neutral pH so that the kinetics of gastric pH reduction *in vivo* could be mimicked. To standardize digestive conditions of all foods and ensure adequate immersion of the pH probe, the water volume was adjusted according to the bolus volume so that the initial volume of the gastric contents was always 40 mL after the bolus addition. Each bolus was introduced into the gastric compartment immediately after preparation where it was mixed with the gastric contents for 30 s. At this point, stirring was

interrupted for another 30 s to allow for particle sedimentation before collecting the first sample (2 mL) from the supernatant. Stirring was then resumed and the gastric phase was initiated by activating the influx of HCl (0.1 M in eSGF) and pepsin (4700 U/mL in eSGF). Practical constraints, imposed by the sample collection and gastric phase preparation procedures, resulted in a 2 min lag between the end of the oral phase and the start of gastric digestion. This was the shortest time possible to complete all the experimental steps. The influx of HCl was automatically controlled by STORM<sup>®</sup> to reduce the pH from 6 (initial pH of chime  $\sim$  5.9) to 2 in 90 min and keep it at 2 for the following 30 min. This was intended to replicate the pH evolution observed during the digestion of main meals (comparable to a lunch or dinner) (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Malagelada, Go, & Summerskill, 1979). The flow rate of the pepsin solution (4705 U/mL), also controlled by STORM<sup>®</sup>, was constant and pre-set to achieve a concentration of  $\approx$  2000 U/mL within 90 min. The total volume of the HCl and pepsin solutions added during the gastric phase was  $22 \pm 2$  mL. This was defined after preliminary tests in order to compensate the volume withdrawn by sampling. A total of 11 more samples were indeed collected from the supernatant at 3, 6, 9, 15, 20, 30, 45, 60, 75, 90 and 120 min using the above-described procedure (interruption of stirring for 30 s immediately before collecting a 2 mL sample). Collected samples were immediately placed in a dry bath (ThermoMixer C, Eppendorf, Hamburg, Germany) (99 °C, 150 rpm, 5 min) for enzyme inactivation, cooled down on ice, and conserved at -20°C until further analysis.

### 2.3.3. INTESTINAL PHASE

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Intestinal digestion was conducted in the same DiDGI<sup>®</sup> compartment. 22 mL of a Simulated Intestinal Fluid electrolyte solution (eSIF), 10 mL of water and 80  $\mu$ L of 0.3 M CaCl<sub>2</sub> were added to the chime remaining after the gastric phase ( $38 \pm 2$  mL). The pH of the mixture was adjusted to 7.0 with 1M NaOH and the intestinal phase was initiated by adding 10 mL of a pancreatin solution in eSIF (tryptic activity of 800 U/mL) that ensured a trypsin activity of  $103 \text{ U/mL} \pm 3 \text{ U/mL}$  and a total volume close to 80 mL at the start of the intestinal phase. STORM<sup>®</sup> was programmed to automatically maintain the pH at 7.0 by adding 0.02 M NaOH (also prepared in eSIF). Supernatant samples were collected at 4, 9, 15, 20, 30, 60, 90 and 120 min employing the same procedure described in section 2.3.2.

## 2.4. DIGESTION MONITORING AND CHEMICAL ANALYSES

All digestion experiments were monitored qualitatively (visual analysis of the devolution of food macrostructure) and quantitatively (dry mass and proportions of starch released and hydrolysed into oligosaccharides). Before analysis, samples were thawed at 4 °C and centrifuged (10 min, 3000 g and 4 °C) to separate the suspended fine particulate fragments released during food

disintegration from the liquid medium containing starch hydrolysis products, including oligosaccharides. Both the fine particle pellets and supernatants were recovered and kept at -20 °C until further analysis. All the analyses of the centrifuged fractions were performed after thawing (4 °C).

#### 2.4.1. DEVOLUTION OF FOOD MACROSTRUCTURE AND FINE PARTICLE PELLETS DRY MASSES

The devolution of food macrostructure was followed through camera pictures taken at the end of the oral phase, and after 60 min and 120 min of both the gastric and intestinal phases of digestion (i.e. t = 0, 60, 120, 180 and 240 min of gastro-intestinal digestion). Additionally, the dry masses of the fine particle pellets (18h at 110 °C in an oven) were used as a quantitative indicator of food degradation throughout digestion and to correct for losses of undigested/unreleased starch during sampling assuming that the fraction of starch in the fine particle pellet was inversely proportional to the fraction of released starch (section 2.4.2). Undigested starch in the fine particle pellets was estimated as follows:

$$\text{Undigested starch lost (g)} = \frac{\text{TS dry food}}{100} \times \text{dry pellet} \times \frac{S_{\text{pellet}}}{100}$$

where TS dry food is the proportion (%) of total starch in the dry food sample, dry pellet is the dry mass of fine particle pellet (g) obtained after centrifugation of the digestion sample, and S pellet is the proportion of starch in the fine particle pellet (%), estimated as 100 – % of total starch released (determined after analysis of the corresponding supernatant fraction as described in section 2.4.2.).

#### 2.4.2. STARCH RELEASE FROM THE FOOD MATRIX

Released starch refers to all solubilised starch fractions and products of starch hydrolysis. This starch fraction was measured as glucose after incubation with amyloglucosidase and centrifugation, as previously described (section 2.2.3). Released starch was expressed as the percentage of total starch released over time, after correction for starch losses during sampling (section 2.4.1).

#### 2.4.3. OLIGOSACCHARIDES RELEASE

The proportion of starch hydrolysed into oligosaccharides, with degrees of polymerization of up to 10 (Hoebler, et al., 1998), was estimated as follows. 50 µL of supernatant were added to 400 µL of 80 % (v/v) ethanol and an overnight incubation followed (16 h, 4 °C) (Ballance, et al., 2013; Hoebler, et al., 1998; Mishra, Hardacre, & Monro, 2012). After centrifugation (10 min, 1000 g, 4 °C), the oligosaccharide fraction, recovered in the supernatants was quantified as glucose after incubation with amyloglucosidase and centrifugation, as previously described (section 2.2.3). The oligosaccharide content of the samples was calculated as mg of glucose × 0.9. The

oligosaccharide release was expressed as the percentage of total starch over time, after correction for starch losses during sampling (section 2.4.1).

## 2.5. *IN VITRO* PREDICTION OF THE GLYCEMIC INDEX

The glycemic index (GI) of each food was estimated using the *in vitro* procedure described by (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). Briefly, 50 mg of food samples were incubated (1 h, pH 1.5, 37 °C) with pepsin (5531 U/mL in the final mixture) to simulate gastric proteolysis, their pH was then adjusted to 6.9 and they were incubated at 37 °C for 180 min with pancreatic  $\alpha$ -amylase (0.43 IU/mL in the final mixture) to simulate intestinal amylolysis. During this second incubation, 1 mL samples were collected from the supernatant every 30 min and immediately transferred to a boiling water bath to inactivate  $\alpha$ -amylase (100 °C, 5 min). All samples were stored at 4 °C for 24 h before being analysed using the procedure described in section 2.2.3 for solutions of solubilised starch. Partially hydrolysed starch was calculated as mg of glucose  $\times$  0.9. The rate of starch digestion was expressed as the percentage of total starch released as a function of time and the predicted GI was calculated based on the % of starch released at 90 min ( $H_{90}$ ) using the equation  $GI = 39.21 + 0.803 (H_{90})$ . Each food was analysed in triplicate.

### 3. RESULTS

#### 3.1. FOOD CHARACTERIZATION

The water, starch and protein contents of each food is presented in Table 7. Globally, bread contained less water (45.7 %) than wheat and gluten-free pasta (65-69 %), but had a higher starch content (47.2 vs. 27-28 %). The proportions of resistant starch in bread and GF pasta were similar (between 3.4 % and 4.2 % of total starch) and about three times lower than in wheat pasta (13.4 %). The protein content of wheat spaghetti ( $\approx$  5 %, w/w) was about twice that of GF spaghetti.

Table 7- Composition of the studied foods. All values were experimentally obtained except for the protein content of bread which was estimated from the French food composition table (French Agency for Food Environmental and Occupational Health & Safety, 2017).

	Bread	Wheat spaghetti	Gluten-free spaghetti
<b>Water</b> (g/100 g of food)	45.7 $\pm$ 0.4	65.4 $\pm$ 0.1	68.8 $\pm$ 8.8
<b>Protein</b> (g/100 g of food)	7.4	5.0	2.4
<b>Total starch</b> (g/100 g of food)	47.2 $\pm$ 1.1	27.4 $\pm$ 1.3	27.9 $\pm$ 0.2
<b>Digestible starch</b> (g/100 g of total starch)	96.6 $\pm$ 0.6	86.6 $\pm$ 1.1	95.6 $\pm$ 0.7
<b>Resistant starch</b> (g/100 g of total starch)	3.4 $\pm$ 0.6	13.4 $\pm$ 1.1	4.4 $\pm$ 0.7

#### 3.2. DYNAMIC *IN VITRO* DIGESTION

Dynamic digestions of bread, wheat and GF spaghetti comprising oral (2.5 min), gastric and intestinal phases (120 min each) were carried out in triplicate. Both qualitative and quantitative methods were employed to monitor (1) the devolution of food macrostructure, (2) the starch release from the food matrix, and (3) its hydrolysis into oligosaccharides.

##### 3.2.1. DEVOLUTION OF FOOD MACROSTRUCTURE

The visual appearance of the chimes during the digestion experiments is presented in Figure 43. The digestion of bread was characterized by a somewhat homogeneous chime. Individual particles could hardly be distinguished in the beginning and were completely dissolved by the end of the gastric phase. In contrast, particles of both types of pasta were clearly identifiable until the end of gastric digestion even though their degradation evolved differently. Despite equivalent initial particle size and shape, wheat pasta tended to be degraded faster during gastric digestion. During the first 60 min, wheat pasta particles were broken down into

numerous, visibly smaller, pieces, with only a few remaining at 120 min. Particles of GF pasta seemed more resistant with many particles having retained their shape and size after 60 min, and some relatively large pieces were still visible at 120 min. During the intestinal phase, the opposite trend was observed. The remaining particles of GF pasta were rapidly disintegrated leading to a macroscopically homogenous solution, comparable to the bread chime, whereas the particles of wheat pasta appeared to be more resistant to intestinal digestion, with a number of them still visible at the end of the experiments.

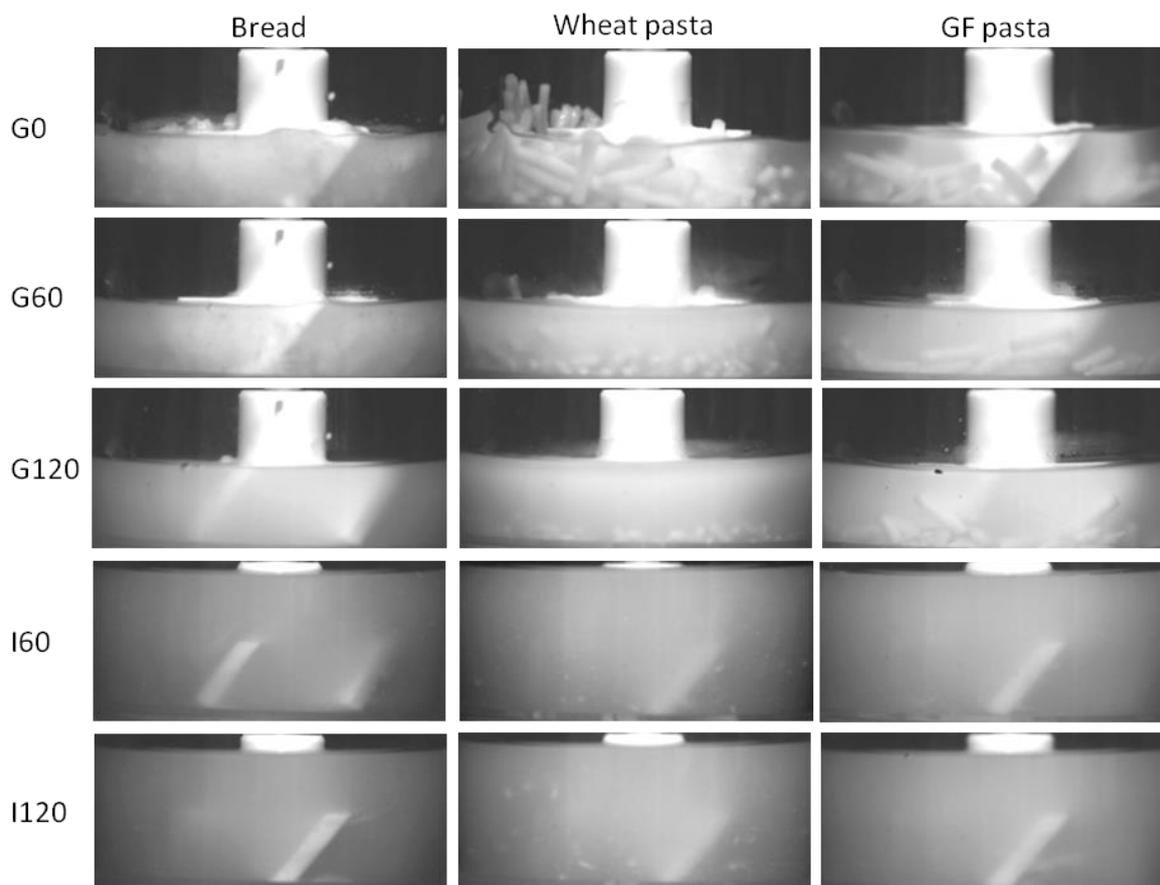


Figure 43- Devolution of food macrostructure. Pictures taken at the end of the oral phase/start of the gastric phase (G0), and 60 and 120 min of after the start of both the gastric (G) and intestinal (I) phases of digestion (t = 0, 60, 120, 180 and 240 min).

### 3.2.2. DRY MASS OF THE FINE PARTICULATE FRACTION

The dry masses of the fine particulate material recovered in the 3000 g pellets from the liquid phase of the digestion, presented as percentages of the total dry mass of the corresponding food at the start of digestion, are presented in Figure 44 (white circles) and can be used as a complementary indicator of the rate of food degradation. For bread, there was a steep rise during the very first minutes of digestion up to  $\approx 1.4\%$ , followed by a reduction between 30 and 60 min that stabilised at about  $0.7\%$  resulting in a plateau that lasted until the end of the gastric phase. The data for wheat and GF pasta showed a more steady increase during the first 60 min up to a plateau that lasted until the end of gastric digestion. The plateau reached with wheat

pasta was close to the first one observed for bread, at about 1.5 %, whereas it was lower than 1 % for GF pasta. The results obtained during the intestinal digestions were similar for both types of pasta and close to those obtained for bread, with stable plateau values in the range of 0.3 - 0.7 %.

The cumulative fine particle pellet mass is also represented in Figure 44 (dashed line) for two main purposes. First, it clearly illustrates the overall tendency that can be drawn from these measures: the rate of bolus disintegration into small pieces (small enough to be sampled out but large enough to be recovered in the fine particle pellet upon centrifugation) during gastric digestion seemed to increase in the following order: GF pasta < wheat pasta < bread, a finding that correlates well with the previously described visual observations (Figure 43). Second, it shows that a significant amount of food was sampled out during the course of the experiments (from 10 to 16 %) and represented loss of substrate that needed to be taken into account in the calculations of released starch and oligosaccharides.

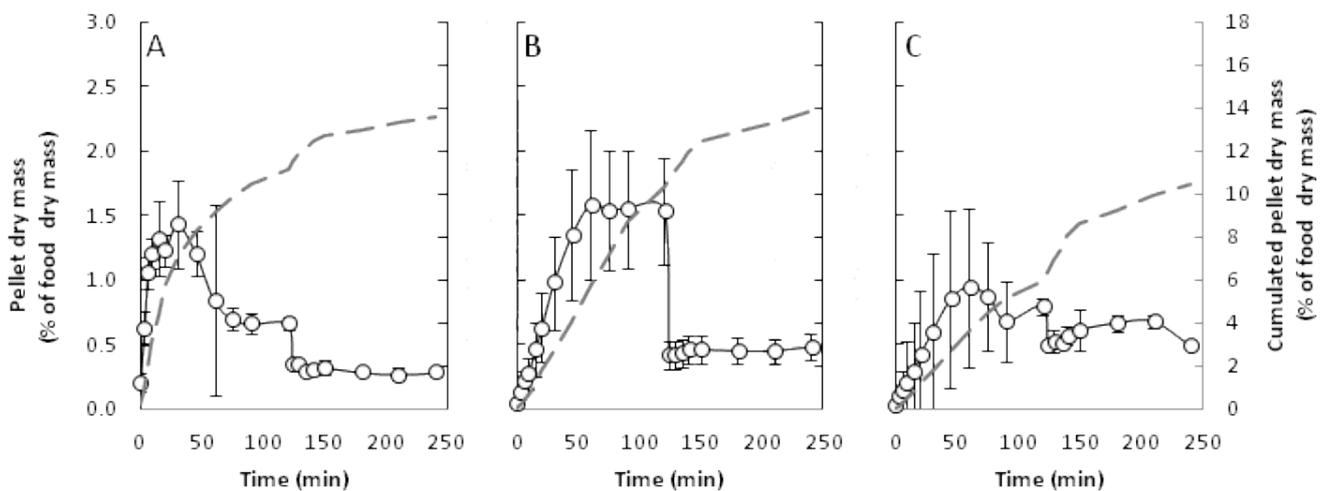


Figure 44 - Dry masses of fine particle pellets. Incremental (-○-, left hand y-axis) and cumulative (- - -, right hand y-axis) dry mass of the fine particle pellet of centrifuged samples, expressed as percentage of the initial dry mass of food, during the course of oro-gastro-intestinal digestions *in vitro*: (A) Bread, (B) Wheat spaghetti and (C) Gluten-free spaghetti. Average ± SD, 3 rpt.

## 3.2.3. STARCH RELEASE AND HYDROLYSIS INTO OLIGOSACCHARIDES

Figure 45 shows the kinetics of starch release and hydrolysis into oligosaccharides during digestion of bread (A), wheat pasta (B) and GF pasta (C). Released starch refers to the proportion of starch that was released from the food matrix into the chime and recovered in the sample supernatants regardless of its level of degradation. In contrast, released oligosaccharides reflect an extensive hydrolysis of starch, constituting a good indicator of the extent of the amyolytic process. The first point in each graph ( $t = 0$  min) corresponds, simultaneously, to the end of the oral phase (2.5 min) and the beginning of gastric digestion, and the point at 120 min marks the transition to the intestinal phase. Starch release and its hydrolysis into oligosaccharides were initiated during the oral phase. During the gastric phase both phenomena exhibited “rise-plateau” profiles, with a 30-45 min long initial increase followed by a plateau that lasted until the start of the intestinal phase ( $t = 120$  min). Immediately after the addition of pancreatin, the release of starch and oligosaccharides showed a second rise before new plateaus were reached. While these “rise-plateau” digestion profiles during gastric and intestinal processing were common to all foods, the contribution of each digestive phase differed: bread starch was mainly released and hydrolysed during the gastric phase (Figure 45A), whereas most of that in pasta was digested during the intestinal phase (Figure 45B and C).

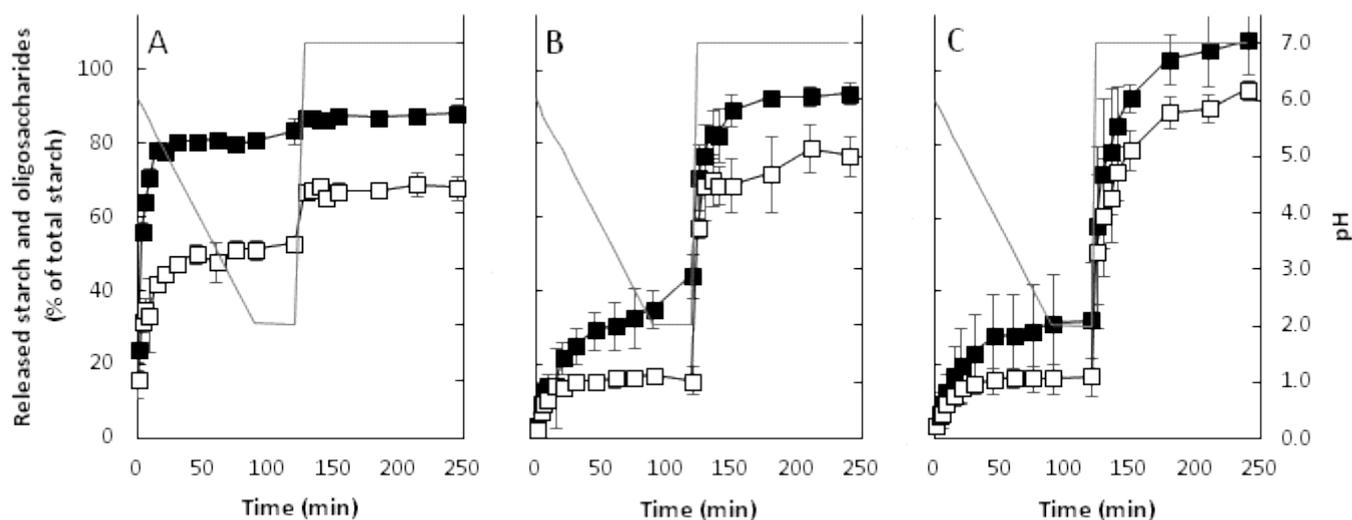


Figure 45- Starch digestion. Released starch (-■-), oligosaccharides (-□-), and pH of the chime (—) during the course of *in vitro* oro-gastro-intestinal digestions: (A) Bread, (B) Wheat spaghetti, (C) Gluten-free spaghetti. Average  $\pm$  SD, 3 rpt.

According to the starch release curves (dark squares), oral digestion caused the release of 24 % of bread starch, a value that increased to 80 % within only 20 min of gastric digestion. Oral and

gastric digestion of both pastas appeared slower and less extensive. Starch released during the oral phase amounted to around 3.5 % of the total, and the plateau, reached later than for bread, corresponded to only  $\approx 30$  % of the total starch. Consequently, intestinal digestion of bread starch was characterized by a very slight increase, of about 7 % (final plateau at 87 %), whereas intestinal digestion of both pastas showed a fast and intense release of the remaining starch (measured at 94 and 100 % of total starch in wheat and GF pasta, respectively, at the end of the experiment).

According to the results in Figure 45 (white squares), 16 % of bread starch and almost all of the starch released from both pastas, was hydrolysed into oligosaccharides during the oral phase. During gastric digestion, more than half of the starch released from all foods was hydrolysed into oligosaccharides within 30-45 min. During intestinal digestion, starch hydrolysis into oligosaccharides was resumed and progressed rapidly for all foods, leading to plateaus corresponding to about 67-95 % of the total starch.

### 3.3. *IN VITRO* PREDICTION OF THE GLYCEMIC INDEX (GI)

The starch release kinetics obtained using the *in vitro* protocol for GI prediction (Goñi, Garcia-Alonso, & Saura-Calixto, 1997) are presented in Figure 46 (white symbols). According to these results, bread exhibited the highest hydrolysis rate with a plateau corresponding to approximately 83 % of the total starch reached within 60 min. Despite the different compositions of wheat and gluten-free pastas, no differences were observed between their starch digestion rates. In both cases, almost linear curves were obtained and starch was released slowly. More than 75 % of the starch had not yet been released after 180 min. The predicted GIs estimated from these data were  $100 \pm 1.9$ ,  $47.6 \pm 4.0$  and  $47.4 \pm 1.6$ , for bread, wheat and GF pastas, respectively.

The starch release kinetics during the gastric phase of our digestions, obtained using the same sample analytical procedures, are also plotted in Figure 46 (grey symbols) for comparison purposes. Although the enzymes and experimental conditions are not the same, the results were highly comparable, being possible to observe, in both cases, a much faster starch release for bread, and a similar (much lower) rate for both types of spaghetti.

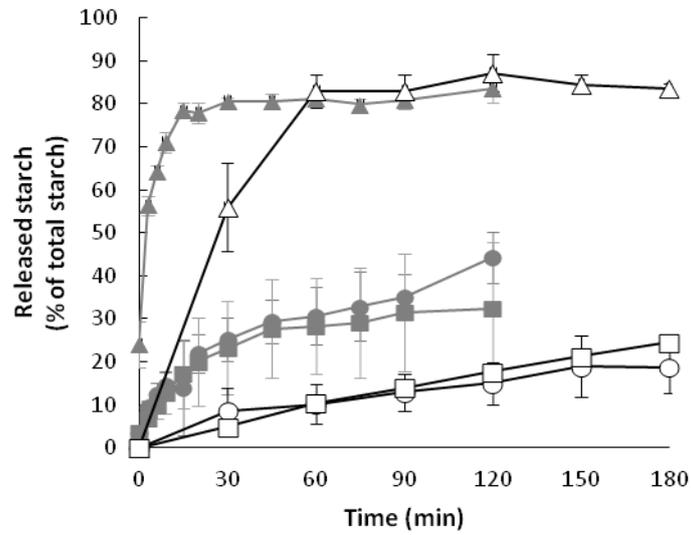


Figure 46 - Comparison of digestion protocols. Kinetics of starch obtained during our gastric digestions (grey symbols), and with the protocol of Gofii et al. (1997) commonly used to predict the GI of foods (white symbols). Starch release during digestion of white wheat bread (-▲- and -△-), wheat spaghetti (-■- and -□-) and gluten free spaghetti (-●- and -○-).

## 4. DISCUSSION

The role of Human salivary  $\alpha$ -amylase (HSA) in starch digestion is often overlooked in favour of that of pancreatic  $\alpha$ -amylase. Conducting *in vitro* digestions that mimic *in vivo* reality in terms of gastric pH and chronology of enzymatic action, we have recently shown that salivary amylase can, in fact, play a preponderant role in the gastric digestion of bread starch before being inactivated by acidity (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). In the present study, we intended to widen our investigations to complementary sample analyses and to other highly consumed starchy foods (spaghetti) throughout the course of semi-dynamic oro-gastro-intestinal *in vitro* digestions.

### 4.1. GENERAL CONSIDERATIONS

We may first highlight the good agreement between the different methods used to monitor the digestion experiments. For example, bread particles were the smallest after the oral phase (Figure 43), rapidly becoming small enough to be sampled with 1 mL graduated pipettes (inner diameter of 1.5 mm) during the gastric phase. This explains the faster increase of the fine particle pellet dry mass (Figure 44), and is also in agreement with the much higher starch release and hydrolysis rates observed for bread than for wheat and GF spaghetti during gastric digestion (Figure 45). The overall behaviour of both types of pasta was similar but a close inspection of the slight differences between them appears to show consistent trends among the different sets of results. Wheat pasta particle size was visibly reduced faster than that of GF pasta during gastric digestion, accordingly, the dry fine particle pellet mass for wheat pasta tended increase faster at this stage. The reverse tendency was then observed during the intestinal phase, only wheat pasta fragments were still visible at the end of the experiments what correlates well with the lower extents of starch release and hydrolysis observed for this food (Figure 45B).

### 4.2. GASTRIC AND INTESTINAL CONTRIBUTIONS TO STARCH DIGESTION

The shape of the starch and oligosaccharide release kinetics (Figure 45), with 2 successive increases for all foods, is explained by the chronology of enzymatic activity. The first rise corresponds to the action of HSA before it is completely inactivated by the increasing acidity of the chime. Indeed, we have previously shown that the amylolytic activity of saliva is completely lost below pH 3.5, but is still close to 50 % of its optimal activity at pH 4.0 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). According to the gastric pH reduction kinetics used here, based on *in vivo* data following a full meal (Malagelada, Go, & Summerskill, 1979), pH 4.0 was reached at 45 min, and pH 3.0 at 67 min. Therefore it seems that the released starch and oligosaccharides plateaued slightly before complete HSA inactivation, suggesting that the limiting factor could be the impaired accessibility of the remaining starch to HSA. Porcine pepsin, in turn, starts to be

active below pH 4.0 (Kondjoyan, Daudin, & Santé-Lhoutellier, 2015; Pletschke, Naudé, & Oelofsen, 1995), when HSA is about to be totally inactivated. The hydrolysis of the protein network that entraps the remaining starch can therefore be considered to take place during the second hour of gastric digestion only. During this period, the amount of released starch tended to increase slightly for pasta (Figure 45B and C) but our results indicate that pepsin action had limited consequences. The second rise, observed during the intestinal phase, can be attributed to the presence of pancreatic enzymes. Pancreatic  $\alpha$ -amylase further advances the amylolytic process and benefits from the preceding (pepsin) and concomitant (pancreatic proteases) hydrolysis of the protein network that improves the accessibility to starch. According to these considerations, the ratio between the two plateaus is therefore indicative of the gastric and intestinal contributions to the digestion of starch from each of the studied foods.

The fastest starch hydrolysis rate was observed for bread. We had recently shown that as much as 80 % of bread starch can be released during the first 20 min of gastric digestion (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). Our current results therefore confirm that HSA can be responsible for an extensive amylolysis before the chyme is emptied into the small intestine. Moreover, they also show that starch hydrolysis was more important during the gastric phase, where  $\approx$  60 % of starch was degraded into oligosaccharides, than during intestinal digestion (a further increase of  $\approx$  20 %). Between our current and previous investigations, conducted with different salivas, breads and pH reduction kinetics, we have covered a wide range of scenarios from what could be considered physiologically fast to slow gastric pH reduction kinetics (from pH 6 to 2 in 30 to 90 min) and low to high levels of saliva incorporation in the bolus (from 0.2 to 0.5 mL per gram of bread crumb). We may therefore confidently state that the contribution of HSA to the digestion of bread starch is highly relevant in a wide range of conditions.

Wheat pasta elicited a lower starch digestion rate than wheat bread, in agreement with previous *in vitro* (Berti, Riso, Monti, & Porrini, 2004) and *in vivo* (Packer, Dornhorst, & Frost, 2000) studies. The contribution of HSA during the gastric digestion of wheat pasta, although not negligible, was considerably lower, representing a little more than a third of that for wheat bread. A first, and obvious, cause lies in the distinct saliva contents of the boluses that, with the intention of replicating those found *in vivo* (Hoebler, Devaux, Karinthe, Belleville, & Barry, 2000; Jourden, et al., 2016), were 4 times higher for bread than pasta. But the different saliva incorporation levels and amylolysis rates of each food can be related to their structural properties. At a macroscopic level, the porous structure of bread facilitates water infiltration (Mathieu, et al., 2016), and thus impregnation of saliva and secretions carrying digestive enzymes. In contrast, pasta is a densely packed product through which water penetration is limited, even during boiling (Cunin,

Handschin, Walther, & Escher, 1995). At a molecular level, the starch in bread crumb is found in a rather uniform gelatinization state, forming a continuous network that contains swollen starch granules and starch that leached out from the granules (J. A. Gray & J. N. Bemiller, 2003). In turn, the level of starch gelatinization in cooked wheat spaghetti decreases progressively towards the centre of the spaghetti strand, and the density of the protein network is increasingly higher (Cunin, Handschin, Walther, & Escher, 1995; Fardet, et al., 1998). Because granule swelling and dispersion of starch polysaccharides during gelatinization render starch increasingly more accessible to  $\alpha$ -amylase action (Mishra, Hardacre, & Monro, 2012), starch in bread crumb is much more accessible than starch in pasta for which the vulnerability to hydrolysis gradually lowers towards the centre of spaghetti strands.

The rate of starch hydrolysis during gastric digestion of wheat spaghetti was similar to that of GF spaghetti, but it was slightly lower during the intestinal phase. In parallel, despite the same initial particle size and digestive conditions, particles of wheat pasta were degraded faster than those of GF pasta in the gastric phase, whereas the opposite was observed in the intestinal phase (Figure 43). The gluten network has been reported to slowdown enzymes' accessibility to starch (Fardet, et al., 1998). Therefore, one could have expected a constantly higher digestion rate from GF pasta, as previously observed *in vitro* (Jenkins, et al., 1987) and *in vivo* (Jenkins, et al., 1987; Johnston, Snyder, & Smith, 2017). The recommended cooking times are slightly different for wheat (9 min) and GF pasta (10 min). By cooking both foods at the same temperature for 11 minutes, it is possible that starch gelatinization in wheat pasta was slightly higher, further disrupting the surrounding gluten network. This might be the reason why we observed a faster degradation of wheat pasta during the gastric phase. However, wheat spaghetti had twice as much protein as GF spaghetti and, as mentioned above, it is also known that the density of the gluten network increases towards the interior of wheat spaghetti strands. As a consequence, wheat pasta could be expected to exhibit an increasing resistance to degradation, as we observed during the intestinal phase. There are, however, other relevant factors, such as product formulation and processing techniques, that can counterbalance the potential effects of wheat protein removal (Pellegrini & Agostoni, 2015) to generate GF formulations that do not affect the glycemic response adversely, as it was previously found *in vitro* (Berti, Riso, Monti, & Porrini, 2004) and *in vivo* (Packer, Dornhorst, & Frost, 2000).

#### 4.3. *IN VIVO* DATA SUPPORTING OUR FINDINGS

On the one hand, it is clear that the structural properties of food can strongly impact the rate of starch digestion and consequently the glycemic indexes. On the other hand, it is also evident that food structure can be extensively disrupted in the digestive stages that precede intestinal

digestion, hence diminishing potential differences derived from the structural properties of each food. This is indeed what our results show: important differences in the extent of oro-gastric starch digestion for bread and pasta that nearly vanished within 30 min of intestinal digestion only. As it was shown above, these results are consistent with both *in vitro* digestibility assays to predict the glycemic response and *in vivo* data. One may therefore question the assumption that pancreatic amylase always plays the predominant role in the digestion of starchy foods driving the attention to HSA, which is the most susceptible of being influenced by food structure.

To the best of our knowledge, the main arguments serving as a justification for the limited extent of HSA's contribution to starch digestion are the short duration of the oral phase and the erroneous idea that this enzyme is quickly inactivated once food boluses are swollen. Although HSA has been extensively studied, its usefulness to digestion is still questioned (des Gachons & Breslin, 2016; Fernández & Wiley, 2017) even though strong hints that its role is more relevant than what is generally considered can be recurrently found. Oral food processing is indeed brief but, as we have discussed above, HSA can continue hydrolysing starch in the stomach for long periods of time. For instance it has been observed that after the consumption of a liquid meal, the pH was still above 5.0 at half-gastric emptying time ( $48 \pm 28$  min.) and reached 3.0 only after more than 80 % of the gastric contents were emptied (Carrière, Renou, Ville, Grandval, & Laugier, 2001). This can explain Bergeim's findings, who found that more than half of the starch in mashed potatoes and bread was extensively digested in the stomach (Bergeim, 1926). Additionally, it has also been shown that swallowing high- (ex. potato or corn) but not low-starch (apple) foods without chewing resulted in the reduction of starch digestibility, and the associated glycemic response (Read, et al., 1986). This is also in line with a number of evolutionary concepts suggesting that increased HSA expression would have improved the efficiency of starch digestion, constituting a fitness advantage that drove a positive selection phenomenon in at least some high starch-populations (Perry, et al., 2007). Accordingly, a positive association between a high HSA activity and populations consuming a diet consisting predominantly on carbohydrates, has been repeatedly highlighted (des Gachons & Breslin, 2016; Perry, et al., 2007; Squires, 1953). Our observations, together with the work of all these research teams, undeniably strengthen HSA's role in the digestive process and also imply that this enzyme's contribution to gastric digestion is highly likely to play a part in the definition of the glycemic response elicited by starchy foods.

## CONCLUSION

The present study not only confirms the important role played by HSA during the gastric digestion of bread, but also demonstrates that, despite the more compact structure of pasta and a much lower level of saliva incorporation during bolus formation, the contribution of salivary amylase to the digestion of starch in pasta is far from being negligible. The starch digestion rates during gastric digestion were well correlated with a widely used *in vitro* protocol to estimate the GI of foods and with other *in vivo* studies. Given HSA's relevant role in starch digestion, it is highly likely that factors influencing its amylolytic performance, other than the structural properties of a food, may translate into an altered glycemic response. This could open new opportunities and strategies to attenuate the high glycemic response to some starchy foods by inhibiting HSA.

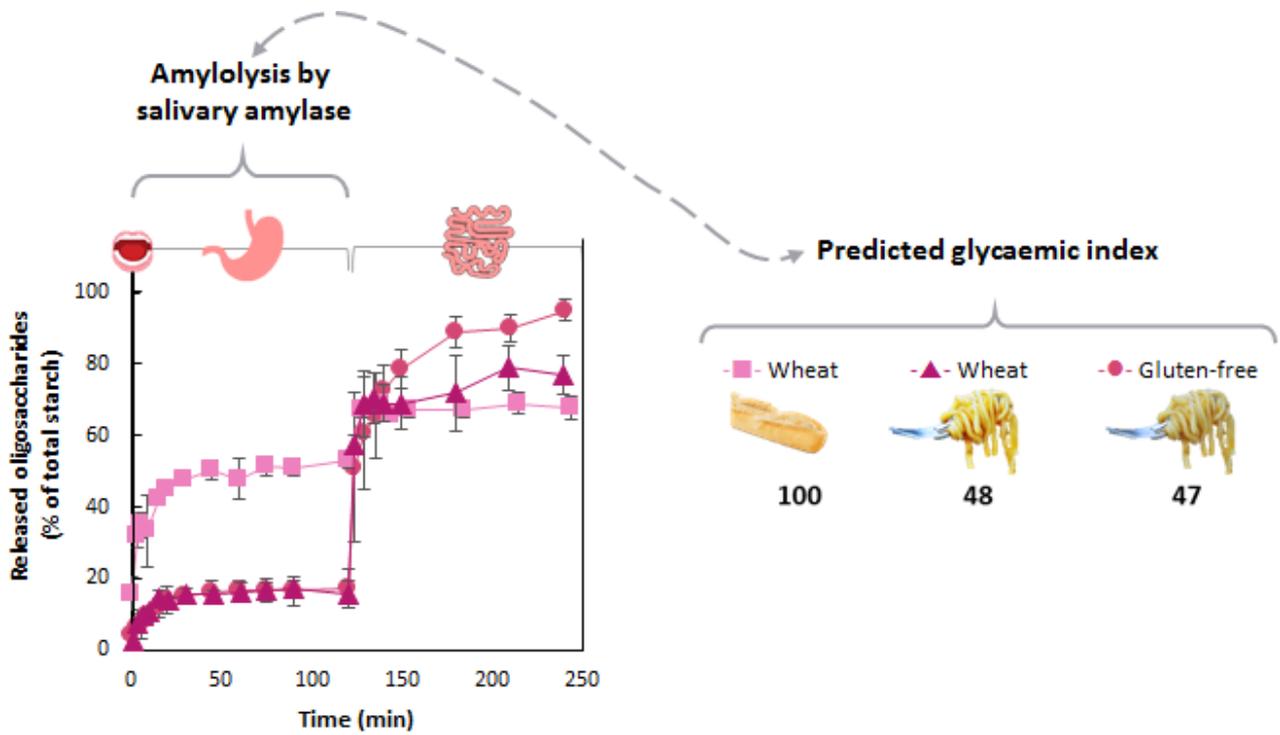
## ACKNOWLEDGMENTS

We would like to thank David Forest and Thomas Cattenoz for their technical help in the laboratory. We would like to acknowledge the IDEX-Paris Saclay consortium for partially supporting this research through a PhD grant and we are grateful to François Boué and Evelyne Lutton for their important help in obtaining this doctoral grant and helpful discussions.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

**KEY MESSAGES**



- We studied starch hydrolysis during dynamic *in vitro* digestions of bread and pasta
- The contribution of salivary  $\alpha$ -amylase (HSA) and pancreatic  $\alpha$ -amylase was assessed
- HSA released 30 % (pasta) to 80 % of starch (bread) during the gastric phase
- 50 % of the released starch was hydrolysed into oligosaccharides by HSA
- Amylolysis by HSA is likely to influence the glycaemic response to starchy foods



II. *IN VITRO* STUDIES

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# CHAPTER 3

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## ACID INDUCED REDUCTION OF THE GLYCAEMIC RESPONSE TO STARCH-RICH FOODS: THE SALIVARY AMYLASE INHIBITION HYPOTHESIS

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### Informal presentation

Our previous results demonstrated the importance of salivary amylase in the digestion of different starch-rich foods, and its level of contribution was well correlated with the predicted glycaemic response. For example, its role was more preponderant in the gastric digestion of bread than of pasta, and accordingly, the predicted glycaemic response was higher for bread than pasta.

This was intriguing...

**CAN WE SLOWDOWN THE DIGESTION OF STARCH BY  
PREMATURELY INHIBITING THIS ENZYME?**

**COULD THIS BE PART OF THE LINK BETWEEN MEAL ACIDITY  
AND THE ATTENUATION OF THE GLYCAEMIC RESPONSE TO  
STARCH-RICH FOODS?**

**LET US MAKE A FIRST TEST IN THE LAB!**

## ABSTRACT

---

Numerous studies have reported that the glycaemic response to starch-rich meals can be reduced by 20-50% with acidic drinks or foods. A number of candidate explanations have been put forward, but this phenomenon still remains vaguely understood. This study intends to demonstrate the remarkable effect of acid inhibition of salivary  $\alpha$ -amylase during oro-gastric hydrolysis of starch, shedding light on this often overlooked mechanism.

Oro-gastric digestions of bread, wheat and gluten-free pastas, combined with either water or lemon juice were performed using a dynamic *in vitro* system that reproduces gastric acidification kinetics observed in humans. In the presence of water, large proportions of starch (25-85%) and oligosaccharides (15-50%) were released from all foods within the first hour of gastric digestion (pH > 3.5). In the presence of lemon juice (pH < 3.5 at all time), starch release was about twice as low, and amylolysis into oligosaccharides was completely interrupted.

Acid-inhibition of salivary  $\alpha$ -amylase may explain, at least in part, the reduction of the blood glucose response through acidification of starch-rich foods/meals. This offers new perspectives for the development of strategies to improve the glycaemic response elicited by starch-rich diets.

## 1. INTRODUCTION

A growing body of evidence relating high glycaemic index diets with increased risk of type-2 diabetes, coronary heart disease and some types of cancer (Bhupathiraju, et al., 2014; Mirrahimi, et al., 2012; Mullie, Koechlin, Boniol, Autier, & Boyle, 2016) has fuelled research on the development of strategies to reduce the glycaemic response to certain foods. Starch-rich foods have been drawing special attention because they supply up to 50% of our energy intake (Stylianopoulos, 2012), thereby playing a major role in the post-prandial glycaemic responses elicited by our diets. Because of the positive correlation between the rate of starch hydrolysis for a certain food and the corresponding glycaemic response (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; D. J. A. Jenkins, et al., 1982), extensive work has been dedicated to identify ways of slowing down starch digestion.

An efficient strategy to reduce the glycaemic response to a starchy meal in normal (Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Leeman, Östman, & Björck, 2005; H. Liljeberg & Björck, 1998; Elin Östman, Granfeldt, Persson, & Björck, 2005; Sugiyama, Tang, Wakaki, & Koyama, 2003), insulin resistant (Carol S Johnston, Kim, & Buller, 2004), and diabetic subjects (Liatis, et al., 2010; Mitrou, et al., 2010) is to lower its pH by pairing it with an acidic food or drink (typically vinegar). The same holds true for processes that result in the acidification of the food itself, sourdough bread fermentation for example (Bo, et al., 2017; Lappi, et al., 2010; H. Liljeberg & Björck, 1996; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009). According to the above studies, vinegar supplementation and sourdough fermentation of bread can reduce the postprandial glycaemic response by 20-50% and 20-30%, respectively. The underlying mechanism has naturally intrigued researchers and various hypotheses have been put forward. In our view, starch hydrolysis by salivary  $\alpha$ -amylase (HSA) is the mechanism most likely to be altered by meal acidity, but in recent reviews, this hypothesis is hardly addressed (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016; Poutanen, Flander, & Katina, 2009), or is not even considered (Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014).

To understand the basis of this hypothesis it is important to remind that the process of starch digestion relies on the action of two key enzymes: HSA and pancreatic  $\alpha$ -amylase. HSA initiates amylolysis in the mouth and can remain active in the stomach until it is inactivated by the increasing acidity (S. S. Gropper & J. L. Smith, 2013), between pH 3 and 4 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. H. Meyer, 1987). For purified HSA, inactivation appears to be gradual in the presence of starch, as other researchers have observed

that it can have a protective effect, preserving up to 56% of its activity after 1 h at pH 3 (Rosenblum, Irwin, & Alpers, 1988). Pancreatic  $\alpha$ -amylase and brush border enzymes then complete amylolysis in the small intestine, where glucose is finally absorbed (S. S. Gropper & J. L. Smith, 2013). Because of the short duration of the oral phase and the general assumption that post-prandial gastric acidification is fast, among others, it is generally believed that HSA plays a minor role and that most starch is digested by pancreatic  $\alpha$ -amylase (des Gachons & Breslin, 2016). If this was the case, however, there would be a very limited margin to influence enzymatic performance through increased meal/food acidity: any effect on HSA would be of minor relevance, meanwhile it is unlikely that pancreatic amylase activity would be affected as the pH of the chyme is rapidly neutralised in the duodenum. The physiological reality is that after consumption of a meal, gastric pH rises to 4.5 – 6.7 and it is reduced steadily, taking 75-107 min to reach  $\text{pH} \leq 2$  (Dressman, et al., 1990; Gardner, Ciociola, & Robinson, 2002; Malagelada, Go, & Summerskill, 1979). This implies that there is a large time window during which HSA can hydrolyse starch. By the end of the gastric phase of digestion, hydrolysis of 59% (Bergeim, 1926) to 80% (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b) of bread starch has indeed been observed *in vivo* and *in vitro*, respectively. According to these considerations, it is straightforward that any means of prematurely acidifying gastric contents will influence the performance of HSA and can constitute an opportunity to slow down amylolysis and ultimately improve the glycaemic responses elicited by starchy foods. The aim of the present study was to demonstrate this effect through dynamic *in vitro* digestions of white bread, wheat and gluten-free pastas, combined with either water or lemon juice.

## 2. MATERIALS AND METHODS

All experimental methods and analytical procedures have been described and justified in detail in one of our previous studies. Therefore, only the main experimental steps, their principles, and relevant adaptations will be described here. Interested readers are invited to refer to (Freitas & Le Feunteun, 2018b) for further details.

### 2.1. MATERIALS

Three starchy foods were studied: French wheat baguette supplied by Lesaffre (France), wheat pearl pasta (Cerealis, Portugal) and n°5 gluten-free (GF) spaghetti (Barilla Alimentari, Italy). Mineral water (Evian, France) and lemon juice (Polenghi Group, Italy) were bought at a local supermarket.

Frozen human saliva pooled from 10 donors (Normal saliva in 5mL aliquots, ref. 991-05-P-5) was purchased from Lee Biosolutions (United States), and porcine pepsin (P-6887) from Sigma-Aldrich (France). Amyloglucosidase from *Aspergillus Niger* (ref 10 102 857 001, Roche, Switzerland) and D-Glucose kits (ref. 103.21, BioSenTec, France) were used in the analysis of digestion samples.

### 2.2. PREPARATION

The same bread was previously studied by our team and only the crumb, separated from the crust as described in (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b), was used. Wheat pearl pasta was cooked for 25 min and GF spaghetti for 11 min.

### 2.3. ORO-GASTRIC *IN VITRO* DIGESTION

Saliva was thawed at 4°C, and warmed to 37°C in a water bath for 10 min before use. Oral processing was manually performed to produce boli that were submitted to gastric digestions in DiDGI®, a computer-controlled dynamic *in vitro* system (Ménard, et al., 2014). The composition, volumes and enzyme concentrations of gastric solutions were based on the standardized method for static *in vitro* digestions (Minekus, et al., 2014a) with the exception that simulated gastric fluid electrolyte solution (eSGF) was prepared at a neutral pH. Neutralized eSGF was used so that the pattern of gastric acidification in humans could be mimicked (Malagelada, Go, & Summerskill, 1979). Two different digestions were carried out in triplicate for each food with either (1) water or (2) lemon juice (pH = 2.3).

**ORAL PHASE.** Bread was processed in a food chopper (Multi-moulinette 400 CW, Moulinex, France), GF spaghetti strands were chopped into 1 cm long pieces and wheat pasta beads (3-5 mm diameter) were left intact. Each bolus was formed by mixing for 30 s bread or pasta (the equivalent to 3 g of starch) with 20% or 5% of saliva (mass of saliva / mass of food), respectively.

The amylolytic activity of the pooled saliva sample, as determined by Bernfeld's method (Peter Bernfeld, 1955), was  $345 \pm 37$  U/mL.

**GASTRIC PHASE.** DiDGI's gastric compartment was set-up with an operating temperature of 37 °C, a stirring speed of 10 rpm, 20 mL of eSGF, 10 µL of 0.3 M CaCl<sub>2</sub> and 7 mL of either mineral water or lemon juice. A pre-determined volume of deionised water was then added to reach a final volume of 40 mL after introducing the bolus.

Immediately after being prepared, each bolus was introduced in the gastric compartment where stirring continued for 30 s before it was interrupted for another 30 s and a first sample (2mL) was collected from the supernatant. Stirring was then resumed and the gastric phase was initiated 2 min after the introduction of the bolus by activating the fluxes of HCl (0.1 M in eSGF) and pepsin (4705 U/mL in eSGF). The flux of HCl was automatically adjusted to reduce the initial pH of the chime to 2.0 by decreasing it 1 unit every 22.5 min, and keep it constant thereafter. The initial pH of the chimes was  $6.0 \pm 0.5$  with water and  $2.5 \pm 0.5$  with lemon juice. The pepsin flux was set to reach  $\approx 2000$  U per mL of chime at 90 min of gastric digestion in both cases.

Additional samples, 2 mL each, were collected from the chime's supernatant at 3, 6, 9, 15, 20, 30, 45, 60, 75, 90 and 120 min, after a 30 s interruption of stirring to allow particles to sediment. Immediately after being collected, samples were transferred to a dry bath (99 °C, 150 rpm, 5 min) for enzyme inactivation and kept at -20 °C until required for further analysis.

#### 2.3.1. ANALYSIS OF DYNAMIC DIGESTION SAMPLES

After centrifugation (10 min, 3000 g and 4 °C), sample supernatants were analysed for released starch and oligosaccharides. Released starch was quantified as glucose equivalent (D-Glucose kits) after complete hydrolysis with amyloglucosidase. Oligosaccharides with degrees of polymerization of up to 10 (Hoebler, et al., 1998) were quantified the same way after selective precipitation of polysaccharides in an 80% (v/v) ethanol solution.

#### 2.4. STATISTICAL ANALYSIS

The proportions (% of total starch) of starch released and hydrolysed into oligosaccharides were plotted against time. The incremental area under the curves (AUC) was calculated using the trapezoidal method. Single factor ANOVAs were performed to evaluate if replacing water with lemon juice produced statistically significant differences on AUC and at each time-point of the curves. Statistically significant effects were accepted at the 95% confidence interval. All data are presented as means  $\pm$  SDs.

### 3. RESULTS

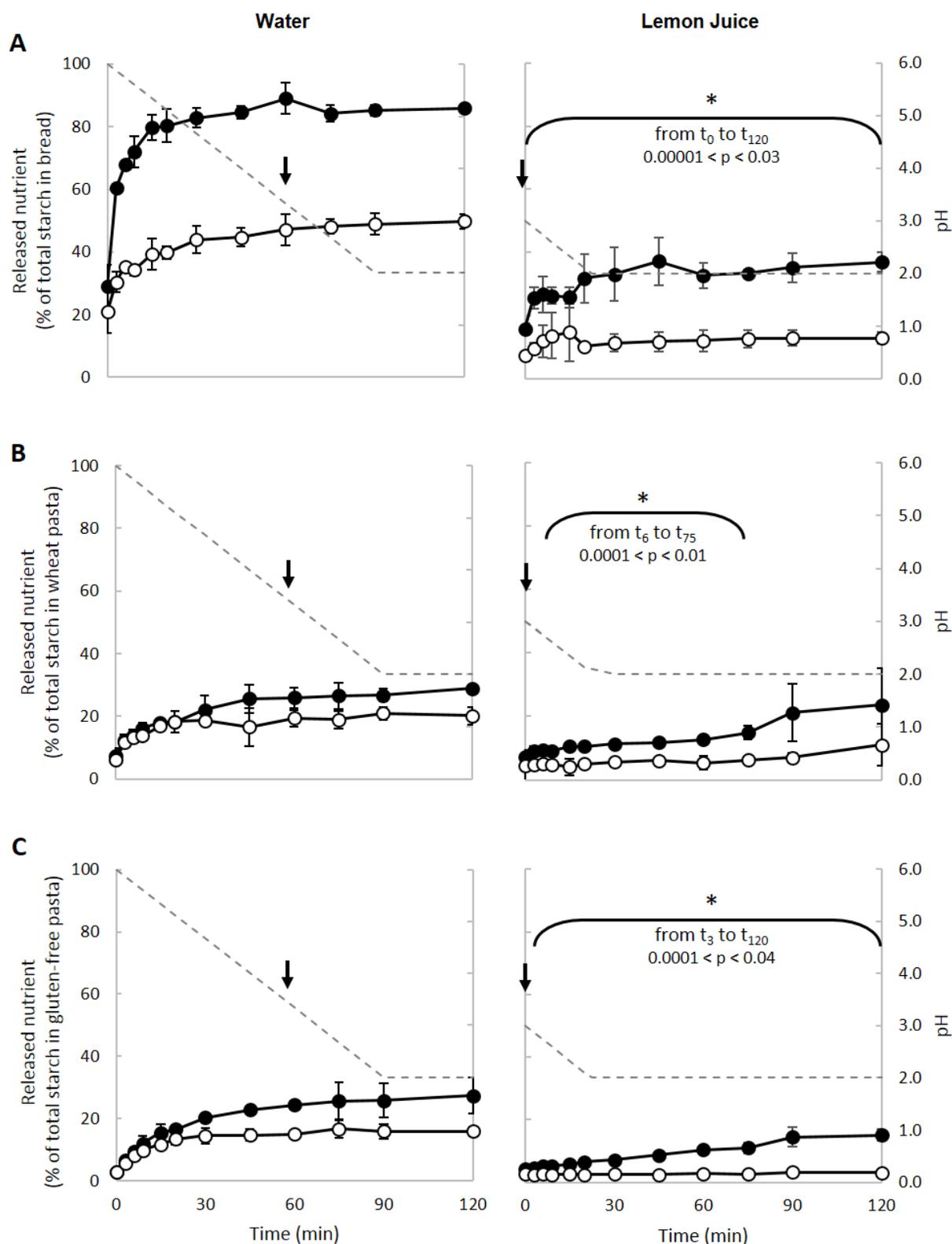
Figure 47 shows the kinetics of starch release and hydrolysis into oligosaccharides during *in vitro* oro-gastric digestions of bread (A), wheat pearl pasta (B) and GF spaghetti (C) with either water (left column) or lemon juice (right column). Released starch refers to all the starch fractions released during digestion regardless of their level of degradation. Conversely, released oligosaccharides refer to short chain amylolysis products. The first point in each graph ( $t = 0$  min) marks the beginning of the gastric phase of digestion launched 2.5 min after the food was brought into contact with saliva, and 2 min after the bolus was introduced in the gastric compartment containing either water or lemon juice. The incremental AUCs are presented in Figure 48.

#### CONSUMPTION OF STARCH-RICH FOODS WITH WATER (Figure 47, left column)

Starch release and hydrolysis were initiated during the oral phase for all foods. At the beginning of the gastric phase, bread starch was about three times more degraded (29% of released starch, 70% thereof in the form of oligosaccharides) than that of wheat and GF pasta (about 7 and 3% of released starch, respectively, with oligosaccharides representing more than 80% thereof). After 30 to 60 min of gastric digestion, amylolysis reached a plateau that was about 3 times higher for bread (84% of released starch) than for wheat and GF pastas (25-27% of released starch). In all cases, 60-70% of the starch released had been hydrolysed into oligosaccharides by HSA.

#### CONSUMPTION OF STARCH-RICH FOODS WITH LEMON JUICE (Figure 47, right column)

Released starch and oligosaccharides were significantly lower for all foods in the presence of lemon juice, as it is indicated by the respective AUCs in Figure 48. As indicated in Figure 47, the reduction of the proportion of released starch and oligosaccharides with lemon juice was also statistically significant ( $\alpha = 0.05$ ) at most time-points (62 over the 72), with p-values ranging from 0.04 down to 0.000006. This was already observable at  $t = 0$  for bread (16% of released starch), less than 2 min after the bolus had been introduced in the gastric compartment containing lemon juice. The effect of this beverage became more obvious during the course of gastric digestion, particularly with bread, for which the starch release plateau remained at around 35%, *i.e.* approximately 2.5 times lower than in the presence of water. The interruption of amylolysis as soon as the bolus was brought into contact with lemon juice was also evident, and led to oligosaccharide release plateaus about 3 times lower than with water for all foods.



**Figure 47-** Oro-gastric *in vitro* digestions of starchy foods. Release of starch (●) and oligosaccharides (○) during gastric digestion of bread (A), wheat pasta (B) and GF-pasta (C) with either water (left column) or lemon juice (right column). Average  $\pm$  SD, 3 rpt. The dotted line represents the pH evolution, and down arrows (▼) denote HSA inactivation ( $\text{pH} \leq 3.5$ ). Asterisk (\*) symbols indicate the points on the starch and oligosaccharide release curves for which the effect of lemon juice was statistically significant ( $\alpha = 0.05$ ).



**Figure 48-** Incremental area under the curves of released starch (AUC<sub>Starch</sub>) and oligosaccharides (AUC<sub>Oligosaccharides</sub>) during gastric *in vitro* digestion of bread, wheat and GF pasta paired with water or lemon juice. Average  $\pm$  SD, 3 rpt. Asterisk (\*) symbols indicate statistically significant differences: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

## 4. DISCUSSION

The optimum pH of HSA in saliva is close to 6, and its activity is progressively lost as pH lowers, with approximately 50% left at pH 4, and complete inactivation below pH 3.5 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. H. Meyer, 1987). This explains the relevant contribution of HSA to starch digestion in presence of water, as pH conditions enabling amylolytic activity persisted for about one hour (Figure 47, left column). The previously reported protective effect of starch on HSA (Rosenblum, Irwin, & Alpers, 1988) (preserving up to 56% of its activity after 1 h at pH 3) was not observed under our experimental conditions. Actually, despite the presence of starch, amyolysis was clearly interrupted as soon as the bolus was introduced in the gastric compartment containing lemon juice (Figure 47, right column).

The results obtained with water are very consistent with our previous study in which we analysed the differences between similar types of starch-rich foods (Freitas & Le Feunteun, 2018b). Although such comparisons are outside the scope of the present study, it remains important to emphasize here the important contribution of HSA to the amyolysis of starch in bread and, though less extensive, also in pasta, during gastric digestion. The novelty of our current results lies in the demonstration that because HSA plays an important role in starch hydrolysis, the consequences of its premature inhibition due to meal/product acidification should be reevaluated. This mechanism is likely to explain, at least in part, the attenuation of the glycaemic response observed in the following 15 studies (Bo, et al., 2017; Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Kim, & Buller, 2004; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Lappi, et al., 2010; Leeman, Östman, & Björck, 2005; Liatis, et al., 2010; H. Liljeberg & Björck, 1996, 1998; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008; Mitrou, et al., 2010; Elin Östman, Granfeldt, Persson, & Björck, 2005; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009; Sugiyama, Tang, Wakaki, & Koyama, 2003). The possibility that this effect results from a reduced rate of starch digestion and/or impaired enzymatic activity has been investigated before, but the results were sometimes contradictory and conclusions unclear (H. G. Liljeberg, Lönner, & Björck, 1995; Elin Östman, Nilsson, Elmståhl, Molin, & Björck, 2002; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009). This is most probably due to the protocols used, which tend to standardize the pH in a way that is not always representative of human physiology. Amyolysis by HSA during oro-gastric digestion is the digestive process most likely to be affected by the pH of a food or meal. However, this enzyme is rarely considered in experimental protocols, and even when protocols comprise an oral phase, an immediate acidification is almost always used to simulate the gastric phase at a constant pH

of 3 or less. Consequently, almost all of the starch is digested during the intestinal phase that is not influenced by the initial pH of the food. Enzymatic performance is thereby always conditioned in a way that is not representative of the chronology of enzymatic actions *in vivo* nor accounts for the possible influence of the pH of the food item itself.

Still, it is possible to find hints that strengthen our results and hypothesis in the literature. For example, it has been observed that the pH of common breads is in the range of 5.6–6, whereas that of acid breads eliciting a reduced glycaemic response is typically between 3.9 and 4.6 (Lappi, et al., 2010; H. Liljeberg & Björck, 1998; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008). Moreover, whenever comparisons were made between meal supplementation with a source of acetic acid, like vinegar, and a neutral-pH equivalent, the effect on the glycaemic response was lost (Brighenti, et al., 1995; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010). Additionally, it has been reported that the effect observed with increased acidity is lost in three other cases: (1) when vinegar was consumed 5 h before the test meal (Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010), and when the starch-rich food was substituted with (2) a low glycaemic index one (Liatis, et al., 2010), or (3) dextrose (Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010). Each of these cases reflects a situation in which any effect on HSA activity would be: (1) irrelevant due to the obvious chronological mismatch, (2) less significant because of the lower contribution of HSA to starch hydrolysis (as shown for pasta in Figure 1), or (3) not applicable at all.

To conclude, it is also important to evaluate the viability of applying these results in real-life settings. Our experiments were conducted with 7 mL of water or lemon juice, and bread portions containing the equivalent to 3 g of starch. Considering that bread is composed of about 50% of starch (Stauffer, 2007), for a 50 g serving of an ordinary bread (approximate weight of two regular-sized slices of sandwich bread), 58 mL of lemon juice, would be needed to maintain the proportion used in this study, *i.e.* close to a fifth of a 250 mL glass. This seems to be a reasonable volume to consume as part of a meal. Additionally, there are other widely consumed foods, such as such as vinegars, grapefruit juice, and wine which have similar acidity levels (all classically showing  $\text{pH} \leq 3.5$ ) that can be suspected to exert a significant effect on starch hydrolysis by HSA. Achieving acidity levels similar to those in this study therefore appears viable in real-life settings. This strongly supports the feasibility of designing strategies, based on this approach, which should be applicable to both in *in-vivo* feeding situations and in the context of product development.

## CONCLUSION

We have demonstrated that pairing starch-rich foods with an acidic drink (lemon juice) results in an important reduction of starch hydrolysis at the gastric level due to HSA inhibition by premature gastric acidification. It is highly likely that this mechanism can explain, at least in part, the attenuation of the glycaemic response with increased meal/food acidity that is persistently observed *in vivo*. This work also offers new perspectives for the development of strategies aiming at improving the glycaemic response elicited by starch-rich diets.

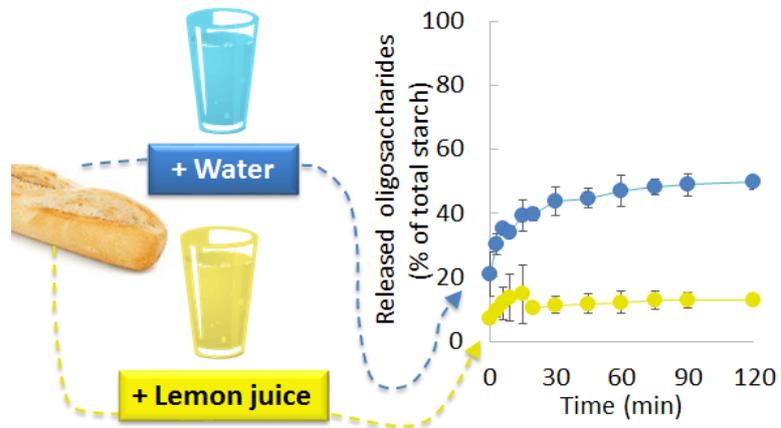
## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

Key messages



- It is unclear how acid drinks/foods lower the glycaemic response to starchy meals
- We compared oro-gastric *in vitro* digestion of starchy foods with/without lemon juice
- Gastric release and hydrolysis of starch were largely reduced with lemon juice
- Acid inhibition of salivary amylase has a greater effect than generally considered
- This mechanism, often overlooked, can help explaining related *in vivo* observations

# CHAPTER 4

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INHIBITORY EFFECT OF BLACK TEA, LEMON JUICE,  
AND OTHER BEVERAGES ON SALIVARY AND  
PANCREATIC AMYLASES: WHAT IMPACT ON  
BREAD STARCH DIGESTION? A DYNAMIC *IN VITRO*  
STUDY.

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*Journal article draft*

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Informal presentation

A strategy as simple as matching starch rich foods with others would be easy to adopt by the general population.

We have compared water with lemon juice, and our first tests strongly suggest that this might be a suitable strategy to slowdown starch digestion during the gastric phase, and it seems that it could also explain the outcomes of numerous clinical studies.

**BUT WHAT IS THE EXTENT OF THE EFFECT OF LEMON JUICE ON AMYLOLYTIC ENZYMES? AND WHAT ABOUT OTHER BEVERAGES OR CONDIMENTS WHICH COULD ALSO INFLUENCE THE ACTIVITY OF THESE ENZYMES?**

**ALSO, WHAT IS THE OVERALL IMPACT OF THIS TYPE OF MIX-AND-MATCH STRATEGIES ON DIGESTION (I.E. INCLUDING THE INTESTINAL PHASE)?**

## ABSTRACT

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The inhibition of digestive amylases can slow down starch digestion, thereby constituting an opportunity to delay the uptake of starch-derived glucose and improve postprandial glycaemic responses. Certain properties of foods such as their acidity or polyphenols can confer them such inhibitory capacity.

The aim of this work was twofold: (i) to screen some commonly available beverages and condiments for their capacity to inhibit the amylolytic activity of saliva and pancreatin, and (ii) to study the effect of two of them, in comparison with water, on the starch hydrolysis kinetics during dynamic *in vitro* digestion of a commercial sandwich bread.

Beverages and condiments screened for their inhibitory capacities were: instant coffees (2), green (2) and black (9) teas, wines (rosé, red and white wine), vinegars (from white wine, red wine and apple cider), and lemon juice. A black tea and lemon juice were then selected and their impact on bread starch digestion was investigated in dynamic *in vitro* oro-gastro-intestinal conditions.

Coffees, green and black teas reduced the amylolytic activity of saliva by 5-10%, 20-45% and 30-70%, correspondingly. At their native pHs, wines, vinegars and lemon juice exhibited a strong inhibitory capacity (from 90% to 100%). Upon pH neutralisation, these acidic solutions largely lost their inhibitory capacity, with the exception of red wine, which still inhibited > 80% of salivary amylase's activity. Similar levels of inhibition were found on the amylolytic activity of pancreatin for each product category. *In vitro* digestions performed in the presence of black tea and water (control experiment) led to similar results with the interesting exception that the proportion of released oligosaccharides was about 20% lower with black tea by the end of the intestinal phase. Lemon juice had a very strong effect on both released starch and oligosaccharides during the gastric phase, and also led to a  $\approx$  20% reduction of the proportion of oligosaccharides by the end of the intestinal phase.

These results provide a strong biochemical rationale for the development of dietary strategies aiming at improving the glycaemic response elicited by starch-rich foods that could be tested *in vivo*.

## 1. INTRODUCTION

Hyperglycaemia has been associated with a higher risk of developing metabolic diseases such as obesity, type 2 diabetes mellitus and cardiovascular disease (Blaak, et al., 2012). Tight glycaemic control is therefore necessary to maintain health and prevent disease (Blaak, et al., 2012). Starch-rich foods constitute a key source of exogenous glucose. By supplying up to 50% of our energy intake (Stylianopoulos, 2012), they can play a determinant role on postprandial glycaemic excursions. The digestion of starch is initiated in the mouth by salivary  $\alpha$ -amylase (HSA), which starts hydrolysing starch molecules (S. S. Gropper & J. L. Smith, 2013). This enzyme can carry on the amylolytic process in the stomach until it is inactivated by the increasing acidity, between pH 3 and 4 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. H. Meyer, 1987). Pancreatic  $\alpha$ -amylase and brush border enzymes then resume amylolysis in the small intestine, where glucose, the final product of starch digestion, is finally absorbed (S. S. Gropper & J. L. Smith, 2013).

The inhibition of digestive amylases can slow down starch digestion, thereby constituting an opportunity to delay the uptake of starch-derived glucose and improve postprandial glycaemic responses. Indeed, this strategy has been considered effective in contexts where glycaemia management represents an even bigger challenge than in healthy populations. An example is the treatment of type 2 diabetes via the administration of acarbose, which inhibits amylolytic enzymes in the digestive tract (Salvatore & Giugliano, 1996). A simple dietary strategy to achieve a lower glycaemic response by pairing starch-rich foods, or meals, with food products that can somehow exert similar inhibitory effect might therefore benefit both healthy individuals and those for whom glycaemia management poses a challenge.

We have previously hypothesised that pairing starch-rich foods with an acidic food product could slow down starch digestion by premature acid inhibition of HSA (Freitas & Le Feunteun, 2018a), and that this mechanism could even contribute to elucidate a number of results from clinical studies in which the glycaemic response to starch-rich meals was reduced in presence of an acidic product (Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Leeman, Östman, & Björck, 2005; H. Liljeberg & Björck, 1998; Elin Östman, Granfeldt, Persson, & Björck, 2005; Sugiyama, Tang, Wakaki, & Koyama, 2003). Additionally, there are other properties of foods which can confer them such inhibitory properties. An example is the reported effect of tea and tea extracts due to their polyphenolic composition (Y. Hara & Honda, 1990; Kashket & Paolino, 1988; Kwon, Apostolidis, & Shetty, 2008; Quesille-Villalobos, Torrico, & Ranilla, 2013; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015; Zhang & Kashket, 1998).

The different sites of action of amylolytic enzymes in the digestive tract are determinant to their vulnerability. HSA for example is present during the oral and gastric phases. It is therefore exposed not only to the food components, but also to their pH and structural characteristics. In turn, pancreatic  $\alpha$ -amylase, is more likely to be affected by the composition of food only, since the pH of the chyme is rapidly neutralised in the duodenum and the structure of the food has already been largely disrupted at this stage. Logically, the extent of their contribution to the digestive process will also influence the magnitude of the effects that can be achieved.

The aim of this work was to screen commonly available beverages and condiments for their capacity to inhibit the amylolytic activity of saliva and pancreatin, before studying the effect of two of them (selected for their observed inhibitory capacity) during dynamic *in vitro* digestions a commercial sandwich bread.

## 2. MATERIALS AND METHODS

### 2.1. MATERIALS

Crustless, white, wheat-based sandwich bread (Harry's 100% mie Nature, Barilla France S.A.S, Boulogne-Billancourt, France), 2 instant coffees, 3 green teas, 9 black teas, 1 rosé wine, 1 white wine, 1 red wine, and 3 vinegars (from white wine, red wine and apple cider), 1 lemon juice and mineral water were bought at a local supermarket. For simplification purposes, all products were coded with randomly allocated letters that do not reflect the brand of the products. All these details can be found in the Supplementary data - Table 1.

Frozen human saliva pooled from 10 donors (Normal saliva in 5 mL aliquots, ref. 991-05-P-5) was purchased from Lee Biosolutions (Maryland Heights, United States). Porcine pepsin (P-6887) and pancreatin (P1750, 4USP) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Amyloglucosidase from *Aspergillus Niger* (ref 10 102 857 001) was from Roche (Rotkreuz, Swizerland). The substrate used for the determination of HSA activity was potato starch (ref. 101 253, from Merck, Darmstadt, Germany). All other chemical reagents were of classical analytical grade.

#### 2.1.1. CHARACTERISTICS OF BREAD AND ENZYMES

The composition of bread is presented in Table 8. The water, total, digestible and resistant starch contents of the bread used in the digestion experiments was determined as previously described (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). The product label was used as reference for the other components.

Table 8- Composition of the white, wheat-based sandwich bread used in the study.

<b>Fat</b> <sup>1</sup> (g/100g of bread)	4.3
<b>Water</b> <sup>2</sup> (g/100g of bread)	36.1 ± 0.7
<b>Total Starch</b> <sup>2</sup> (g/100g of bread)	38.7 ± 4.2
<b>Digestible starch</b> <sup>2</sup> (% of total starch)	94.4 ± 1.5
<b>Resistant starch</b> <sup>2</sup> (% of total starch)	5.6 ± 1.5
<b>Fibre</b> <sup>1</sup> (g/100g of bread)	3.8
<b>Protein</b> <sup>1</sup> (g/100g of bread)	7.0

<sup>1</sup> According to the label of the product

<sup>2</sup> Experimentally determined in our lab as previously described (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b)

The amyloglucosidase activity provided by the manufacturer (14 U/mg of protein) was used as reference. The activities of saliva (345 ± 37 U/mL), pepsin (3319 ± 70 U/mg) and pancreatin (Trypsin activity of 3.42 ± 0.04 U/mg), measured in our lab according to previously described

methods (Minekus, et al., 2014), were used as a reference for digestion experiments and analytical assays. D-Glucose kits (ref. 103.21) were acquired from BioSenTec (Auzeville Tolosane, France).

## **2.2. PREPARATION OF BEVERAGES AND CONDIMENTS**

Teas and coffees were freshly brewed (1% w/v) with mineral water (100 °C). Teas were infused for 15 min before the tea bags were removed. Teas, coffees, all other beverages and the vinegars were used at room temperature.

## **2.3. IMPACT OF BEVERAGES AND CONDIMENTS ON THE AMYLOLYTIC ACTIVITY OF SALIVA AND PANCREATIN**

The amylolytic activity of salivary  $\alpha$ -amylase in saliva, of porcine pancreatin in simulated intestinal fluid electrolyte solution (eSIF) (Minekus, et al., 2014b), as well as the inhibitory effects of the products listed in table 1 (supplementary data) were determined using an adapted version of Bernfeld's method (Peter Bernfeld, 1955), as further described.

### 2.3.1. AMYLOLYTIC ACTIVITY OF SALIVA AND PANCREATIN

A 2% (w/v) starch solution was prepared in 0.32 M sodium acetate buffer (pH = 4.75). Four glass tubes were prepared with 0.25 mL starch solution and 0.25 mL of deionized water. Each tube was then incubated (3 min, 20 °C) with a different volume (0, 50, 70 or 100  $\mu$ L) of either saliva (10-fold diluted (w/w) in deionized water), or pancreatin (a 0.75 mg/mL solution prepared in eSIF). The enzymatic reaction was interrupted by adding 0.5 mL of DNS solution (consisting of 96 mM 3, 5-Dinitrosalicylic Acid, 0.5 M NaOH and 0.16 M of potassium tartrate). The content of enzymatic solution of each tube was then standardized to 100  $\mu$ L, and all tubes were placed in a boiling water-bath for 15 min. After cooling to room temperature on ice, 4.95 mL of water was added. Finally, a spectrophotometric measurement at 540 nm (UV-Vis spectrometer Evolution 201, Thermo Fisher Scientific, Massachusetts, United States) was performed and the concentration in maltose equivalents was determined from a previously established calibration curve. Enzymatic activity was defined as follows: 1 unit liberated 1.0 mg of maltose equivalents in 3 min at pH 6.9 at 20 °C.

### 2.3.2. INHIBITORY EFFECT OF BEVERAGES AND CONDIMENTS ON THE AMYLOLYTIC ACTIVITY OF SALIVA AND PANCREATIN

The impact of each of the 21 beverages and condiments listed in section 2.1 on the amylolytic activity of saliva and pancreatin was investigated by substituting the 0.25 mL of deionised water by 0.25 mL of each product in the previously described procedure. Coffees and teas (pH 6.5  $\pm$  0.5), were used as prepared. Acidic products (wines, vinegars and lemon juice) were studied in both acidic and neutralised forms with saliva, but only in the neutralised form with pancreatin.

Indeed, the pH of the chyme is quickly neutralized in the duodenum, so that it is unlikely that pancreatic enzymes are affected by the initial pH of a food.

#### **2.4. STABILITY OF HSA IN SALIVA AFTER EXPOSURE TO ACID ENVIRONMENT**

The enzymatic activity of HSA following exposure of saliva to acidic environments was investigated as follows. Seven tubes containing 4 mL of simulated gastric electrolyte fluid (eSGF) (Minekus, et al., 2014b) at different pHs were prepared (using diluted HCl), and incubated at 37°C. 1 mL of saliva (10 fold diluted in deionised water), pre-warmed to 37°C, was then added to each tube, the final pH of the mixtures ranged from 1.5 to 3.9. Samples (750 µL) were collected from each tube at 30 s and at 15, 30, 60 and 120 min. 750 µL of NaOH solutions of pre-calibrated concentrations were immediately added to neutralize the pH of the samples to  $6.9 \pm 0.5$ . Samples were conserved at -20 °C until analysis was carried out using the control assay for determination of the amylolytic activity of saliva.

#### **2.5. ORO-GASTROINTESTINAL *IN VITRO* DIGESTION**

Bread digestion protocols can be divided into two groups: (1) control digestions, which aimed at studying the respective contributions of the two amylolytic enzymes to starch hydrolysis, and (2) experimental digestions, conducted to assess the impact of tea and lemon juice on starch hydrolysis. With the exception of the control digestion with no saliva nor pancreatin, all digestion experiments were performed in triplicate. They comprised a manual oral phase that immediately preceded gastrointestinal digestions performed with a dynamic *in vitro* system, DiDGI® (INRA, Grignon, France), controlled and monitored by the software STORM® (INRA, Grignon, France). A standardized static *in vitro* digestion method (Minekus, et al., 2014a) was used as reference to define the composition and activities of the gastrointestinal secretions, and the volumes were set to preserve the enzymatic activities and electrolyte concentrations recommended in the same standardized protocol. We have previously described our dynamic digestion protocol and the sample analysis procedure in detail, therefore, only the main experimental steps, key principles and adaptations are presented here. Interested readers are invited to refer to (Freitas & Le Feunteun, 2018b) for further information. The digestion protocol was the following.

**ORAL PHASE.** Bread was processed in a food chopper (Multi-moulinette 400 CW, Moulinex, Ecully, France) and each bolus was formed by mixing bread (6 g) with saliva (1.2 mL, pre-heated to 37 °C) for 30 s with a spatula.

**GASTRIC PHASE.** Immediately after preparation, each bolus was introduced in DiDGI®'s gastric compartment containing 20 mL of neutralised eSGF, 10 µL of 0.3 M CaCl<sub>2</sub> and 12.8 mL of deionized water and set-up, beforehand, with an operating temperature of 37 °C and a stirring speed of 10 rpm. The chime was stirred for 30 s, stirring was interrupted for another 30 s and a first sample (2 mL) was collected from the supernatant. Stirring was then resumed and the gastric phase was initiated by activating the fluxes of pepsin (4705 U/mL in eSGF) and HCl (0.1 M in eSGF). The pepsin flux was set to reach ≈2000 U per mL of chime at t = 90 min, and was stopped afterwards. The flux of HCl was automatically adjusted to decrease the pH of the chime by 1 unit every 22.5 min until pH 2 was reached, and keep it constant thereafter, in order to replicate the kinetics of gastric acidification observed in humans (Malagelada, Go, & Summerskill, 1979). The gastric phase lasted 120 min.

**INTESTINAL PHASE.** 22 mL of eSIF, 10 mL of deionised water and 80 µL of 0.3 M CaCl<sub>2</sub> were added. The pH of the chime was adjusted to 7.0 with 1M NaOH and the intestinal phase was initiated by adding 10 mL of a pancreatin solution in eSIF to reach twice the final volume of the gastric chime and a trypsin activity of 100 U/mL. DiDGI® was programmed to automatically maintain the pH at 7.0 by adding 0.02 M NaOH (also prepared in eSIF) and preserve stirring (10 rpm) and temperature (37 °C) settings. The intestinal phase lasted 120 min.

**SAMPLING.** In addition to the first sample (collected from the gastric compartment 1 min after the oral phase, and approximately 30 s before gastric digestion started), additional samples were collected from the chime's supernatant at 5, 15, 30, 60, 120, 125, 135, 150, 180 and 240 min. Each sample (2 mL) was collected after interrupting stirring for 30 s to allow particles to sediment, and was immediately transferred to a dry bath (ThermoMixer C, Eppendorf, Hamburg, Germany) (99 °C, 150 rpm, 5 min) for enzyme inactivation. Samples were kept at -20 °C until required for further analysis.

#### 2.5.1. BREAD STARCH DIGESTION - CONTROL EXPERIMENTS.

Three different experiments were carried out: (1) with no saliva nor pancreatin, (2) with saliva but without pancreatin, (3) without saliva but with pancreatin. To do so, saliva and the pancreatin solution were replaced with deionised water and eSIF, respectively, when needed.

#### 2.5.2. BREAD STARCH DIGESTION IN PRESENCE OF WATER, BLACK TEA AND LEMON JUICE.

Three different digestions were carried out. In each experiment, 7.4 mL of deionised water out of the 12.8 mL added at the start of the gastric phase were replaced with the same volume of either (1) mineral water, (2) black tea, or (3) lemon juice (pH = 2.3).

#### 2.5.3. ANALYSIS OF DYNAMIC DIGESTION SAMPLES

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All samples were centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) (10 min, 3000 *g* and 4 °C), and their supernatants were analysed to quantify the released starch and oligosaccharides. Released starch was quantified as glucose equivalents (D-Glucose kits) after complete hydrolysis with amyloglucosidase. Oligosaccharides with degrees of polymerization of up to 10 (Hoebler, et al., 1998) were quantified in the same way after selective polysaccharide precipitation in an 80% (v/v) ethanol solution.

### 3. RESULTS AND DISCUSSION

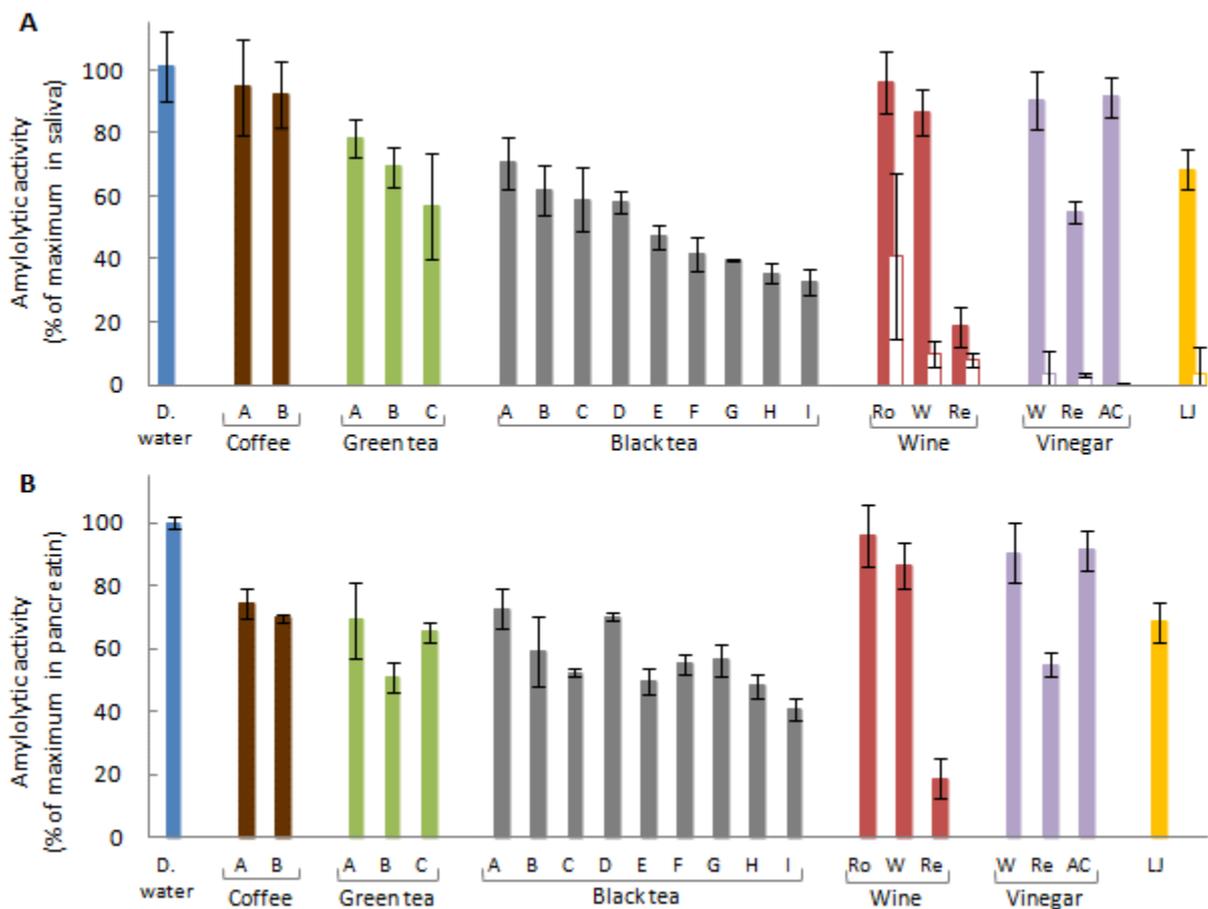
#### 3.1. INHIBITORY EFFECT OF BEVERAGES AND CONDIMENTS ON THE AMYLOLYTIC

##### ACTIVITY OF SALIVA AND PANCREATIN

The amylolytic activity of saliva was  $345 \pm 37$  U/mL this is close to the results that we (352 U/mL) (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b) and other researchers (410 U/mL) (Peter Bernfeld, 1955) have previously found. The amylolytic activity of pancreatin was  $24 \pm 1$  U/ mg. The inhibitory effect of the tested products on the amylolytic activity of saliva is presented in Figure 49A. Starting with the neutral pH products, coffees, green teas and black teas reduced HSA's activity by 5-10%, 20-45% and 30–70%, correspondingly, in our assay conditions. When tested at their native pHs, wines, vinegars and lemon juice exhibited a very high inhibitory capacity, which ranged between 90% and 100% of inhibition. With the exception of red wine and red wine vinegar, which still inhibited  $\approx 82\%$  and  $45\%$  of HSA's activity in their neutralised form, acidic foods largely lost their inhibitory capacity upon pH neutralisation, thereby reflecting a major effect of acidity. As it can be observed in Figure 49B, comparable inhibitory effects were obtained with pancreatin in terms of levels of inhibition within each product category.

Coffee did not influence the activity of neither of these amylolytic enzymes. To the best of our knowledge, there are no studies about the effect of coffee itself, but there is some evidence in the literature showing that some substances isolated from coffee beans exert an inhibitory effect on pancreatic amylase (Funke & Melzig, 2005; Narita & Inouye, 2009). It is possible that the concentration of such substances in the coffees tested was too low for an inhibitory effect to be produced. The effect of tea and tea extracts on the amylolytic activities of salivary  $\alpha$ -amylase and pancreatic  $\alpha$ -amylase has been widely studied by different teams of researchers. Most have found similar results to ours, confirming the capacity of different teas to inhibit salivary (Y. Hara & Honda, 1990; Kashket & Paolino, 1988; Zhang & Kashket, 1998) and pancreatic (Kwon, Apostolidis, & Shetty, 2008; Quesille-Villalobos, Torrico, & Ranilla, 2013; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015)  $\alpha$ -amylases *in vitro*, and reporting that black teas have a higher capacity to inhibit both of these enzymes (Quesille-Villalobos, Torrico, & Ranilla, 2013; Zhang & Kashket, 1998). The same has been observed for tea extracts or purified tea polyphenols (Fei, et al., 2014; Forester, Gu, & Lambert, 2012; K. Hara, et al., 2012; Miao, Jiang, Jiang, Zhang, & Li, 2015; Sun, Gidley, & Warren, 2018; Sun, Warren, Netzel, & Gidley, 2016; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). This reduction of the catalytic activity can be characterized as a mixed-type inhibition as both competitive inhibition (by binding to the active site) (Sun, Warren, Netzel, & Gidley, 2016) and non-competitive inhibition (by binding with the enzyme-substrate complex) have been identified (Y. Hara & Honda, 1990). It is also

interesting to note that some teams have observed that this inhibitory capacity is generally higher for the brush-border enzyme  $\alpha$ -glucosidase (Kwon, Apostolidis, & Shetty, 2008; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012) and that it can also impair intestinal glucose uptake as it also applies to specific transporters in the intestinal epithelial wall (Shimizu, Kobayashi, Suzuki, Satsu, & Miyamoto, 2000). Recently, however, some contradictory results have been published, showing that the inhibitory effect of tea polyphenols and tea extracts can either be lost or enhanced below a certain polyphenol concentration (Tong, Zhu, Guo, Peng, & Zhou, 2018; Yang & Kong, 2016).



**Figure 49** - Impact of beverages and condiments. Amylolytic activity (% of maximum) of saliva (A) and pancreatin (B) in the presence of coffee, green and black teas, wines, winegars and lemon juice (LJ) at their native (full bars) and neutralized (open bars) pH. The maximum amylolytic activity of saliva was  $345 \pm 37$  U/mL, and for pancreatin it was  $24 \pm 1$  U/ mg. Data are mean  $\pm$  SD, 3 rpt.

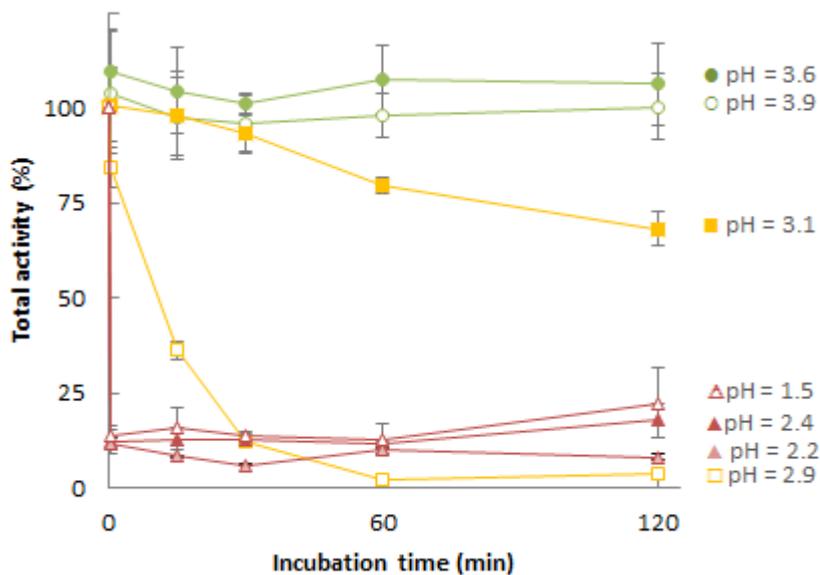
Acidic foods were tested at their native pHs and after pH neutralization. For acidic foods, experiments conducted at their native pH intended to reflect the influence these might exert during processes that are most likely to be affected by the pH of foods (specifically oral and gastric activity of HSA). Neutralisation of the pH of acidic foods intended to reproduce intestinal conditions.

The almost complete inhibition of HSA with wines, vinegars and lemon juice at their native pH shows the predominant effect of acidity over other factors, such as their polyphenol content, on its activity for such acidic beverages or condiments. The optimum pH of HSA in saliva is close to 6, and as the pH is lowered, its activity is progressively lost, with approximately 50% left at pH 4, and complete inactivation below pH 3.5 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. Meyer, 1987). With the exception of red wine (pH = 3.7), all other investigated acidic solutions' pH was below this inactivation threshold (between 2.3 and 3.2), hence explaining their capacity to almost completely inactivate HSA, even after being mixed with the starch solution at a 1:1 volume ratio. We may remind here that pancreatic  $\alpha$ -amylase is likely to be largely protected from this effect because of the rapid neutralisation of the pH once the chyme is propelled into the duodenum, reason why the impact of acidic foods on the amylolytic activity of pancreatin was only tested after pH neutralisation. Red wine was the only product that preserved almost its inhibitory capacity almost completely at neutral pH, suggesting an important effect of its polyphenol content. Important losses of inhibitory capacity were observed for all other acidic foods showing the predominant effect of acidity over other factors such as the polyphenol content for example. Research on the other tested products is less abundant, but the observed inhibitory effect of grape seed extracts on HSA (Yilmazer-Musa, Griffith, Michels, Schneider, & Frej, 2012) and of wine on pancreatic  $\alpha$ -amylase (Kwon, Apostolidis, & Shetty, 2008) is also in agreement with our results.

### **3.2. RESIDUAL AMYLOLYTIC ACTIVITY OF SALIVA AFTER EXPOSURE TO AN ACID ENVIRONMENT**

These experiments aimed at evaluating the residual amylolytic activity of saliva at neutral pH after exposure to *in vitro* gastric conditions at different pHs. During digestion, saliva is exposed to an increasing acidic environment within the stomach before being emptied in the small intestine. It is therefore uncertain how much of the amylolytic activity of saliva can be restored during the intestinal phase of digestion, and thus contribute to the intestinal hydrolysis of starch.

According to the results, presented in Figure 50, 3 cases can be distinguished:  $\text{pH} > 3.5$ ,  $\text{pH} \approx 3.0$ , and  $\text{pH} < 2.5$ . When HSA was exposed to  $\text{pH}$  3.6 or 3.9, the amylolytic activity of saliva was fully recovered after  $\text{pH}$  neutralisation, regardless of the period of incubation. When the  $\text{pH}$  was close to 3 (2.9 or 3.1), the residual amylolytic performance was dependent on exposure time. For short (30 s) exposure, 90% to 100% of activity was still observed after incubation at  $\text{pH}$  2.9 and 3.1, correspondingly. For longer exposures, the amylolytic activity was progressively, and irreversibly, lost at a  $\text{pH}$  dependent rate. Finally, for  $\text{pH} < 2.5$ , HSA activity was irreversibly lost, regardless of exposure time.



**Figure 50** – Impact of exposure of saliva to acidic environments. Residual amylolytic activity of saliva after exposure to  $\text{pH} > 3.5$  (all circles),  $\text{pH} \approx 3.0$  (all squares), and  $\text{pH} < 2.5$  (all triangles). Each sample was analysed 3 times for remaining HSA activity at  $\text{pH}$  6.9. Plotted values refer to mean  $\pm$  SD.

Our results are in agreement with the findings of Bernfeld and colleagues (1948) who conducted similar experiences with purified HSA, exposing it to  $\text{pH}$  ranging from 3 to 12 for 30 min, 2 h and 20 h. They observed that between  $\text{pH}$  4 and 11, 100% of activity was recovered, regardless of exposure time. Between  $\text{pH}$  3 and 4, the activity of HSA was still recovered after 30 min but as exposure time increased, an increasing proportion of HSA was irreversibly lost. It has previously been shown that the optimum  $\text{pH}$  of HSA in saliva is close to 6-7, and its activity declines as the  $\text{pH}$  lowers, with approximately 50% of the maximum activity left at  $\text{pH}$  4 and complete inactivation found below  $\text{pH}$  3.5 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b; M. Fried, S. Abramson, & J. Meyer, 1987). The results of our stability assays, in agreement with the available literature, point towards the existence of an irreversible, time-dependent inactivation

threshold around pH 3. However, they also show that despite an impaired amylolytic activity at acidic pH, whenever HSA is not exposed to pH  $\approx$  3 or below, this effect is reversible. This may be particularly relevant in the context of human carbohydrate digestion. Depending on the properties of the meal, it can take more than 45 min to lower the pH of the gastric chime to 4, and close to 1 h to further reduce it to 3 (Malagelada, Go, & Summerskill, 1979). Therefore, HSA in the gastric chime propelled into the small intestine in the first 45 – 60 min of digestion might fully recover its enzymatic activity and resume amylolysis in combined action with pancreatic  $\alpha$ -amylase. This is indeed consistent with previous reports that HSA comprised as much as 14% of the total amylase in the jejunum over a 200 min period after meal consumption (M. Fried, S. Abramson, & J. Meyer, 1987).

### **3.3. DYNAMIC *IN VITRO* DIGESTION OF BREAD STARCH IN PRESENCE OF WATER, BLACK TEA AND LEMON JUICE**

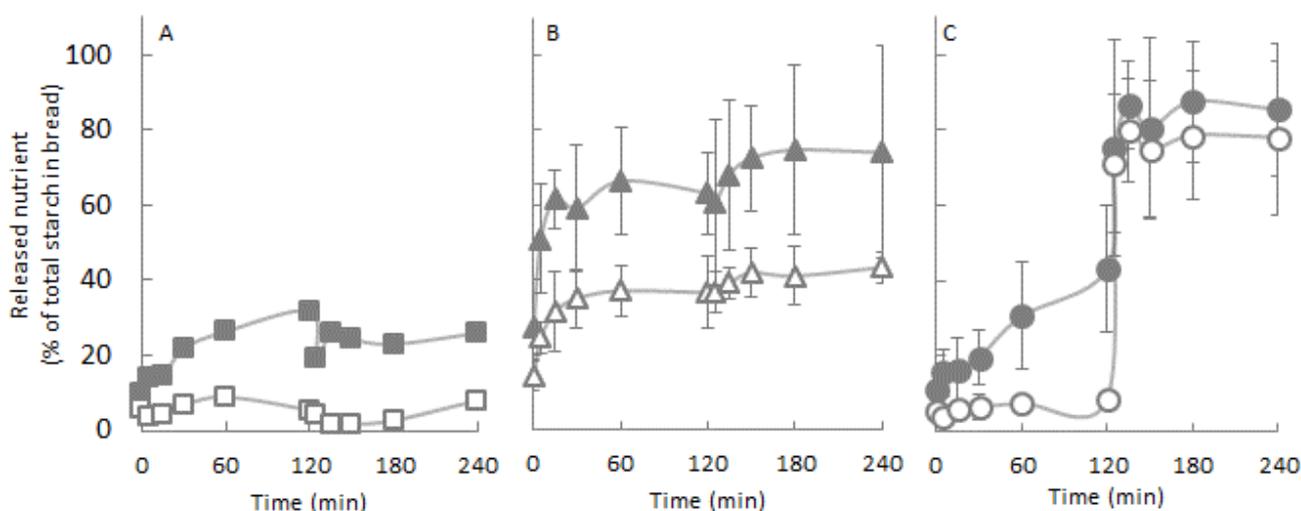
#### **3.3.1. BREAD STARCH DIGESTION - CONTROL DIGESTIONS**

The results of the control digestions carried out with no saliva nor pancreatin, with saliva alone, and with pancreatin alone are presented in Figure 51A, B and C, correspondingly. In the absence of amylolytic activity (Figure 51A), about 15% of the total starch was solubilised in the chime within the first minutes of gastric digestion. As the pH progressively decreased, the proportion of total starch released into the chime increased within the first 15 min reaching a plateau at 25-30% that lasted until the end of the intestinal phase. This starch release profile can be attributed to a rapid disintegration of bread particles within the gastric medium (Freitas & Le Feunteun, 2018b) and the subsequent hydrolysis of the gluten network by pepsin as the pH lowers (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). Indeed, pepsin is inactive in the initial pH conditions of the gastric phase so that only the effects of mixing and starch diffusion towards the surrounding can explain the initial rise of about 15%. The second increase of about 15% may, however, be essentially akin to the pepsin action as previously showed (Freitas, Le Feunteun, Panouillé, & Souchon, 2018b). Since pepsin is inactive at pH 7 and pancreatin was not used, the extent released starch remained stable throughout the intestinal phase. The oligosaccharide fraction, estimated between 4% and 9% of the total starch content of the bread throughout digestion (Figure 51A), corresponds to the signal arising from the bread content in simple sugars since no amylase was used in this control experiment.

When HSA was the only amylolytic enzyme present (Figure 51B), considerably more starch and oligosaccharides were released. After 15 min, released starch and oligosaccharides plateaued at about 60-65% and 37%, correspondingly. A further increase of 4-5% seemed to occur within in the first minutes of intestinal digestion. More experiments would be needed to confirm this

small apparent increase, which was unexpected according to our results showing an irreversible inactivation of HSA after exposure to pH < 3 and the pH = 2.0 conditions set for the last stage of our gastric digestion. We may nevertheless note that the presence of starch has been reported to have a protective effect on HSA, preserving up to 56% of the activity of purified amylase after 1 h at pH 3 (Rosenblum, Irwin, & Alpers, 1988). It is therefore possible that part of HSA activity was recovered during the intestinal phase.

In the absence of saliva but in presence of pancreatin (Figure 51C), the gastric starch and oligosaccharide release profiles were the same as those already described for the experiment free from amylolytic enzymes (Figure 51A). As soon as the intestinal phase was initiated, the proportion of total starch released into the chyme increased almost instantaneously, plateauing close to 85% within 15 min with almost all of it hydrolysed into oligosaccharides. This experiment therefore clearly illustrates the great efficiency of pancreatin to rapidly and extensively hydrolyse starch present in the chyme emptied from the stomach.



**Figure 51** - Dynamic *in vitro* digestion of bread – control experiments. Proportion of total starch released (full symbols) and hydrolysed into oligosaccharides (open symbols) during the course of control *in vitro* oro-gastro-intestinal digestions of equal portions of bread performed with no saliva nor pancreatin (A), with saliva but without pancreatin (B), and without saliva but with pancreatin (C). The first point in each curve corresponds to the end of the oral phase and the start of the gastric phase. The gastric and intestinal phases lasted 120 min each. Average  $\pm$  SD, 3 rpt.

### 3.3.2. BREAD STARCH DIGESTION IN PRESENCE OF WATER, BLACK TEA AND LEMON JUICE

The effect of pairing water, lemon juice or tea with bread on starch digestion is shown in Figure 52A, B and C, respectively. In the presence of water (Figure 52A), approximately 15% of the total

starch was released into the form of oligosaccharides after the oral phase. After the start of the gastric phase, amylolysis proceeded and plateaued after about 30 min to 72% of the total starch, with more than half thereof in the form of oligosaccharides. During the intestinal phase, starch release appeared to have only slightly increased close to 80% of the total starch, and most of the amylolytic activity consisted in hydrolysing into oligosaccharides the polysaccharides released during the gastric phase. This “rise-plateau-rise-plateau” profile was preserved when water was replaced with black tea and lemon juice (Figure 52B and C) but with different patterns. In presence of tea (Figure 52B), released starch and oligosaccharides plateaux were about 10% lower during the gastric phase. During the intestinal phase, released starch reached the same level as with water, but hydrolysis into oligosaccharides remained impaired, stagnating at about 50% of the total starch. In presence of lemon juice (Figure 52C), released starch corresponded to about 40% of the total bread starch at the gastric stage, and hydrolysis into oligosaccharides was totally interrupted after the oral phase. During the intestinal phase, the proportion of total starch released rapidly matched that of the other two experiments. Hydrolysis into oligosaccharides remained close to that obtained in the presence of tea, i.e. slightly lower the level observed with water.

Starch hydrolysis during the digestion of bread with water was similar to the results we obtained previously with other types of bread (Freitas & Le Feunteun, 2018a, 2018b). We have previously highlighted the important contribution of the gastric phase to the digestion of starchy foods, (Freitas & Le Feunteun, 2018a, 2018b; Freitas, Le Feunteun, Panouillé, & Souchon, 2018b) and present results provide further confirmation of this. The replacement of water with tea and lemon juice slowed down starch hydrolysis, in agreement with the capacity of these beverages to inhibit the amylolytic activity of HSA and pancreatin (section 3.1.).

This effect was more noticeable with lemon juice (Figure 52C), which almost reproduced the digestive pattern of the control digestion containing only pancreatin (Figure 51C, noting that the early increase of released starch during the gastric phase is probably due to an earlier pH-activation of pepsin) with limited starch release and no hydrolysis into oligosaccharides. The magnitude of the effect of lemon juice during the gastric phase therefore directly reflects its inhibitory capacity (Figure 49A). Indeed, the initial pH of the chyme at the start of the gastric phase was 2.5 in presence of lemon juice, hence inducing a complete inhibition of HSA. These results are in agreement with our previous work showing that pairing 3 starch-rich foods with lemon juice caused an important reduction of starch hydrolysis at the gastric level because of an early acid-induced inhibition of HSA (Freitas & Le Feunteun, 2018a). It is therefore very likely that similar results would have been obtained with the other acidic foods such as vinegar that

exhibited an inhibitory capacity equivalent to that of lemon juice (Figure 49A). In our view, this could indeed explain, at least in part, the effectiveness of the widely studied strategy of supplementing a starch-rich meal with an acidic product (often vinegar) to reduce the glycemic response *in vivo* (Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Leeman, Östman, & Björck, 2005; H. Liljeberg & Björck, 1998; Elin Östman, Granfeldt, Persson, & Björck, 2005; Sugiyama, Tang, Wakaki, & Koyama, 2003).

In the presence of tea, only about 33 % and 41 % of the amylolytic activity of saliva and pancreatin was preserved with the assays we used to investigate the inhibitory capacity of beverages and condiments (Figure 49). During the dynamic *in vitro* digestion black tea did not influence the kinetics of starch and oligosaccharide release in the gastric phase and in the intestinal phase there were no differences for starch release either, but there was a 20% reduction in the proportion of oligosaccharides (Figure 52B). The limited impact of tea in the digestion experiments is most certainly due to the differences between the conditions employed between these experiments (section 2.5) and the enzymatic assays (section 2.3). In particular, 3 conditions of the *in vitro* digestion experiments could have contributed to such results: (1) the temperature was higher (37 °C vs. 21 °C in the enzymatic assays), (2) the tea was more diluted ( $\approx$  50% vol. in the enzymatic assay vs. 32% vol. at the start of the gastric phase and less than 15% at the start of the intestinal phase), and (3) the enzyme activity greater (saliva was  $\approx$  1.5 times more concentrated at the start of the gastric phase, and pancreatin  $>$  80 times more concentrated during the intestinal phase). In parallel, starch concentration during the gastric phase was similar to that in the enzymatic assay, and in the intestinal phase it was reduced to about half. Considering the level of tea dilution in the digestion experiments, the ratio of starch to tea was higher. Because the effect of tea is partly due to competitive inhibition (Sun, Warren, Netzel, & Gidley, 2016), it is very likely that it was partly reversed by the higher substrate concentration (Blanco & Blanco, 2017). Judging by the lower proportion of starch hydrolysed into oligosaccharides in the intestinal phase, it did seem that tea might have somewhat limited the later stages of amylolysis, but it is unsure whether this would be enough to produce an effect *in vivo*. The masses of tea and starch in the meal digested *in vitro* would correspond to approximately 125 mL tea (1% w/v) for one portion of bread containing 50 g of starch ( $\approx$ 100 g of bread). *In vivo* human studies testing food portions containing 50g of carbohydrates are also in agreement with our results. Investigations of the effect of 300 mL of green tea (3% w/v) with a starch-rich breakfast (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010), or of including green tea extract (2% w/w) in a starch confection (Sapper, et

al., 2016) did not observe any effect on postprandial glucose. It seems that higher concentrations of tea/tea extracts or higher proportions of tea to starch would be needed to achieve a relevant inhibitory effect during digestion.

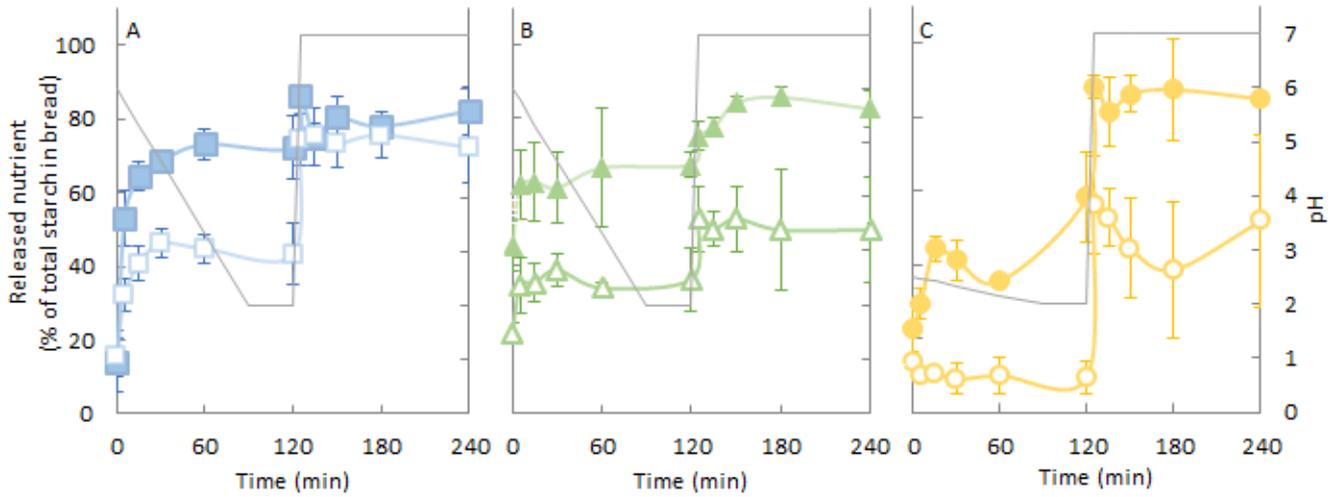


Figure 52 - Dynamic *in vitro* digestion of bread. Released starch (full symbols) oligosaccharides (open symbols) and pH of the chyme (grey line) during the course of oro-gastro-intestinal digestions of equal portions of bread combined with either water (A) tea (B) or lemon juice (C). The first point in each curve corresponds to the end of the oral phase and the start of the gastric phase. The gastric and intestinal phases lasted 120 min each. Average  $\pm$  SD, 3 rpt.

## CONCLUSION

We have shown that green and black teas, red, white and rose wines, vinegars and lemon juice can inhibit the amylolytic activity of both human saliva and porcine pancreatin *in vitro*. The effects were similar in both cases with the pH-neutralised form of these beverages and condiments. However, because saliva is secreted at the very beginning of the digestive system, its amylolytic performance is more susceptible to be impaired by the consumption of inhibitory products, or by the pH of acidic beverages or foods. Our results emphasize the vulnerability of HSA to the pH of tested foods, which resulted in an almost complete inhibition by acidic products such as wines, vinegars and lemon juice at their native pH. These findings were confirmed during dynamic *in vitro* digestions of bread starch in presence of either water, black tea or lemon juice. Both black tea and lemon juice enabled to reduce starch hydrolysis during digestion, but lemon juice exerted a more significant effect than tea, in particular during the gastric phase of digestion. These results provide a biochemical rationale for the development of dietary strategies aiming at improving the glycaemic response elicited by starch-rich foods that could be tested *in vivo*.

## ACKNOWLEDGMENTS

We would like to thank Thomas Cattenoz and Estelle Canellas for their technical help in the laboratory. We would also like to acknowledge the IDEX-Paris Saclay consortium for partially supporting this research through a PhD grant and we are grateful to François Boué and Evelyne Lutton for their help in obtaining this doctoral grant.

## CONFLICT OF INTEREST

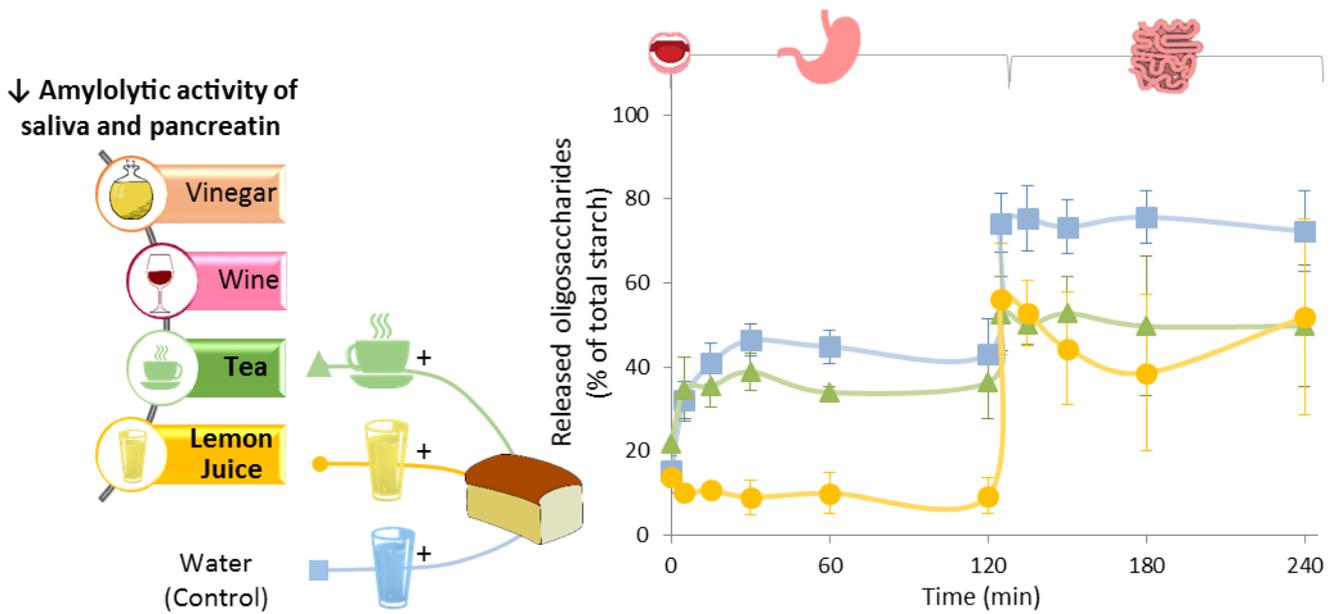
The authors have no conflict of interest to declare.

## SUPPLEMENTARY DATA

Supplementary data - Table 1 – Detailed information about the beverages and condiments screened for their capacity to inhibit the amylolytic activity of saliva and pancreatin.

Products	Codes	Brand-type (Country if available)	Producer (or, if not available, distributor), City, Country	pH
Coffees	A	Nescafé - Nes - instant coffee	Nestlé France, Marne la Vallée, France	6.6
	B	Auchan instant - Extra filtre	Auchan production, Villeneuve d'Ascq, France	6.2
Green teas	A	Twinings of London - Simply pure	Twinings, Andover, United Kingdom	7.2
	B	Désir de Vrai - Gunpowder vert (China)	Destination, Bordeaux, France	7.2
	C	Gorreana (Portugal)	Plantações de Chá Gorreana, Azores, Portugal	7.1
Black teas	A	Twinings of London - Yunnan tea (China)	Twinings, Andover, United Kingdom	6.8
	B	Auchan - Organic Breakfast	Auchan production, Villeneuve d'Ascq, France	7.0
	C	Tetley - English breakfast (Africa/Asia/South America)	Tata Global Beverages GB Limited, Middlesex, England	6.7
	D	Auchan breakfast tea	Auchan production, Villeneuve d'Ascq, France	6.8
	E	Twinings of London - Original English Breakfast (Africa/India/China)	Twinings, Andover, United Kingdom	6.9
	F	Désir de vrai – Breakfast (Sri Lanka)	Destination, Bordeaux, France	6.8
	G	Auchan - Organic from China (China)	Auchan production, Villeneuve d'Ascq, France	6.9
	H	Auchan - Organic	Auchan production, Villeneuve d'Ascq, France	7.1
	I	Lipton yellow label	Unilever France, Rueil Malmaison, France	6.7
Wines	W	Touraine Sauvignon 2016 (France)	Pierre Chainier, Amboise, France	3.2
	Re	Les Fées Pays d'OC -Cabernet Sauvignon 2016 (France)	Jean d'Alibert, Rieux, France	3.7
	Ro	Cabernet d'Anjou 2016 (France)	Les caves des perrières, Mouzillon, France	3.2
Vinegars	W	Auchan - White wine vinegar Champagne-Ardenne Region (France)	Charbonneaux Barbant, Reims, France	2.7
	Re	Auchan - Red wine vinegar aged 2 months in casks 7% acidity	Auchan production, Villeneuve d'Ascq, France	2.6
	AC	Auchan - Cider vinegar 5% acidity	Auchan production, Villeneuve d'Ascq, France	3.1
Lemon Juice	LJ	Lazy lemon	Polenghi, Milan, Italy	2.4

## KEY MESSAGES



- We screened 21 beverages and condiments for their capacity to inhibit salivary and pancreatic amylases
- Coffees, green teas and black teas (all with neutral pH) reduced their activity by 5-10%, 20-45% and 30-70%
- Wines, vinegars and lemon juice (all with acidic pH) inhibited the activity of salivary amylase by 90% to 100%
- Black tea and lemon juice reduced starch hydrolysis during the digestion of bread
- Lemon juice exerted a much more significant effect, in particular during gastric digestion.

# III

## *IN VIVO* STUDIES



**IN THIS PART OF THE MANUSCRIPT THE RESULTS OF THE *IN VIVO* STUDIES WILL BE PRESENTED.**

**THE MAIN GOAL WAS TO ANSWER THE FOLLOWING QUESTIONS:**

**DO THE FOOD PAIRING STRATEGIES TESTED *IN VITRO* TRANSLATE INTO MEASUREABLE DIFFERENCES *IN VIVO*?**

**IS THERE AN IMPACT ON THE GLYCAEMIC RESPONSE AND/OR SATIETY?**

**IF SO, CAN WE PINPOINT THE MECHANISM?**

This work is presented in the form of two extended articles, preceded by an introductory section:

- Introductory section - Preparation of the clinical study
- Chapter I - Lemon juice lowers the postprandial plasma glucose response to bread in healthy volunteers
- Chapter II - Reduction of the glycaemic response to bread in the presence of lemon juice: MRI investigations on gastric processing and emptying

An informal text summarizing the reasoning behind the work presented as well as a scientific abstract were included at the start of each chapter. At the end of each chapter the key results are recapitulated in written and schematic form.



# OUTLINE AND PREPARATION OF THE CLINICAL STUDY

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## 1. STUDY OUTLINE

The outline of the clinical study, which was a 2-branch investigation, is presented in Figure 54. Detailed information about each part of the study can be found in the next two chapters.

The objective of part 1 was to determine the effect of pairing a starch-rich food with water, black tea or lemon juice on postprandial plasma glucose and energy intake in healthy humans. This study was conducted in semi-controlled conditions in our research unit (UMR GMPA, Grignon).

The objective of part 2 was to study the gastric emptying patterns, blood glucose response and satiety perceptions following the consumption of bread with water, tea or lemon juice. This study was conducted at the Service Hospitalier Frédéric Joliot in Orsay, in collaboration with the IR4M research unit (Imagerie par Résonance Magnétique Médicale et Multi-Modalités, UMR8081 Université Paris Sud - CNRS, Orsay, France).

Volunteer recruitment for each part of the study was conducted independently. The test meals were similar, except that the meals were supplemented with 20 peas in part 2, at the very end of the meals, to use them as stomach motility and gastric mixing tracers in MRI images. The data collected in each part of the study was analysed independently.

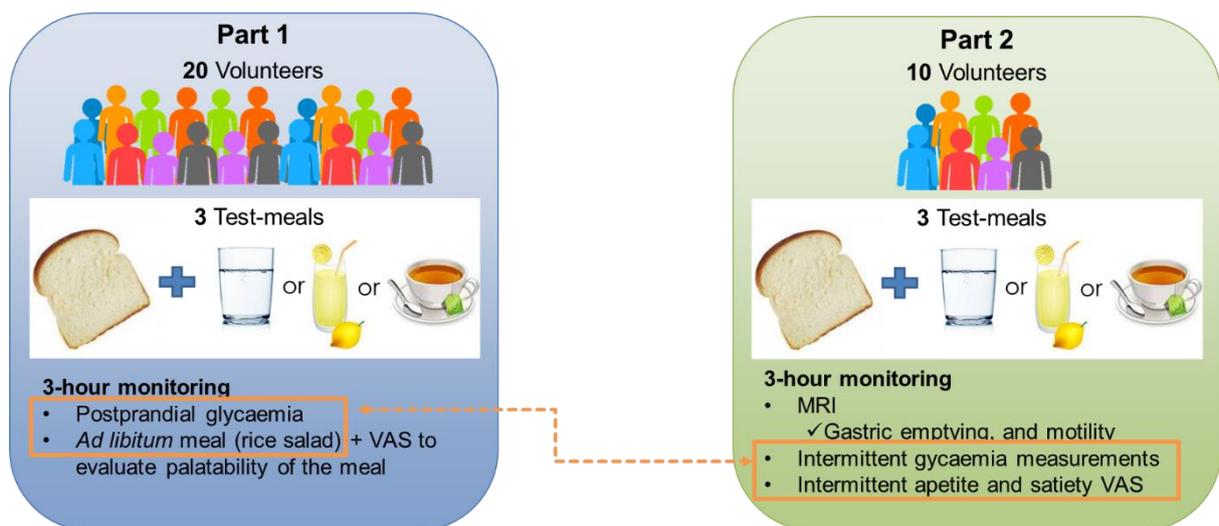


Figure 53 – Schematic description of the clinical study

### 1.1. NOTES ON THE PREPARATION OF THE CLINICAL STUDY

Before detailing the clinical trials and corresponding results, it is worth noting that this was the first clinical trial conducted by our research unit (UMR GMPA). Therefore, in the beginning of my PhD we did not have all the necessary knowledge to be able to plan and execute this project. This was also the first collaboration of our laboratory with both UMR IR4M, specialized in medical and multi-modal MRI, and the CEFRED (Centre d'Exploration Fonctionnelle et de Rééducation Digestive) from the Avicenne Hospital. The design of this study and the recruitment of volunteers were thus complicated by the geographical locations of the different partners, in particular providing that there was a medical visit mandatory at CEFRED (UMR GMPA ~ 40 km west of Paris, UMR IR4M ~ 25 km south of Paris, CEFRED in the north of Paris).

Moreover, during the preparation of this study, the regulations that apply to clinical studies in France were in a process of being changed, with a new law in June 2016 and new decrees becoming effective in November 2016 and May 2017 (Levy, Rybak, Cohen, & Jung, 2017). The new law modified numerous aspects of the process such as the categories of clinical studies, the randomization of the assignment of an ethics committee, among others. Altogether, our initial lack of experience and the changes in the law have created difficulties that could only be surmounted with the support of other teams. A timeline of the project is presented in Figure 54.

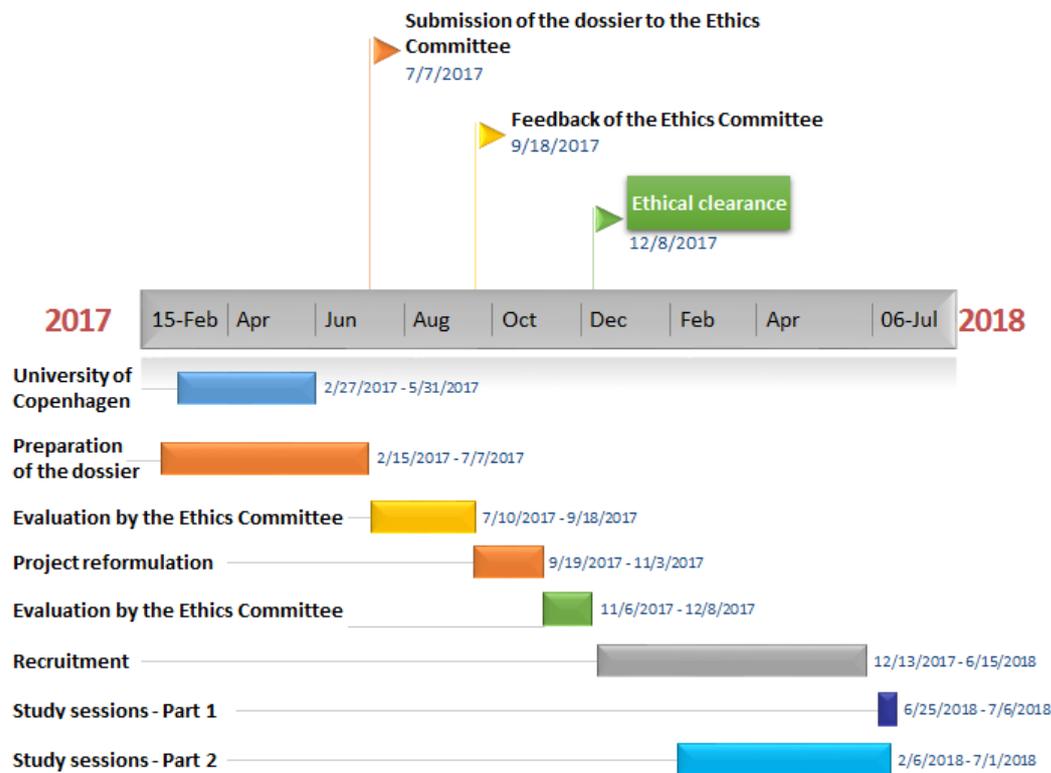


Figure 54- Timeline. Preparation and execution of the clinical study.

Concerning the knowledge I acquired and the help received for the preparation of the dossier, I have spent 3 months within the Obesity Research Group at the Department of Exercise Sports and Nutrition of the University of Copenhagen where I followed clinical trials that were being conducted by Thea Hansen and Professor Anders Sjödin (Figure 54). They have given me files that I used as examples and I have learned with them all the steps that are involved in all the stages of a clinical project, from planning and volunteer recruitment to conducting study sessions. In France, we have collaborated with Robert Benamouzig (Pr., MD, and Principal Investigator), Mourad Benallaoua (PhD.) and Georghe Airinei (MD) from CEFRED (Centre d'Exploration Fonctionnelle et de Rééducation Digestive), Avicenne Hospital. They have extensive experience with the clinical research environment in France, and their help was essential to be able to complete this project. They gave us many advices related with the writing of the research protocol and the new French regulations. They have also proofread the final version of the dossier, and organized all the medical visits for the volunteer recruitment.

Concerning the MRI investigations, we received critical help from the IR4M research unit, in particular from Luc Darrasse (PhD) and Xavier Maître (PhD), who developed the MRI protocol, from Laurène Jourdain (MSc), for her daily support during the MRI sessions, and Rose-Marie Dubuisson (MSc), who coordinated the schedule of all the MRI sessions. Vincent Lebon (PhD, MD, and Co-Investigator), who is at the head of the Service Hospitalier Frédéric Joliot (SHFJ) in Orsay, was the co-investigator in the study-site where we did the MRI sessions. The exams were performed by Laurence Tavassoli, Brigitte Mansalier, Beatrice Lhuillery and Laure Miche from the team of MRI technologists of the Medical Imaging team of the Groupe Hospitalier Nord-Essonne Orsay Hospital.

This collaborative work between three different institutions was initiated by François Boué, my PhD supervisor, who was also the Scientific Coordinator of this clinical research protocol.

#### 1.1.1. NOTES ON THE MRI PROTOCOL AND RESULTS

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During the development of the MRI protocol, different sequences, which allowed for different spatial and time resolutions, and other meals have been tested. Initially, we intended to include 3 bread meals and 3 pasta meals in our clinical study. Considered pasta meals were composed of both pearl-shaped pasta and peas. Pearl pasta were selected to try monitoring the evolution of their particle size during gastric digestion, and peas were planned to be used as particle tracers of the stomach motility and of the gastric mixing, knowing that very good MRI contrasts (*i.e.* white peas and black pasta) could be achieved in high temporal resolution scans. However, following initial feedback from the Ethics Committee considering the level of constraints too elevated for the volunteers, we had to limit the number of meals and decided to keep the 3

bread meals. As these pasta meals could no longer be investigated, we provide two examples of what we could observe with such meal in Figure 55. In this figure, slices of anatomic (A) and motility (B) scans of the gastric region obtained 15 min after consumption are presented. It is possible to observe that in the anatomic scans both individual pieces of pasta and peas, of 3 mm and 5 mm of diameter, respectively, were observed in different shades of grey. In the motility scan the pasta appeared in grey and no individual pieces were discerned, however the peas were

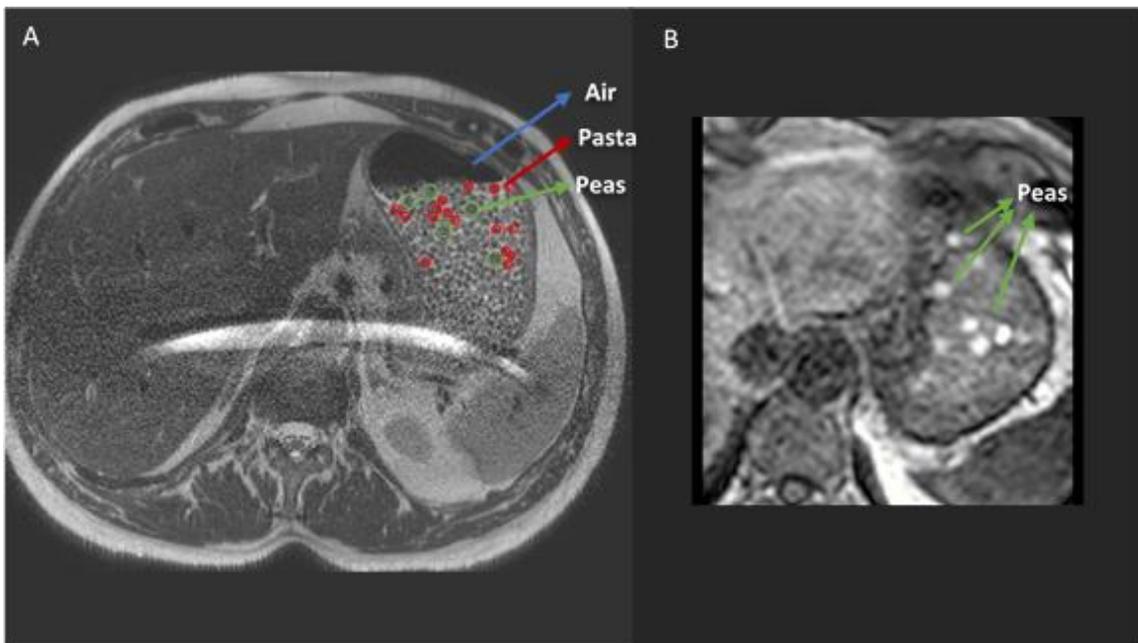


Figure 55- Example of magnetic resonance imaging from the abdominal region of a healthy adult who ingested 150 g of pasta, 20 peas and 250 mL of water. The diameter of pasta and peas was 3 mm and 5 mm, respectively. The subject was lying in supine position and was holding his breath. (A) The image was extracted from an anatomic volume scan performed 15 minutes after the meal was initiated. The meal appears in different shades of grey and the air appears dark at the top. Red and green circles indicate discernable pieces of the pearl-shaped pasta and peas, respectively. (B) The image was extracted from a motility volume scan performed 15 minutes after the meal was initiated. The pasta appears in grey and the peas (indicated by green arrows) appear in white.

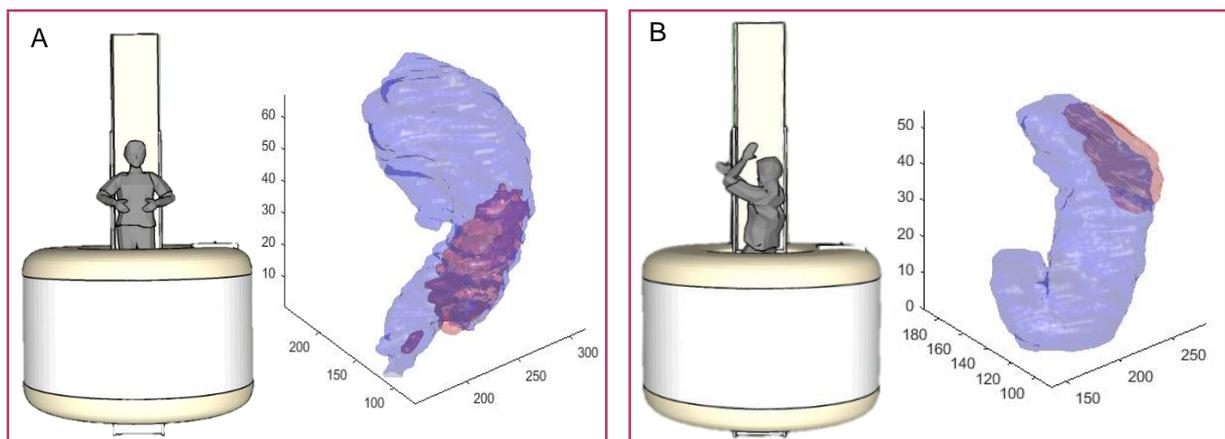
clearly discernable and appeared in white. The parameters of the sequences used are presented in Table 9.

**Table 9- Selected parameters of the MRI sequences used during acquisition of the scans illustrated in Figure 55.**

Imaging parameters	Anatomic volume scan	Motility volume scan
<b>Weighing</b>	T2	T1
<b>Repetition time</b>	“shortest”	“shortest”
<b>Echo time</b>	60 ms	“shortest”
<b>Slice thickness</b>	2 mm	2.6 mm
<b>Number of slices/orientation</b>	90/axial	50/axial (16 scans in total)
<b>Gap</b>	0 mm	0 mm
<b>Matrix</b>	344 x 264	188 x 178
<b>Voxel size</b>	0.90 x 0.90 x 2 mm <sup>3</sup> ≈ 1.62 mL	0.98 x 0.98 x 2.6 mm <sup>3</sup> ≈ 2.5 mL
<b>Flip angle</b>	90 °	30 °

Another point that may be highlighted in here is the influence of the volunteer position within the MRI. By doing 3D reconstructions of the stomach during the MRI protocol development, one issue that was identified was the persistence of air pockets in the lower part of the stomach, close to the pyloric region, due to the position of the subjects (supine) (Figure 56A). This had previously been described and has been related with potential differences in the gastric emptying rate in comparison with the seated position, being suggested that the right decubitus position was more suitable for MRI studies (Treier, et al., 2006). As shown in Figure 56B, our preliminary MRI tests confirmed that the right decubitus position enabled to confine air pockets to the upper region of the stomach.

Taking into account all the developmental work, it was decided to:



**Figure 56-** Influence of subject positioning in the MRI on intragastric meal/air distribution. Examples of 3D reconstructions of the stomach from magnetic resonance imaging exams performed on the abdominal region of two healthy adults in the postprandial state. Images correspond to examinations conducted 30 minutes after the meal started in the supine (A) and right decubitus (B) positions. Blue and red colors illustrate the position and relative volumes of chime and air, correspondingly. Air pockets were present in the lower part of the stomach in the supine position, and in right decubitus they were confined to the upper part.

- Limit breath holding times to 25 s;
- Preserve one anatomic volume scan (high spatial resolution) in each exam for volume reconstructions;
- Include motility volume scans (high temporal resolution) in each exam in which peas have a different contrast, to obtain information about intragastric mixing;
- Include one long motility scan under free-breathing conditions, the spatial resolution was highly compromised but the intent was to try to observe motility patterns over a longer period of time than what would be achievable under breath-holding conditions.

Finally, due to time constraints, not all of these scans could be analysed in time for the results to be included in the thesis manuscript. The postprandial anatomic volume scans were quantitatively analysed and the motility scans (breath hold) were qualitatively analysed. No analysis was yet performed on the long motility scans (free breathing).



III. *IN VIVO* STUDIES

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# CHAPTER 1

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LEMON JUICE LOWERS THE POSTPRANDIAL  
PLASMA GLUCOSE RESPONSE TO BREAD IN  
HEALTHY VOLUNTEERS

*Extended article draft*

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Informal presentation

We mix and match foods every day. Availability, tradition, taste and our perception of the nutrients we need are probably the main authors of our menus.

**BUT WHAT IF THESE CHOICES THAT WE MAKE EVERY DAY CAN ALSO BE AN OPPORTUNITY TO OPTIMIZE THE PHYSIOLOGICAL IMPACT OF THE FOODS WE EAT?**

The results of the *in vitro* studies highlighted the contribution of the oral and gastric phases for the digestion of starch due to the action of salivary  $\alpha$ -amylase, especially in foods associated with elevated postprandial responses, such as bread.

We have also investigated the capacity of a number of food products to inhibit this enzyme. Tea and lemon juice exhibited promising results. Subsequently, we have observed that simulating the consumption of bread with lemon juice vs. water was an effective strategy to slow down starch digestion *in vitro* because of a premature acid inhibition of salivary amylase. The consumption of bread with tea was also simulated but the effect on the extent of starch digestion was less obvious.

Numerous clinical studies have reported an association between lowering the pH of a starch-rich food/meal (with vinegar for example) and an attenuation of the glycaemic response. However, the underlying mechanisms are still not very clear. We hypothesized that the observations of our *in vitro* studies could explain, at least in part, this effect.

**COULD THE FOOD-PAIRING STRATEGIES TESTED *IN VITRO* (CONSUMPTION OF BREAD WITH LEMON JUICE OR WITH TEA) BE EFFECTIVE IN A REAL-LIFE SETTING? AND ARE THE RESULTS SIMILAR TO THOSE OBTAINED WITH OTHER LOW-PH FOODS?**

It has also been debated whether attenuating the glycaemic response *per se* could have an impact on appetite/satiety levels. Therefore, if the above-described strategies effectively reduce the glycaemic response,

**DOES THIS HAVE ANY IMPACT ON SATIETY OR SUBSEQUENT FOOD INTAKE?**

## ABSTRACT

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The inhibition of digestive enzymes that participate in starch digestion could constitute an opportunity to slow down the release of glucose from starch and improve the glycaemic impact of starch-rich foods. Simple dietary approaches consisting in pairing these foods with beverages that have the capacity to inhibit such enzymes could be an easily implementable strategy.

The objective of this work was to test the impact of adding black tea or lemon juice to a starch-rich meal on postprandial plasma glucose and energy intake in healthy humans.

A randomized crossover study was conducted with equal portions of bread (100 g) and 250 mL of either water, black tea or lemon juice. These meals were consumed as breakfast after a 10-h fast in a randomised order. Capillary blood glucose concentrations were self-monitored using the finger-prick method at pre-defined time-points during 180 min. *Ad libitum* energy intake was assessed 3 hours later.

The glycaemic response was similar with the water and tea meals, but was reduced in the presence of lemon juice. Both the  $AUC_{0-90min}$  and the peak blood glucose concentration were significantly lowered by about 30% ( $P < 0.05$ ) with lemon juice compared to water. The peak blood glucose concentration was also reached significantly later (78 vs. 41 min,  $P < 0.01$ ) with lemon juice.

Our results confirm previously reported *in vitro* findings showing that lowering the pH of a starch-rich meal can attenuate the glycaemic response. This could be a simple and effective strategy to reduce the glycaemic response to starch-rich foods in everyday-life meals.

## 1. INTRODUCTION

High glycaemic index diets have been correlated with increased risk of type-2 diabetes, coronary heart disease and some types of cancer (Bhupathiraju, et al., 2014; Mirrahimi, et al., 2012; Mullie, Koechlin, Boniol, Autier, & Boyle, 2016). Starch-rich foods supply up to 50 % of our energy intake (Stylianopoulos, 2012), and therefore play a major role in the post-prandial glycaemic responses elicited by our diets. Actually, the glycaemic response to a certain food can even be predicted from *in vitro* experiments aiming at determining the rate of starch hydrolysis by pancreatic amylase (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Yvonne Granfeldt, Hagander, & Björck, 1995; D. J. A. Jenkins, et al., 1982), and extensive work has thus been dedicated to identify ways of slowing down starch digestion. Inhibiting the digestive enzymes that participate in starch hydrolysis is a strategy that has been considered effective for the treatment of type 2 diabetes, via the administration of acarbose to inhibit amylolytic enzymes (Salvatore & Giugliano, 1996). A simple dietary strategy to achieve a lower glycaemic response could therefore consist in pairing starch-rich foods, or meals, with food products susceptible to exert similar inhibitory effects.

Using *in vitro* enzymatic assays, we have investigated the inhibitory capacity of different beverages and condiments on the activity of salivary and pancreatic  $\alpha$ -amylases (Freitas & Le Feunteun, unpublished), which both play a major role during the digestive process of starch. As previously reported by others (Y. Hara & Honda, 1990; Kashket & Paolino, 1988; Kwon, Apostolidis, & Shetty, 2008; Quesille-Villalobos, Torrico, & Ranilla, 2013; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015), tested products that have a high polyphenol content such as tea, red wine, and were found to inhibit the amylolytic activities of both saliva and pancreatin. The inhibition was in the range of 30 to 80% in our experimental conditions. Moreover, because salivary  $\alpha$ -amylase is completely inactivated below pH 3.5 (Freitas, Le Feunteun, Panouillé, & Souchon, 2018; Fried, Abramson, & Meyer, 1987), the acidic products we tested (vinegars, wines, lemon juice) prevented its action almost completely. Dynamic *in vitro* digestions aiming at evaluating the influence of pairing a bread meal with water, lemon juice and a black tea on starch hydrolysis kinetics were also conducted (Freitas & Le Feunteun, unpublished). In comparison with water, black tea showed no clear effects on starch hydrolysis by HSA during gastric digestion. The quantity of released oligosaccharides was slightly reduced (by  $\sim$  20%), however, during intestinal digestion. The lemon juice drink showed, as expected, a drastic impact during the gastric phase of digestion as the premature acidification of the gastric content resulted in a total inactivation of HSA. The effect of lemon juice during the intestinal

phase of digestion was more subtle, but also led to a slightly reduced amount of released oligosaccharides, as observed with black tea.

The objective of the present work was to determine the effect of consuming a starch-rich food with black tea or lemon juice on postprandial plasma glucose, and on the energy intake in healthy humans, in order to evaluate the potential of the above described strategy in a semi-controlled setting.

Regarding the inhibitory effect of tea on digestive enzymes, and despite encouraging epidemiologic findings and *in vitro* data, two studies have reported that green tea was ineffective in reducing the glycaemic response associated to bread based meals (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010; Sapper, et al., 2016). Nevertheless, our *in vitro* results suggested black teas have a higher capacity to inhibit both salivary and pancreatic  $\alpha$ -amylases, as others before us (Quesille-Villalobos, Torrico, & Ranilla, 2013; Zhang & Kashket, 1998). To the best of our knowledge, the effect of lemon juice has not yet been investigated in humans. There are numerous reports that the postprandial glycaemic response to starch-rich meals can be reduced by 20-50% via an acid-related mechanism, as for example through vinegar supplementation, acid bread fermentation or combination with pickled foods (Bo, et al., 2017; Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Kim, & Buller, 2004; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Lappi, et al., 2010; Leeman, Östman, & Björck, 2005; Liatis, et al., 2010; H. Liljeberg & Björck, 1996, 1998; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008; Mitrou, et al., 2010; Elin Östman, Granfeldt, Persson, & Björck, 2005; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009; Sugiyama, Tang, Wakaki, & Koyama, 2003). In our view, a premature acid inhibition of HSA during gastric digestion is a good candidate mechanism (Freitas & Le Feunteun, 2018a, unpublished) but the debate on the underlying causes remains open (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014; Poutanen, Flander, & Katina, 2009).

Another potentially beneficial effect of slowing down starch digestion is a prolonged satiety, and thus a reduced energy intake during the next meal. Research in this field has not been conclusive (Blaak, et al., 2012), and interestingly, reviews of the literature conducted at the same time have reached contrary conclusions (Pawlak, Ebbeling, & Ludwig, 2002; Raben, 2002). It is possible that it is difficult to conceive test meals that elicit distinct glycaemic responses and are controlled for all the possible factors that may affect satiety. These results are, therefore, often confounded by a lack of control of all the variables (Thomas MS Wolever, Leung, Vuksan, & Jenkins, 2009). If adding tea or lemon juice to a meal without changing its macronutrient or ingredient content

effectively slows down starch digestion *in vivo*, it should also be possible to provide some answers to this matter.

## 2. SUBJECTS AND METHODS

### 2.1. PARTICIPANTS

Eighteen volunteers (7 men and 11 women), participated in the study. The number of subjects was determined by using power calculations on the basis of a previous work (Arvaniti, Richard, & Tremblay, 2000). We calculated that  $n = 15$  would allow us to detect a minimum difference of 260 kJ in energy intake with  $\alpha = 0.05$  and  $\beta = 0.1$  and assuming  $SD = 200$  kJ. A total of 18 participants were recruited, what allowed for a dropout rate close to 20%.

They were recruited through email and poster advertisements. Interested subjects were asked to answer an online pre-screening questionnaire for preliminary assessment of compliance of the following criteria: age, BMI, gender, weight stability, eating habits, medication, food allergies, smoking history, and previous participation in a clinical study < 6 m. Respondents whose answers were in compliance with the eligibility criteria were invited to an information meeting where they received oral and written information about the study. Individuals who attended this meeting and expressed interest in trial participation were scheduled for a medical appointment, at a later date. During the medical appointment they could clarify any eventual questions arising from the information session and sign the consent form. Their physical health and eligibility were confirmed according to the pre-defined inclusion and exclusion criteria, and they received training on self-measurement of blood glucose (SMBG) (Table 10).

Subjects were not aware of the real objectives of the study but were informed that we intended to better understand the digestion of starch-rich meals when ingested in combination with commonly consumed beverages.

The study protocol has been approved by the Ethics Committee Lyon Sud-Est IV, and the study has been registered in the Clinical Trial Registry ([clinicaltrials.gov](https://clinicaltrials.gov); NCT03265392). All volunteers gave their written informed consent after being provided with oral and written information about the aims and protocol of the study.

This study was one part of a 2-branch investigation. The other part of the study was conducted with the same test meals but with different participants and different measurements were performed. The participant characteristics, study details and results presented in this publication are for this part of the study only.

Table 10 –Selection criteria

<p><b>Inclusion Criteria:</b></p> <ul style="list-style-type: none"><li>• To be overall healthy</li><li>• To be between 18 and 60 years old</li><li>• BMI between 18 and 25 (lean)</li><li>• No eating disorders</li><li>• No major weight changes (&gt; 3kg in previous 3 months)</li><li>• Structured eating habits (3 main meals/day, everyday)</li><li>• Not using corticoid drugs, or antidepressives known to influence food consumption, appetite and/or mood.</li><li>• Not using medication known to influence glucose tolerance: acetylsalicylic acid or thyroxin, vitamin or mineral-based dietary supplements, and drugs to control hypertension or osteoporosis are acceptable</li><li>• No allergies to the foods in the test-meals and ad libitum meal</li><li>• No abusive alcohol consumption</li><li>• No intensive exercising habits</li><li>• No participation in a clinical study within the previous 6 months</li><li>• To enjoy the foods in the test-meals and ad libitum meals</li><li>• To be affiliated to a social security system</li><li>• To be available to participate in study sessions</li><li>• To have read and signed the Informed consent form</li></ul>
<p><b>Non-inclusion criteria:</b></p> <ul style="list-style-type: none"><li>• History of diabetes, use of antihyperglycaemic drugs or insulin to treat diabetes or associated conditions</li><li>• Major medical interventions or surgeries requiring hospitalization within the previous 3 months</li><li>• Any medical condition that affects digestion and/or nutrient absorption</li><li>• Use of steroids, protease inhibitors and/or antipsychotic drugs</li><li>• Use of anticoagulation drugs</li><li>• To be participating in another clinical trial, or to be within the exclusion period of a previous clinical trial</li><li>• Having started or stopped smoking within the previous 3 months</li></ul>

## 2.2. STUDY OUTLINE

The study compared the effects of 3 different meals, each of which consisting of sandwich bread paired with a different beverage, on blood glucose and energy intake. Test meals were consumed at breakfast and the participants were free to pursue their usual routines during consumption of the test meal and glucose monitoring.

## 2.3. TEST MEALS

Three test meals were studied: Water, Tea and Lemon juice. Each test meal consisted of 4 slices (100 g) of crustless, wheat sandwich bread (Harry's 100% mie Nature, Barilla S.A.S, Boulogne-Billancourt, France) with 250 mL of either spring water (Mont Roucous, Lacaune-Les-Bains, France), tea or lemon juice. Tea (Lipton Yellow label black tea, Unilever France, Rueil Malmaison, France) was freshly brewed (1% w/v) with spring water (100 °C) and was infused for 15 min before the tea bag was removed. Lemon juice was prepared by mixing 125 mL of commercial lemon juice (Lazy lemon, Polenghi, Milan, Italy) with 125 mL of spring water. The energy and nutrient content of the meals (estimated from the products' labels) is presented in Table 11.

Table 11 - Energy and nutrient content of the meals. Each meal contained equal amounts of white-wheat-bread and 250 mL of either water, tea or lemon juice.

	Water	Tea	Lemon juice
Energy content (kcal)	262.0	262.0	295.8
Energy density (kcal/g)	0.7	0.7	0.8
Energy content (kJ)	1096	1096	1237
Total fat (g)	3.9	3.9	3.9
Total carbohydrate (g)	48.3	48.3	50.8
Sugar (g)	6.0	6.0	8.5
Fibre (g)	2.0	2.0	2.0
Total protein (g)	7.5	7.5	8.0

## 2.4. STUDY DESIGN

A randomized crossover design was used. All subjects consumed were then studied on the 3 test meals for breakfast in a random sequence on 3 different days.

It is known that insulin sensitivity (Pulido & Salazar, 1999), appetite control and eating behaviour (Dye & Blundell, 1997) can vary during the menstrual cycle in healthy women. Additionally, variations in stress levels of both men and women can influence the glucose metabolism (Wing, Blair, Epstein, & McDermott, 1990) and the production of Human salivary  $\alpha$ -amylase (Nater, et al., 2006; Takai, et al., 2004). To try to minimize the biases potentially induced by all these factors, the 3 study sessions of each participant were all scheduled within 4 consecutive days in the same week.

The study visits took place at the Sensory Analysis Department of our research unit (UMR GMPPA, AgroParisTech, INRA, Université Paris-Saclay, 78850, Thiverval- Grignon, France).

## 2.5. PROTOCOL OF THE STUDY SESSIONS

An overview of the study protocol is provided in Figure 57. Participants were asked to refrain from excessive alcohol consumption and any unusual exercise and activity the night before the study sessions. They were instructed to eat their last meal at least 10 h before the study session and to refrain from ingesting anything but water until breakfast. All subjects were provided with a check-list to verify compliance with pre-session requirements, a capillary blood glucose monitoring kit (more details in the “Blood glucose” part of the Measurements section below) and breakfast. They were asked to start answering the check-list and make two baseline blood glucose measurements between 0900 and 0915. Breakfast was to be consumed at a steady pace, within 12 to 15 min, between 0915 and 0930. Subjects were then asked to measure blood glucose again 15, 30, 45, 60, 90, 120, 150 and 180 min after the breakfast starting time. Participants were free to choose the location where breakfast was consumed (they were given the option to consume it at the study site if they desired). They were free to maintain their usual

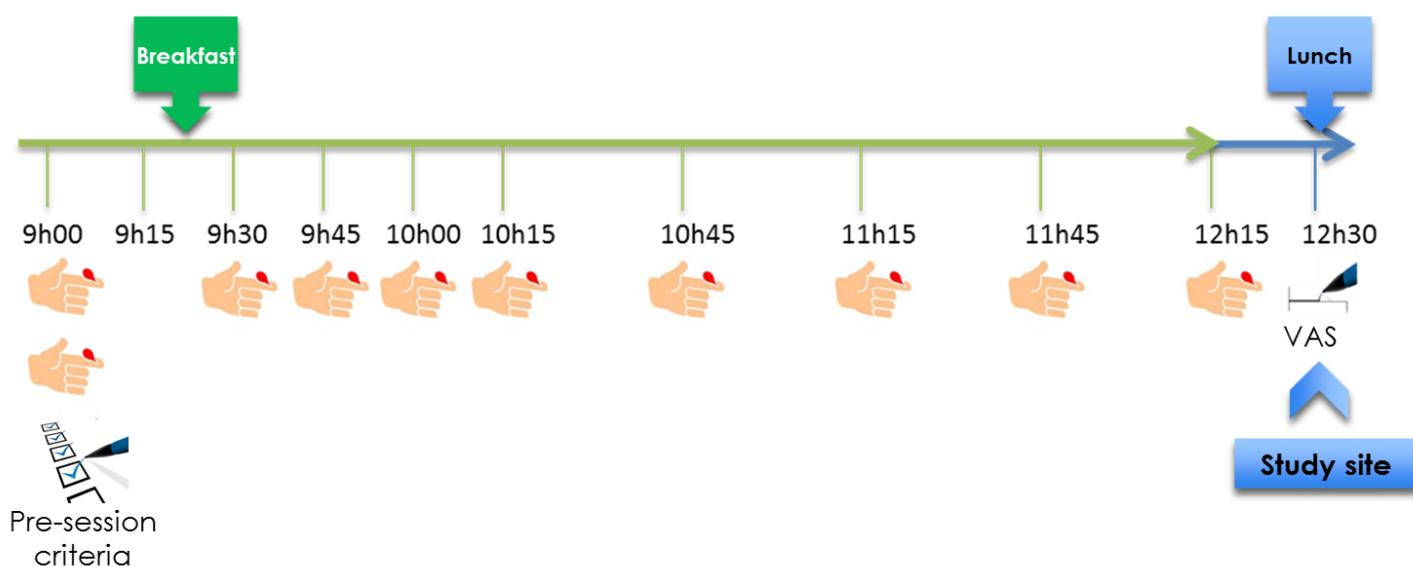


Figure 57- Overview of the study protocol.

routines in the mornings of the study, but were asked not to consume anything between the breakfast and the *ad libitum* lunch, 3 h after the test-meal (1230). Subjects arrived at the study site (sensory Analysis Department of UMR GMPA) between 1215 and 1230 and were served an *ad libitum* meal consisting of a rice salad and spring water.

## 2.6. MEASUREMENTS

### BLOOD GLUCOSE

Each participant self-monitored their capillary blood glucose with the finger prick method, using a capillary blood glucose monitoring kit provided by the research team. The kit was composed of single-use Accu-Chek Safe-T-Pro Plus lancets (Roche Diabetes Care France, Meylan, France), a glucose meter (Accu-Chek Performa Blood glucose Meter, Roche Diabetes Care France, Meylan, France) and corresponding test strips (Accu-Chek Performa test strips, Roche Diabetes Care France, Meylan, France), a paper form to register the measurements times and values and instructions. Sterile compresses and antibacterial spray (Apaisyl Asept, Merck Médication Familiale, Lyon, France) were also provided for cleaning the skin after each measurement.

Capillary blood glucose was measured with the Accu-Chek Performa glucose meter, corresponded to plasma concentration.

Participants performed two repeated measurements at baseline, and then single measurements 15, 30, 45, 60, 90, 120, 150 and 180 min after the start of the test meal. They reported the meal consumption time and the blood glucose concentrations and measurement times in the standard form provided with the glucose measuring kit. Blood glucose concentrations and measurement times were later verified in the memory of the glucose meter.

Time-response curves were constructed and the areas under the glucose response curves (AUC) (mmol · min/L) were calculated using the trapezoidal rule with fasting values as baseline and truncated at zero. Negative areas (below the fasting baseline value) were ignored. Peak values (mmol/L) and time-to-peak (min) were also determined.

### AD LIBITUM MEALS AND ENERGY INTAKE

The *ad libitum* meal consisted of a store-bought ready to eat rice salad with tuna and vegetables (Riz à la Provençale au thon & basilic, Bonduelle, Villeneuve-d'Ascq, France) and a 250 mL bottle of spring water (Mont Roucous, Lacaune-Les-Bains, France). According to the label, 100 g of the *ad libitum* meal provided 132 kcal (553 kJ), 4.5 g of fat, 18 g of carbohydrates (1.5 g of sugars) and 4 g of protein. Participants were provided with one single serving of the *ad libitum* meal

weighing 1200 g, and were instructed to drink all the water served and eat until they were comfortably full. In order to prevent social influences that could affect food intake, the *ad libitum* meal was consumed in individual sensory cabins and all participants were instructed to stay in the room for a minimum of 30 min, even if they had already finished the meal.

The amount eaten (g) was determined by weighing the *ad libitum* meal before and after consumption. The nutritional information provided by the manufacturer was used as reference to calculate the energy (kJ) consumed.

### **PALATABILITY OF THE *AD LIBITUM* MEAL**

Palatability ratings (pleasantness, visual appeal, smell, taste and aftertaste) were obtained for the *ad libitum* meal immediately after consumption. Validated VAS questionnaires (Flint, Raben, Blundell, & Astrup, 2000) consisting of 100 mm visual lines with descriptors anchored at each end describing the extremes (e.g. “How would you rate the smell of this meal? Not good at all/ Extremely good”) were used. Participants rated each characteristic by placing a mark across each line on paper, and participants were not able to refer to their previous ratings when completing the VAS. Analysis of the VAS was conducted by measuring the horizontal distance from the left-hand end of the line to the mark indicated by the participant.

#### **2.7. STATISTICAL ANALYSIS**

All statistical analyses were conducted using the Statistics Toolbox of Excel. Single factor ANOVAs were performed to evaluate the following effects:

- test meals × blood glucose concentration at each time point
- test meals × energy intake
- test meals × palatability ratings
- study day × palatability ratings
- study day × energy intake

Whenever significant effects were detected, paired sample t-test comparison was performed to identify the test meals that elicited the significantly different values. Statistically significant effects were accepted at the 95% level. Data are presented as means ± SEMs unless otherwise specified.

## **3. RESULTS**

### **3.1. PARTICIPANTS' CHARACTERISTICS**

Participants' mean age was 32.8 ± 10.4 (range 20-55). All of them were healthy, with no history of diabetes and no medical condition or use of medications known to affect energy intake,

appetite, gastrointestinal function or blood glucose, as confirmed by means of a full medical history and examination. All subjects had normal body mass indexes ( $23.0 \pm 1.8 \text{ kg/m}^2$ ;  $\bar{x} \pm \text{SD}$ ) and normal fasting glucose ( $5.0 \pm 0.06 \text{ mmol/L}$ ;  $\bar{x} \pm \text{SEM}$ ).

All data from one participant have been excluded due to failure to comply with the pre-session requirements. Another participant failed to attend one of the *ad libitum* meals, therefore the corresponding data (palatability ratings and energy intake) was excluded from the *ad libitum* meal associated results and comparative analyses.

### 3.2. POSTPRANDIAL BLOOD GLUCOSE RESPONSES

Postprandial blood glucose responses elicited by the water, tea and lemon juice meals are presented in Figure 58. Data refers to 17 participants. There was no significant difference in baseline blood glucose concentrations between the three test-meals ( $P = 0.81$ ). Consumption of bread with lemon juice elicited a lower response than when the same quantity was consumed with water or tea, with statistically significant differences from  $t = 15$  to  $t = 45$  min (Figure 58). No significant differences were found between water and tea.

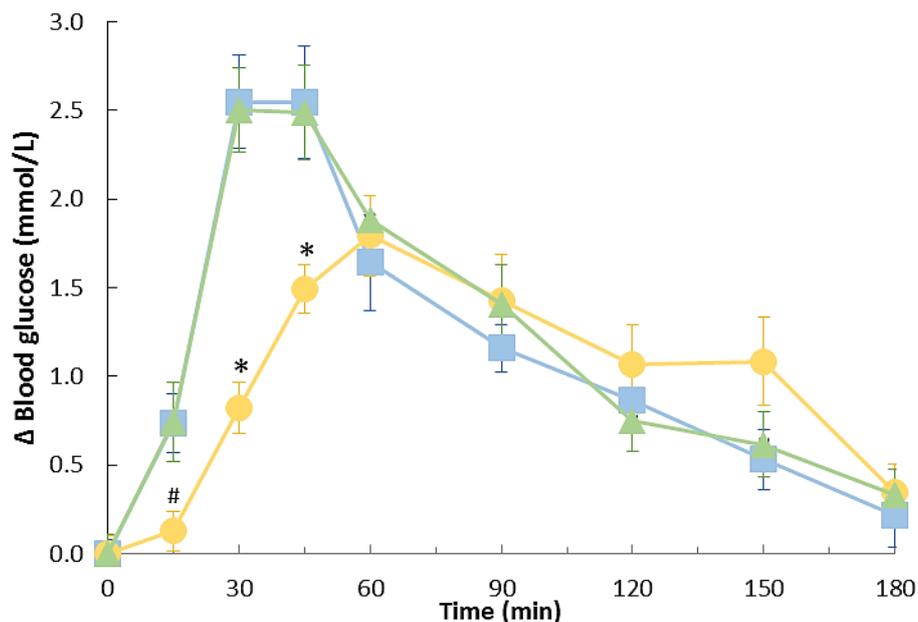


Figure 58 – Postprandial blood glucose responses. Mean ( $\pm$ SEM) incremental changes ( $\Delta$ ) in glucose concentrations in response to equal amounts of carbohydrate from a white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●). Time points at which significant differences were found (ANOVA followed by paired t-test) are identified by # and \* symbols. # indicates a statistically significant difference between lemon juice and water ( $P < 0.01$ ,  $P = 0.051$  between lemon juice and tea). \* indicates statistically significant differences between lemon juice and both water and tea at 30 min ( $P < 0.0001$  and  $0.001$ ) and at 45 min ( $P = 0.014$  and  $0.019$ ).  $n = 17$  healthy adults

The

glycaemic index (GI), peak glucose concentrations, time-to-peak, and mean AUCs (0-90 min and

0-180 min) are presented in Table 12, together with the equivalent changes as percentage of the control experiment (W meal). Again, no differences were found for any of these parameters between the water and tea meals ( $0.66 \geq P \leq 0.96$ ). Despite no significant differences in the GI ( $P = 0.76$ ), lemon juice significantly lowered the peak blood glucose concentrations by 33 % ( $P = 0.03$ ). Moreover, it also significantly ( $P < 0.01$ ) delayed the time to reach the blood glucose concentration peak, by more than 30 min on average (78 vs. 41 min). There were no significant differences in the AUC over the 3-hour monitoring period ( $P = 0.76$ ). It can, however, be interesting to note that a comprehensive analysis of the AUCs at different time points showed that the AUC of the lemon juice meal was significantly lower during the first 90 min ( $P = 0.03$ ), suggesting a delayed, rather than incomplete absorption of glucose from the meal.

Table 12 –Glycemic Index (GI), peak blood glucose concentration, time-to peak, and incremental postprandial areas under the curve (AUC) from 0 to 90 min and 0 to 180 min, after test-meals composed of 100 g of wheat bread and 250 mL of either water, tea or lemon juice<sup>1</sup>

		Water	Tea	Lemon juice
<b>GI</b> <sup>2</sup>		100 ± 9	107 ± 12	77 ± 7
	<i>Change %</i> <sup>3</sup>	-	8	-23
<b>Peak</b>	<i>mmol/L</i>	2.7 ± 0.3	2.9 ± 0.2	1.8* ± 0.3
	<i>Change %</i> <sup>3</sup>	-	7	-33
<b>Time-to-peak</b>	<i>min</i>	41 ± 4	43 ± 3	78* ± 9
	<i>Change %</i> <sup>3</sup>	-	5	90
<b>AUC</b> <sub>0-90 min</sub>	<i>mmol · min/L</i>	145 ± 13	157 ± 18	102* ± 13
	<i>Change %</i> <sup>3</sup>	-	8	-30
<b>AUC</b> <sub>0-180 min</sub>	<i>mmol · min/L</i>	212 ± 18	226 ± 26	199 ± 31
	<i>Change %</i> <sup>3</sup>	-	8	-30

<sup>1</sup>All values are  $\bar{x} \pm \text{SEM}$ . Values within the same row identified with \* are significantly different (ANOVA followed by paired t-test) from the water meal. Peak blood glucose concentration ( $P < 0.05$ ) and time to peak ( $P < 0.01$ ) was significantly lower for the lemon juice than for the water and tea meals. AUC<sub>0-90min</sub> was significantly lower for the lemon juice meal than for the water meal ( $P < 0.05$ ).  $n = 17$  healthy adults.

<sup>2</sup> Calculated from AUC (0 to 120 min) and using the bread + water meal as a reference.

<sup>3</sup> Change as a percentage of the bread + water reference meal.

### 3.3. AD LIBITUM MEAL

An *ad libitum* meal was served 3h after the test meal to analyse whether there was any effect of the test-meals on subsequent energy intake. To rule out a potential saturation effect, due to repeated consumptions of the same meal, participants were asked to evaluate this meal by rating 5 palatability-related attributes using VAS questionnaires during each study session.

#### 3.3.1. PALATABILITY

The palatability results are presented in Figure 59. Overall, ratings of pleasantness, visual appeal, smell, taste and aftertaste over the 3 study days ( $n=16$ ) were respectively  $6.1 \pm 0.4$ ,  $7.1 \pm 0.4$ ,

$6.3 \pm 0.4$ ,  $6.2 \pm 0.5$  and  $6.1 \pm 0.5$  ( $\bar{x} \pm \text{SEM}$ ). Single factor ANOVA ( $\alpha=0.05$ ) did not reveal any significant effect of the test meal ( $0.27 \geq P \leq 0.97$ ) nor of the study day ( $0.76 \geq P \leq 0.91$ ) on palatability ratings.

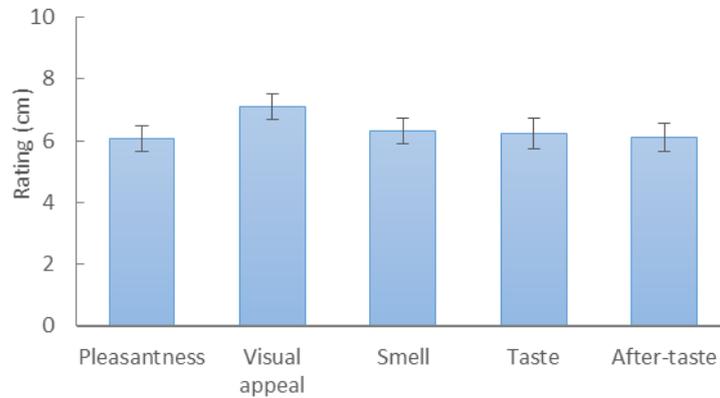


Figure 59 – Palatability of the ad libitum meal. Ratings of pleasantness, visual appeal, smell, taste and after-taste from all participants (n=16) over 3 study sessions  $\pm$  SEM.

### 3.3.2. ENERGY INTAKE

Ad libitum energy intake (kJ) following the water, tea, and lemon juice meals was  $2051 \pm 193$ ,  $1764 \pm 216$  and  $2168 \pm 182$  ( $\bar{x} \pm \text{SEM}$ ), respectively. These results are also presented in Figure 60. The effect of the meal ( $P = 0.34$ ) and of the study day ( $P = 0.70$ ) on *ad libitum* energy intake were both statistically insignificant.

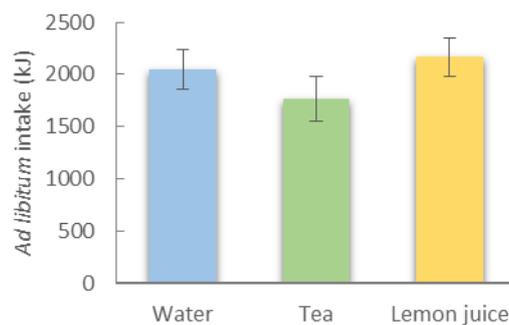


Figure 60 - Ad libitum intake. Energy intake (n=16,  $\bar{x} \pm \text{SEM}$ ) 3 hours after a test-meal composed of 100 g of wheat bread and 250 mL of either water, tea or lemon juice.

## 4. DISCUSSION

Our results show that a lemon juice drink, but not tea, can significantly lower the glycaemic response to a bread meal. The tea used in this study was previously found to inhibit over 60 % of the amylolytic activity of both human saliva and porcine pancreatin, exhibiting the strongest inhibitory capacity out of 9 commercially available black teas (Freitas & Le Feunteun,

unpublished). During dynamic oro-gastro-intestinal *in vitro* digestions of the same bread paired with the same tea, we have observed that the starch release kinetics remained similar to that of a control meal (bread with water), but that the hydrolysis of starch into oligosaccharides was reduced by about 20% during the simulated intestinal phase. In the present study, we have doubled the ratio of tea to substrate (250 mL of tea 1% w/w and 100 g of bread), but no effect could be observed on the postprandial blood glycaemia. Our present findings therefore show that our rather encouraging *in vitro* results, although of limited extent, do not transpose into measurable consequences in *in vivo* conditions. This is in line with previous reports that pairing 300 mL of green tea (3 % w/v) with a starch-rich breakfast, or incorporating green tea extract (2 % w/w) within a starch food did not exert any effect on postprandial blood glycaemia (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010; Sapper, et al., 2016). A great number of possible causes could be put forward to reconcile the *in vitro* literature on the inhibitory capacity of some teas and the lack of observable influence on *in vivo* glycaemic responses, but the most probable explanation is that one would have to consume an unreasonable quantity of tea to obtain significant effects. It has indeed been reported that the inhibitory effect of tea polyphenols and tea extracts can be lost, or may even enhance the catalytic activity of digestive amylases, below a certain polyphenol concentration (Tong, Zhu, Guo, Peng, & Zhou, 2018; Yang & Kong, 2016).

Despite a 3.5% increase in the carbohydrate quantity (Table 11), the effect of lemon juice significantly delayed and lowered the peak blood glucose concentration, with a mean reduction of the AUCs of about 30%. Based on semi-dynamic *in vitro* digestion results with realistic gastric acidification kinetics, we have previously reported that human salivary  $\alpha$ -amylase (HSA) can remain active for a long period within simulated gastric conditions, hydrolysing significant starch quantities from bread ( $\approx$  50%) or pasta ( $\approx$  15-20%) into oligosaccharides, before it is inactivated by the increasing acidity of the gastric content (Freitas & Le Feunteun, 2019; Freitas, Le Feunteun, Panouillé, & Souchon, 2018a). In our view, the contribution of HSA to starch digestion is thus more important than generally considered. The corollary is that acidic foods or meals should lower the contribution of HSA to starch digestion because of a premature acid-induced enzyme inactivation. This is indeed what we observed when studying *in vitro* digestions of starch rich foods paired with lemon juice: the hydrolysis of bread's and pasta's starch into oligosaccharides by HSA was totally prevented during the gastric phase (Freitas & Le Feunteun, 2018a, unpublished). According to our previous results and interpretation, the influence of lemon juice on the glycaemic response would therefore more likely results from its impact on the amylolytic activity of saliva at the gastric stage than from intestinal-related phenomena.

Assuming that the amylolytic activity of saliva was drastically reduced by the low pH of lemon juice (pH  $\approx$  2.3), as observed *in vitro*, the chime emptied from the stomach should contain a large quantity of non-hydrolysed starch, hence delaying the overall starch digestion and absorption process. This could provide more time for the blood glucose regulation metabolism to be effective, thereby lowering the blood glucose concentration peak and the overall glycaemic response. In other words, the reduction of about 30% we observed on the AUCs could essentially reflect the contribution of HSA to the glycaemic response to bread starch. As a matter of course, other mechanisms could be involved, in particular a delayed gastric emptying. This possibility will be investigated in a short future.

As detailed in the introduction, lowering the pH of a starchy meal (either by adding an acidic component or lowering the pH of the food items) has been proven to effectively reduce the glycaemic response in numerous clinical trials (Bo, et al., 2017; Brighenti, et al., 1995; Carol S Johnston & Buller, 2005; Carol S Johnston, Kim, & Buller, 2004; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010; Lappi, et al., 2010; Leeman, Östman, & Björck, 2005; Liatis, et al., 2010; H. Liljeberg & Björck, 1996, 1998; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008; Mitrou, et al., 2010; Elin Östman, Granfeldt, Persson, & Björck, 2005; Scazzina, Del Rio, Pellegrini, & Brighenti, 2009; Sugiyama, Tang, Wakaki, & Koyama, 2003). These studies investigated starch-rich meals in which the pH was lowered through vinegar supplementation, acid bread fermentation or combinations with pickled foods. Although none of them has tested a meal supplemented with lemon juice, the attenuation of postprandial glycaemia ranged from 20–50%, and is therefore in good agreement with the 30 % we observed. As previously discussed (Freitas & Le Feunteun, 2018a), the work of these research teams also provides important hints that support our interpretation. For example, the pH of common breads was in the range of 5.6–6, whereas that of acid breads eliciting a reduced glycaemic response was typically between 3.9 and 4.6 (Lappi, et al., 2010; H. Liljeberg & Björck, 1998; H. G. Liljeberg, Lönner, & Björck, 1995; Maioli, et al., 2008). Additionally, the effect was lost when vinegar was (1) replaced with a neutral-pH equivalent (Brighenti, et al., 1995; Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010), (2) not consumed at the same time as the starch-rich food (Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010) or (3) when the starchy food was substituted with dextrose (Carol S Johnston, Steplewska, Long, Harris, & Ryals, 2010). These cases reflect situations in which (1) HSA would not be inhibited, (2) any effect on its activity would be irrelevant due to the mismatched consumption times or (3) not applicable at all.

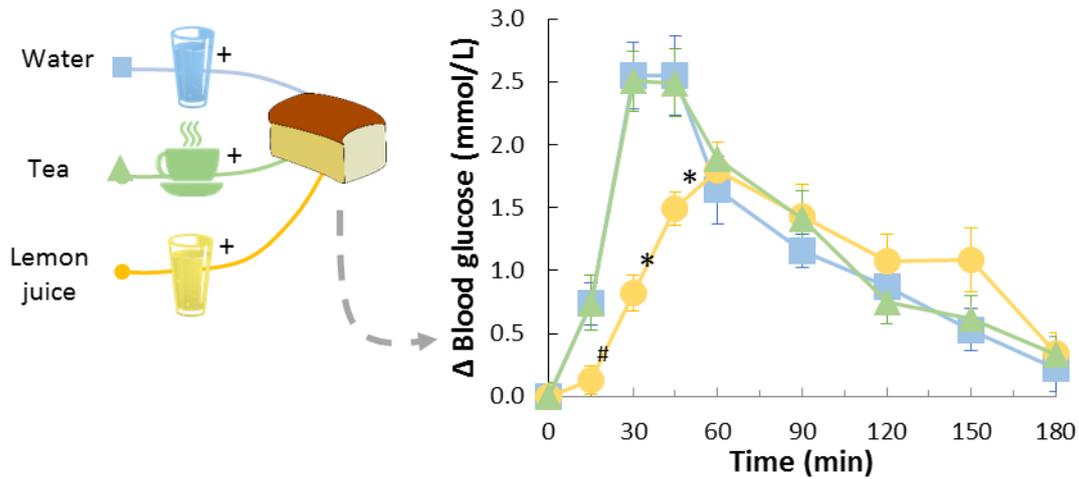
With regards to the energy intake 3 hours after the test meal, we did not observe any effect of tea or lemon juice. The aforementioned studies investigating the effect of low pH meals did not

study energy intake, but satiety perceptions were analysed through visual analogue scales in four of them. The results were inconclusive with 2 studies reporting increased satiety (Bo, et al., 2017; Elin Östman, Granfeldt, Persson, & Björck, 2005) and 2 others finding no effect (H. Liljeberg & Björck, 1996; H. G. Liljeberg, Lönner, & Björck, 1995). A possible explanation is that the extent of the decrease of the glycaemic response was not of sufficient. For instance, the European Food Safety Agency requires a GI reduction of at least 45 units for an increased satiety claim for carbohydrates with a low glycaemic index (EFSA, 2010). As previously reported, it is also possible that lowering the GI per se is not a good indicator of an increased satiety, and that the effects observed in other studies are due to other factors (Thomas MS Wolever, Leung, Vuksan, & Jenkins, 2009).

## CONCLUSION

We have investigated the blood glucose response and energy intake following the consumption of equal portions of bread with either water, tea or lemon juice at breakfast. No effect was observed on energy intake. The glycaemic response was similar between the water and tea meals, but the peak blood glucose concentration was significantly lower and delayed in the presence of lemon juice, leading to a 30% reduction of the area under the glycaemic curve. Our results confirm that lowering the pH of a starch-rich meal can attenuate the glycaemic response. According to some of our previous works, it is likely that this commonly observed phenomenon can be explained, at least partly, by an early acid-induced inhibition of the amylolytic activity of saliva. Moreover, because of the semi-controlled nature of this study, our results strongly suggest that pairing an acid drink with a starchy meal could be a simple and effective strategy to reduce the glycaemic response to starch-rich foods in everyday-life meals.

## KEY MESSAGES



- We investigated the impact of pairing bread with black tea or lemon juice on postprandial plasma glucose and energy intake in healthy humans
- The glycaemic response was similar between the water and tea meals, but was reduced in the presence of lemon juice.
- The peak blood glucose concentration was significantly lowered by about 30% ( $P < 0.05$ ) with lemon juice compared to water.
- The peak blood glucose concentration was also reached significantly later (78 vs. 41 min,  $P < 0.01$ ) with lemon juice.
- This could be a simple and effective strategy to reduce the glycaemic response to starch-rich foods in everyday-life meals.



III. *IN VIVO* STUDIES

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# CHAPTER 2

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REDUCTION OF THE GLYCAEMIC RESPONSE TO  
BREAD IN THE PRESENCE OF LEMON JUICE: MRI  
INVESTIGATIONS ON GASTRIC PROCESSING AND  
EMPTYING

*Extended article draft*

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Informal presentation

We have compared the postprandial glycaemic response to equivalent portions of bread consumed with either water, lemon juice or tea, and have observed that lemon juice significantly attenuated it. Our results were similar to those of numerous other clinical studies investigating the effect of vinegar, sourdough bread fermentation or pickled vegetables consumed with starch-rich foods. It has been reported that a delayed gastric emptying could be part of the explanation for these observations, but despite several investigations, this candidate mechanism remains controversial.

IS THE LEMON JUICE EFFECT INDUCED BY A DELAYED OR SLOWER GASTRIC EMPTYING? OR CAN IT BE MORE LIKELY EXPLAINED BY A SLOWER STARCH DIGESTION RATE WITHIN THE STOMACH?

By the way, about that question in the state of the art... Perhaps that was not the right choice of words:

HOW FASCINATING IS IT TO ~~THINK THAT WE CAN~~ ACTUALLY SEE BEYOND SIGHT?

## ABSTRACT

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We have observed that lemon juice significantly attenuates the glycaemic response to bread. Our results are in line with numerous clinical studies reporting that the postprandial glycaemic response to starch-rich meals can be reduced by 20-50% by lowering the pH of a meal (through supplementation with vinegar, acid bread fermentation or combination with pickled foods). Despite extensive reviews on this subject, the underlying mechanism remains to be clarified. Based on our previous *in vitro* findings, we hypothesized that such effects could, at least in part, be due to a premature acid-induced inhibition of salivary amylase. However, a potential delay of gastric emptying could also be part of the explanation.

The main objective of the present work was therefore to investigate whether the gastric emptying patterns remain, or not, the same when a bread meal is paired with water, tea, and lemon juice.

Three meals, containing portions of bread and 250 mL of either water, tea or lemon juice were included in this study. All meals were supplemented with 20 peas, which the subjects were asked to swallow at the end (with half of the beverage provided) without chewing. We have studied the gastric phase of digestion by MRI. To ensure comparability with previous findings we have also included measurements of blood glucose concentrations and assessed satiety perceptions.

Overall, the blood glucose measurements were comparable to those in our previous study, and no effect was observed on satiety perceptions. The meals containing water and tea produced similar results for all the studied parameters. The postprandial volume of gastric contents 30 min after the start of the meal was about 50% higher when bread was consumed with lemon juice compared with the water meal ( $454.0 \pm 18.6$  vs.  $298.4 \pm 19.5$  mL,  $\bar{x} \pm \text{SEM}$ ). The overall gastric emptying rate was also about 50% faster for the lemon juice meal ( $1.8 \pm 0.2$  for water vs.  $2.7 \pm 0.1$  mL/min for lemon juice,  $\bar{x} \pm \text{SEM}$ ). Both of these differences were statistically significant with  $P < 0.00001$  and  $P < 0.01$ , respectively.

The reduction of the glycaemic response when the pH of a meal is lowered is more likely to derive from an interruption of starch hydrolysis during the gastric phase of digestion (due to premature inhibition of salivary  $\alpha$ -amylase) than from a lower gastric emptying rate.

## 1. INTRODUCTION

The present study corresponds to the second part of a 2-branch clinical study. In the first part of this work, we have investigated the impact of pairing bread with water, tea or lemon juice on blood glucose response and energy intake. We observed no effect of the drink on energy intake, and the glycaemic response was similar between the water and tea meals. However, the peak blood glucose concentration was significantly lower and reached later in the presence of lemon juice, leading to a 30% reduction of the area under the glycaemic curve. Numerous studies have already reported similar findings, with reductions of the postprandial glycaemic response of 20-50% obtained by lowering the pH of starch-rich meals through vinegar supplementation, acid bread fermentation or combination with pickled foods. The literature on the underlying causes of this effect is extensive, but there is no clear consensus yet (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014; Poutanen, Flander, & Katina, 2009). As gastric emptying rates influence the appearance of nutrients within the blood stream, a reduced and/or delayed gastric emptying with acidic meals has been identified as a good candidate explanation (H. Liljeberg & Björck, 1998). However, other researchers who investigated this possibility, reached the conclusion that the effect of native vinegar on blood glucose is to be explained by a mechanism related to acidity but not to gastric emptying (Brighenti, et al., 1995). In our previous works (Freitas & Le Feunteun, 2018a, unpublished), we brought light to the idea that such effects could, at least in part, be due to a premature acid-induced inhibition of salivary amylase. Using magnetic resonance imaging (MRI), the main objective of the present work was therefore to determine whether the gastric emptying patterns remain the same, or not, when a bread meal is paired with water, tea, and lemon juice. The blood glucose response and satiety perceptions were also measured at different intervals to confirm that previously observed effects were preserved during the MRI investigations.

## 2. SUBJECTS AND METHODS

### 2.1. PARTICIPANTS

Ten male volunteers, participated in the study. The number of subjects was determined by using power calculations on the basis of previous work (Deane, et al., 2010; Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999; Kwiatek, et al., 2009; Marciani, Gowland, Spiller, et al., 2001; Marciani, et al., 2013). We calculated that a sample size of 7 participants would allow us to detect a minimum difference of 15 min in the half-gastric emptying time with  $\alpha = 0.05$  and  $\beta = 0.10$ , assuming a standard deviation (SD) of 7 min. We increased the sample size to 10 to allow for a 25% dropout rate.

Because insulin sensitivity (Pulido & Salazar, 1999), appetite control and eating behaviour (Dye & Blundell, 1997) can vary during the menstrual cycle in healthy women, only men were recruited. Recruitment was initiated through email and poster advertisements. Interested subjects were asked to answer an online pre-screening questionnaire for preliminary assessment of compliance of the following criteria: age, body mass index (BMI), gender, weight stability, eating habits, medication, food allergies, smoking history, and previous participation in a clinical study < 6 months. Respondents whose answers were in compliance with the eligibility criteria were invited to an information meeting where they received oral and written information about the study. Individuals who attended this meeting and expressed interest in trial participation were scheduled for a medical appointment, at a later date. During the medical appointment they could clarify any eventual questions arising from the information session and sign the consent form, and their physical health and eligibility were confirmed according to the pre-defined inclusion and exclusion criteria (Table 13).

Subjects were not aware of the real aim of the study as this could affect the outcome of the study. They were informed that the aim was to understand better the digestion of starch-rich meals when ingested in combination with commonly consumed beverages.

The study protocol was approved by the French "Ethics Committee Lyon Sud-Est IV" and the study was registered in the Clinical Trial Registry (Clinicaltrials.gov; NCT03265392). All volunteers gave their written informed consent after being provided with oral and written information about the aims and protocol of the study.

This study was one part of a 2-branch investigation. The other part of the study was conducted with the same test meals but with different participants and different measurements were performed. The participant characteristics, study details and results presented in this publication are therefore specific to this part of the study.

Table 13 – Selection criteria

**Inclusion Criteria:**

- To be overall healthy
- To be between 18 and 60 years old
- BMI between 18 and 25 (lean)
- No eating disorders
- No major weight changes (> 3kg in previous 3 months)
- Structured eating habits (3 main meals/day, every day)
- Not using corticoid drugs, or antidepressives known to influence food consumption, appetite and/or mood.
- Not using medication known to influence glucose tolerance: acetylsalicylic acid or thyroxin, vitamin or mineral-based dietary supplements, and drugs to control hypertension or osteoporosis are acceptable
- No allergies to the foods in the test-meals and ad libitum meal
- No abusive alcohol consumption
- No intensive exercising habits
- No participation in a clinical study within the previous 6 months
- To enjoy the foods in the test-meals and ad libitum meals
- To be affiliated to a social security system
- To be available to participate in study sessions
- To have read and signed the Informed consent form
- To be a male
- Ability to be in an MRI scan without moving
- Ability to hold breath for 25s (a requirement for certain MRI exams)

**Non-inclusion criteria:**

- History of diabetes, use of antihyperglycemic drugs or insulin to treat diabetes or associated conditions
- Major medical interventions or surgeries requiring hospitalization within the previous 3 months
- Any medical condition that affects digestion and/or nutrient absorption
- Use of steroids, protease inhibitors and/or antipsychotic drugs
- Use of anticoagulation drugs
- To be participating in another clinical trial, or to be within the exclusion period of a previous clinical trial
- Having started or stopped smoking within the previous 3 months
- Any contraindications to an MRI exam: claustrophobia, tattoos, presence of ferromagnetic elements, having a pacemaker, metallic prosthesis, cochlear implants, vascular clips, insulin pumps
- Any condition or disease that might affect the results of MRI exams.

## 2.2. STUDY

The study compared the effects of 3 different meals, each of which consisting of sandwich bread paired with a different beverage, on gastric emptying, blood glucose and appetite perceptions.

### 2.2.1. TEST MEALS

Three meals were studied: Water, Tea and Lemon juice. Each test meal consisted of 4 slices (100 g) of crustless, wheat sandwich bread ("Harry's 100% mie Nature", Barilla France S.A.S, Boulogne-Billancourt, France) with two 125 mL glasses of either spring water (Mont Roucou, Lacaune-Les-Bains, France), tea or lemon juice. The water meal was the reference meal. Tea (Lipton Yellow label black tea, Unilever France, Rueil Malmaison, France) was freshly brewed (1 % w/v) with spring water (100 °C) and was infused for 15 min before the tea bag was removed. Lemon juice was prepared by mixing 125 mL of commercial lemon juice (Lazy lemon, Polenghi, Milan, Italy) with 125 mL of spring water.

All test meals were supplemented with 20 frozen peas (Petits pois extra fins surgelés, Auchan Production, Villeneuve-d'Ascq, France), which were intended to be used as tracers of the chime homogenization. The energy and nutrient content of the meals (estimated from the products' labels) is presented in Table 14.

Table 14 - Energy and nutrient content of the meals. Each meal contained equal amounts of white-wheat-bread, 20 peas and 250 mL of either water, tea or lemon juice (lemon juice).

	Water	Tea	lemon juice
<b>Energy content (kcal)</b>	265.4	265.4	299.1
<b>Energy density (kcal/g)</b>	0.75	0.75	0.84
<b>Energy content (kJ)</b>	1110	1110	1251
<b>Total fat (g)</b>	3.9	3.9	3.9
<b>Total carbohydrate (g)</b>	48.6	48.6	51.1
<b>Sugar (g)</b>	6.2	6.2	8.7
<b>Fiber (g)</b>	2.3	2.3	2.3
<b>Total protein (g)</b>	7.8	7.8	8.3

### 2.2.2. STUDY DESIGN

A randomized crossover design was used. Subjects went to a first medical appointment for examination of complete health history and overall health status, and confirm absence of MRI contraindications. All subjects participated in 3 study sessions, separated by  $\geq 2$  weeks, in which they received the 3 test meals for breakfast in a random sequence. As per their request, two volunteers had 2 sessions scheduled within a 2 week period due to availability constraints. The study visits took place at the Service Hospitalier Frédéric Joliot (Orsay, France).

2.2.3. STUDY PROTOCOL

An overview of the study protocol is provided in Figure 61. Participants were asked to refrain from excessive alcohol consumption and any unusual exercise and activity the night before the study sessions and throughout the study period. On each study day, each participant was scheduled to arrive at the study facilities at 07:30 am after a 10-h overnight fast. Compliance with pre-session requirements was verified and participants were asked to answer an MRI safety form, and a Visual Analogue Scale (VAS) to evaluate appetite perceptions. A baseline capillary blood glucose concentration measurement was performed twice and followed by a first MRI exam initiated at 08:30 am.

Volunteers were asked to eat the bread and drink 1 glass of the beverage first, and to swallow the peas at the end, without chewing, with the second glass of beverage. Meals were consumed between 08:45 am and 09:00 am, and subjects were instructed to eat at a steady pace, and finish the meal in 12 to 15 min. For 3 hours after the start of the breakfast, MRI exams, blood glucose measurements and Visual analog scales (VAS) evaluation of hunger and satiety were completed at pre-defined time-points, as presented in Figure 61.

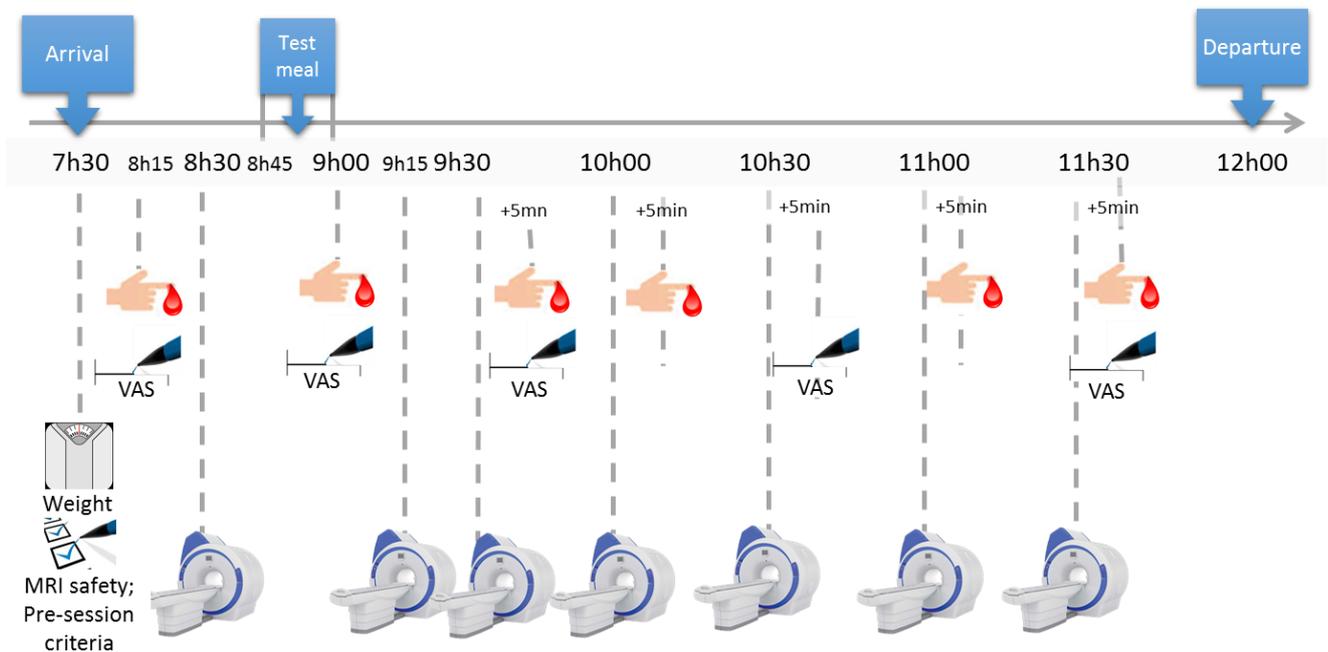


Figure 61- Overview of the study protocol.

## 2.3. DATA ACQUISITION AND MEASUREMENTS

### 2.3.1. MRI ACQUISITION

All MRI acquisitions were performed in a clinical, whole-body 1.5 T system (Achieva, Philips Healthcare, Best, The Netherlands) using a 32-channel SENSE torso/cardiac coil (Phillips Healthcare, Best, The Netherlands). Participants were also equipped with earplugs to reduce noise. A respiratory sensor (Phillips Healthcare, Best, The Netherlands) was secured under the top antenna, on the epigastric region, with a Velcro strap. This sensor, connected to a wireless transmitter (Phillips Healthcare, Best, The Netherlands), was used to monitor respiratory movements. All subjects were examined in the right decubitus position, feet-in-first, in order to minimize the differences with the seated position (Treier, et al., 2006).

Each MRI exam was composed of a series of 6 acquisitions, all covering the complete gastric region (volume scans): 1 survey, 1 anatomic scan (25 s, breath-hold), 3 motility scans (22 s, breath hold) and another motility scan (1 min 25 s, free breathing). An overview of the scans performed within each MRI exam is presented in Figure 62, and the MRI parameters applied in both types of scan are presented in Table 9.

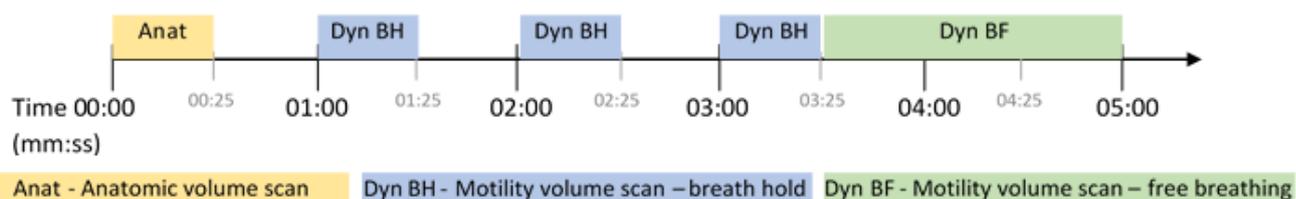


Figure 62- Magnetic resonance imaging. Sequence of the scans performed during each exam.

Table 15- Selected parameters of the MRI sequences used for imaging of the gastric region.

Imaging parameters	Anatomic volume scan	Motility volume scan – breath hold; motility volume scan – free breathing
<b>Weighing</b>	T2	T1
<b>Repetition time</b>	345 ms (single-shot 2D mode)	2.8 ms
<b>Echo time</b>	60 ms	0.89 ms
<b>Number of slices/orientation</b>	72/axial	3D
<b>Matrix</b>	192 x 148 x 1 (RL x AP x FH)	108 x 86 x 36 (RL x AP x FH)
<b>Voxel size</b>	1.7 x 1.7 x 3 mm <sup>3</sup> ≈ 8.6 mL	3.0 x 3.0 x 6.0 mm <sup>3</sup> ≈ 54 mL
<b>Scan time</b>	24.8 s	3.5 s
<b>Flip angle</b>	90 °	5 °

The first MRI exam was conducted before meal consumption, and 6 others were performed 30, 45, 75, 105, 135, and 165 min after the start of the meal (Figure 62). The volunteers exited the MRI after each exam (except between the 30 and 45 min exams) for blood glucose

measurements and/or answering a VAS to evaluate appetite perceptions, after which they had a 15-20 min break. The coils were removed from their bodies for at least 2 of the breaks, unless the participant preferred to keep them. Participants were free to stand and take short walks within the facilities of the study centre but were asked to refrain from smoking, eating, drinking or doing any activities related to food or exercise.

**ANATOMIC VOLUME SCAN – BREATH HOLD.** A set of contiguous 2D slices of the abdominal region of the subject, with a high spatial resolution, was acquired to enable a 3D volume reconstruction of the stomach. Echo-planar images were obtained using a single-shot turbo spin echo (TSE) sequence (effective echo time of 60 ms, acquisition bandwidth of 702 Hz/pixel). Parallel imaging sensitivity encoding (SENSE) with an acceleration factor of 2 and half-scanning with a factor of 0.71 in the fold-over (anterioe-posterior) direction were used to increase the slice acquisition rate. Interleaved acquisition mode was used to acquire a total of 72 axial slices covering a field of view of 320 x 253 x 216 mm<sup>3</sup> (right- left x anterior-posterior x feet-head), with no gap, within a single 25 s breath hold. The acquisition matrix size was 192 x 148 thus a native in-plane resolution (pixel size) of 1.67 x 1.71 mm<sup>2</sup> and slice thickness of 3 mm. Following interpolation, reconstructed voxel size was 0.83 x 0.83 x 3 mm<sup>3</sup>.

**MOTILITY VOLUME SCAN – BREATH HOLD.** A dynamic series of 6 consecutive 3D images of the abdominal region of the subject, with a high temporal resolution, was acquired to assess gastric motility. A multi-shot turbo field echo (TFE) fast imaging mode was used (TFE factor of 18, effective echo time of 0.89 ms, acquisition bandwidth of 500 Hz/pixel), along with SENSE acceleration factors of 2 x 2 in the anterior-posterior and feet-head directions 2 respectively. Each 3D set covered a field of view of 320 x 261 x 216 mm<sup>3</sup> (right-left x anterior-posterior x feet-head), with no gap. Each 3D image was scanned in 3.5 s, leading to a total duration of 22 s for the whole dynamic series, within a single breath hold. The matrix size was 108 x 86 x 36 and the native voxel size was of 2.96 x 3.03 x 6 mm<sup>3</sup>. Following interpolation, reconstructed voxel size was 1.4 x 1.4 x 3.0 mm<sup>3</sup>.

**MOTILITY VOLUME SCAN – FREE BREATHING.** The only difference with the Motility volume scan – breath hold, is that 24 consecutive 3D images were acquired instead of 6, leading to a total duration of 1 min and 25.3 s (free breathing).

**DATA ANALYSIS** Anatomic volume scans were used to determine the volume of the gastric contents, as illustrated in Figure 63. MATLAB (MathWorks, Massachusetts, United States) was used to manually define regions of interest (ROI) around the gastric contents in each image of a multislice set. The area of each ROI was then multiplied by the slice thickness and the results of all slices were summed to determine the total volume of gastric contents.



Figure 63 - Determination of the volume of gastric contents using anatomic volume scans.

To avoid unwanted biases during data treatment, the correspondence between the MRI exams of each study session and the experimental conditions (that is, the test meal consumed by the volunteer on that day) was established only after determination of all the gastric volumes.

Within the above described ROIs, highly contrasted zones could often be observed, with voxel intensities varying by a factor 10 or more, as illustrated in Figure 64. According to the round shape of these dark zones, and because bread is a rather dry food filled of air, these areas of low MRI signal (T2-weighted) were attributed to bread boli that were still poorly filled with water by the beverage, saliva and/or gastric secretions. Although we did not use any reference material that would have enabled us to correct the MRI signal for inter-exam or daily signal variations, we performed a preliminary analysis of these contrasted zones using a relatively homogeneous

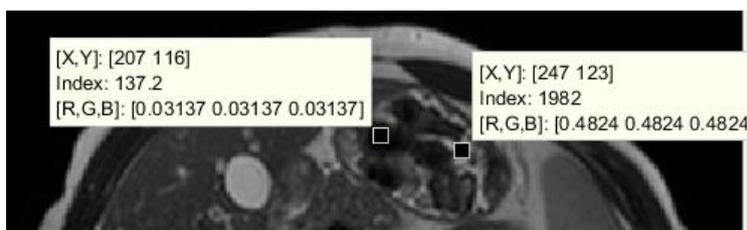


Figure 64 - Example of MRI extracted from an anatomic volume scan showing variations in pixel intensity within the stomach.

fraction of the raw MRI data. To do so, signal intensity within the ROIs was segmented into two using a threshold of 400. This threshold was defined by a visual appreciation of segmented images, and it corresponds, approximately, to the mode of the ROI's intensity histogram distributions 30 min after the start of the meal. For the sake of simplicity, the so-recovered dark and white zones will be further referred to as the solid and liquid phases of the chime. A total of 19 over the 30 study sessions were analysed because the others had abnormally low average intensities, approximately 100 times lower, we did not have time to solve this issue yet, but this will be done in the near future.

The motility volume scans acquired under breath hold can later be used to study the homogenization of the chime in more detail. For the purpose of the current work, only a qualitative description of our main observations will be provided. The motility volume scans acquired under free breathing conditions turned out to be extremely blurry. Therefore, these data have not been analysed so far.

#### 2.3.2. BLOOD GLUCOSE

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Capillary blood samples obtained by finger prick were used to measure plasma glucose concentration with a glucose meter (Accu-Chek Performa Blood glucose Meter, Roche Diabetes Care France, Meylan, France). Two consecutive measurements were performed in the fasted state to establish a baseline, and single measurements were then repeated 15, 55, 85, 145 and 180 min after the start of the test meal.

Time-response curves were constructed and the area under the curve (AUC) ( $\text{mmol} \cdot \text{min}/\text{L}$ ) was calculated using the trapezoidal rule with fasting values as baseline. Negative areas (below the fasting baseline value) were ignored. Peak values ( $\text{mmol}/\text{L}$ ) and time-to-peak (min) were also determined.

#### 2.3.3. APPETITE PERCEPTIONS

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Perceptions of hunger, satiety, fullness and prospective food consumption were quantified with validated VAS questionnaires (Flint, Raben, Blundell, & Astrup, 2000). Thirst and nausea were also assessed. VAS were composed of 100-mm lines with descriptors anchored at each end describing the extremes (e.g. "How hungry are you? Not at all/Extremely"). Participants rated each characteristic by placing a mark across each line on paper, and they were not able to refer to their previous ratings when completing the VAS. Analysis of the VAS was conducted by measuring the horizontal distance from the left-hand end of the line to the mark indicated by the participant.

#### 2.3.4. STATISTICAL ANALYSIS

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All statistical analyses were conducted using the Statistics Toolbox of Excel (Microsoft Corporation, Washington, United States). Single factor ANOVAs were performed to evaluate the effect of the test meals on the volume of gastric contents, blood glucose, and appetite perceptions at each time point. Whenever significant meal x volume of gastric contents or meal x blood glucose effects were detected, paired sample t-test comparison was performed to identify the test meals that elicited the significantly different values. Statistically significant effects were accepted at the 95% level.

### 3. RESULTS

#### 3.1. PARTICIPANTS' CHARACTERISTICS

Participants' mean age was  $26.8 \pm 6.7$  (range 18 - 47). All participants were healthy, with no history of diabetes and no medical condition or use of medications known to affect energy intake, appetite, gastrointestinal function or blood glucose, as confirmed by means of a full medical history and examination. All subjects had normal fasting glucose ( $4.9 \pm 0.1$  mmol/L;  $\bar{x} \pm$  SEM) and normal body mass indexes ( $20.7 \pm 18.3$  kg/m<sup>2</sup>;  $\bar{x} \pm$  SD).

#### 3.2. GASTRIC CONTENTS – QUALITATIVE ANALYSIS

Slice-images extracted from anatomic scans of different participants (each also a different meal) are presented in Figure 65. All gastric contents (contoured in orange) provided enough contrast without the need for contrast enhancers. Because the subjects were lying down in the right decubitus position, the chime was on their right side and the air was confined into “pockets” above, on their left side. Therefore, on the images, the chime appears on the left and the air on the right side. The contrast was similar for all meals, so that that it was not possible to distinguish the meals during the image analysis. It is however possible to discern some boli from the surrounding liquid phase of the chime. These appear darker probably because of a lower hydration level or a shorter T2. They can be distinguished individually, and either appeared relatively scattered (Figure 65A), or in the form of agglomerates (Figure 65B and C) that tended to fall upon gravity towards the bottom of the stomach (right-side of the stomach, but on left side in the images).

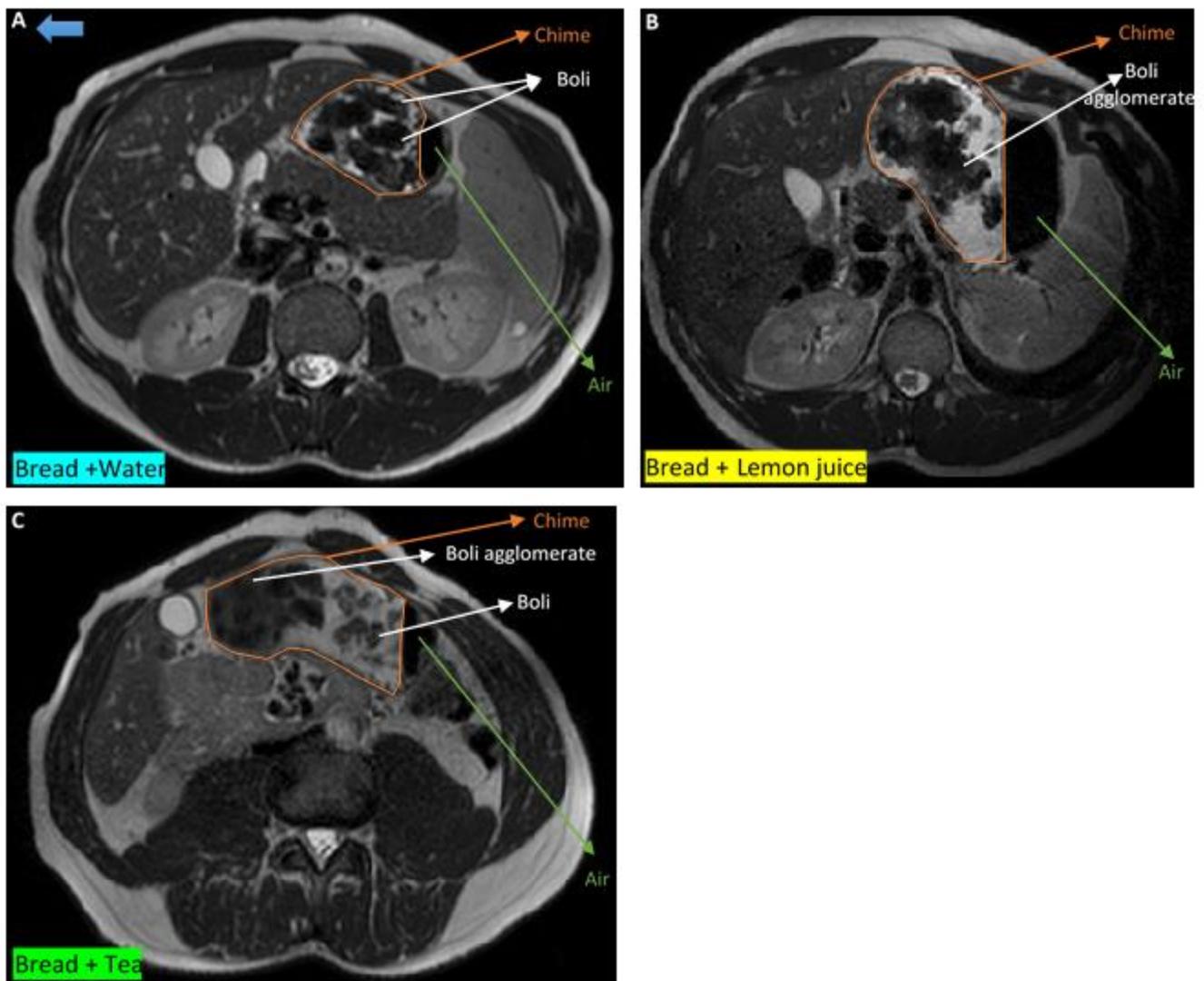


Figure 65 - Examples of magnetic resonance imaging from the abdominal region of healthy adults who ingested equal amounts of white-wheat-bread, 20 peas and 250 mL of either of water (A), lemon juice (B) or tea (C). Subjects were lying in right decubitus and the images were extracted from scans conducted a 30 to 45 min after the time the meal was initiated. The meal/chime appears left in the stomach in different levels of grey and the air appears dark at the right side. Boli are visible within the chime, they appear darker than the surrounding phase scattered or in agglomerates. The thick blue arrow on the top left indicates de direction of gravity.

By individually delineating the chime and the air within the stomach, it was also possible to obtain 3D reconstructions which are illustrative of the relative position and proportion of each component (as presented in Figure 66). As intended, air remained confined to the upper gastric regions throughout the MRI monitoring. Additionally, by following the size of the blue and red areas (corresponding to the chime and air) these reconstructions also indicate that the ratio of air to chime within the stomach increased as the digestion progressed.

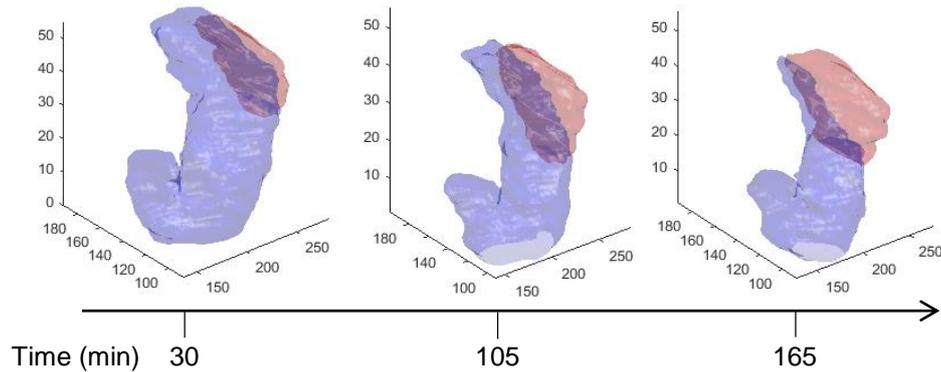


Figure 66 - Examples of a 3D reconstruction of the stomach from magnetic resonance imaging exams performed on the abdominal region of a healthy adult in the postprandial state. From left to right, images correspond to examinations conducted 30, 105 and 165 minutes after the meal started (or 15 to 20 min after it ended). Blue and red colors illustrates the position and relative volumes of chime and air, correspondingly.

### 3.2.1. VISUAL ANALYSIS OF THE CHIME

**INTRAGASTRIC CHIME DISTRIBUTION.** As it can be observed in Figure 65 and Figure 67, intragastric chime distribution was not homogeneous. Regardless of the type of meal consumed (Figure 65) two phases were distinguished: (1) a more solid phase composed of single boli or boli agglomerates and (2) a more liquid phase containing the beverages and gastric secretions and probably particles of bread. Boli agglomerates concentrated near the right-side of the stomach because of the positioning of the subjects, but single boli appeared somewhat scattered. As digestion proceeded, single boli appeared to be mostly unified into larger agglomerates that seemed to have increasingly lighter grey shades (Figure 67). By the end of the study sessions, the total volume of the stomach had been reduced and contained mostly secretions (that appear in white).

**MIXING.** Examples of the images obtained with the motility volume scans are presented in Figure 68. These scans allowed for a good contrast of the peas in the meal, which appear white within the grey chime. They also allowed to observe, in few cases, the gastric contractions, as

previously described by others (Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999). The data presented in Figure 68 is mostly aimed at qualitatively illustrating the mixing of peas, consumed at the very end of the meal, as a function of digestion time. At 30 min (i.e. 15-20 min after the meal ended), most peas seemed to be concentrated in the lower-mid region of the stomach. In most cases, they were observed in the bottom half of the stomach according to the position of the subject (in Figure 68, this is indicated at 30 and 75 min by the white arrows pointing to peas discernable on the left side and middle of the stomach). Because the peas were consumed last, one could indeed have expected to see most peas confined to the upper part of the stomach, especially at 30 min. Our data therefore show that a layering effect did not occur. Instead, it suggests that the mixing of peas with the bread chime was rather efficient. We may also notice that some peas retained their shape until the late stages of the digestive process, preserving also their contrast properties (Figure 68, 135 and 165 min).

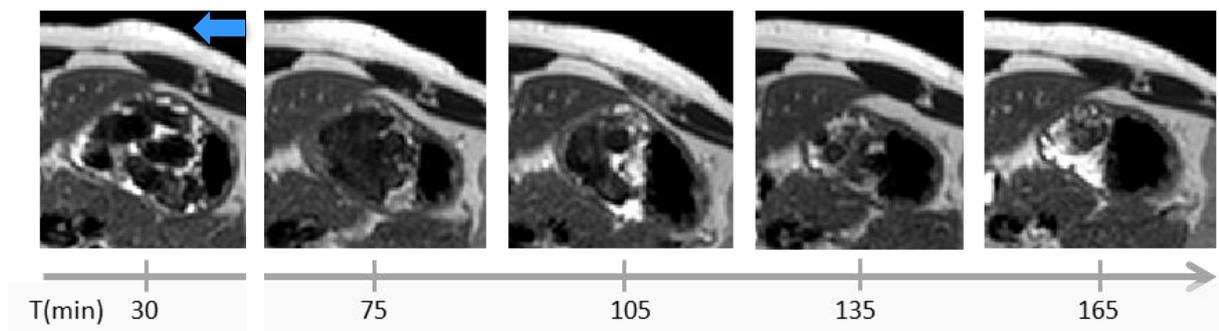


Figure 67 - Examples of magnetic resonance imaging of the gastric region of one healthy adult who ingested 100 g of white-wheat-bread, 20 peas and 250 mL of water. The images correspond to axial slices in the same anatomical region were extracted from anatomic volume scans performed 30, 45, 75, 105, 135 and 165 min after the time the meal was initiated. The chime appears left in the stomach containing visible boli or boli agglomerates that appear darker than the surrounding chime and the air appears black at the right side. Subject was lying in right decubitus. Blue arrow on the top left indicates de direction of gravity.

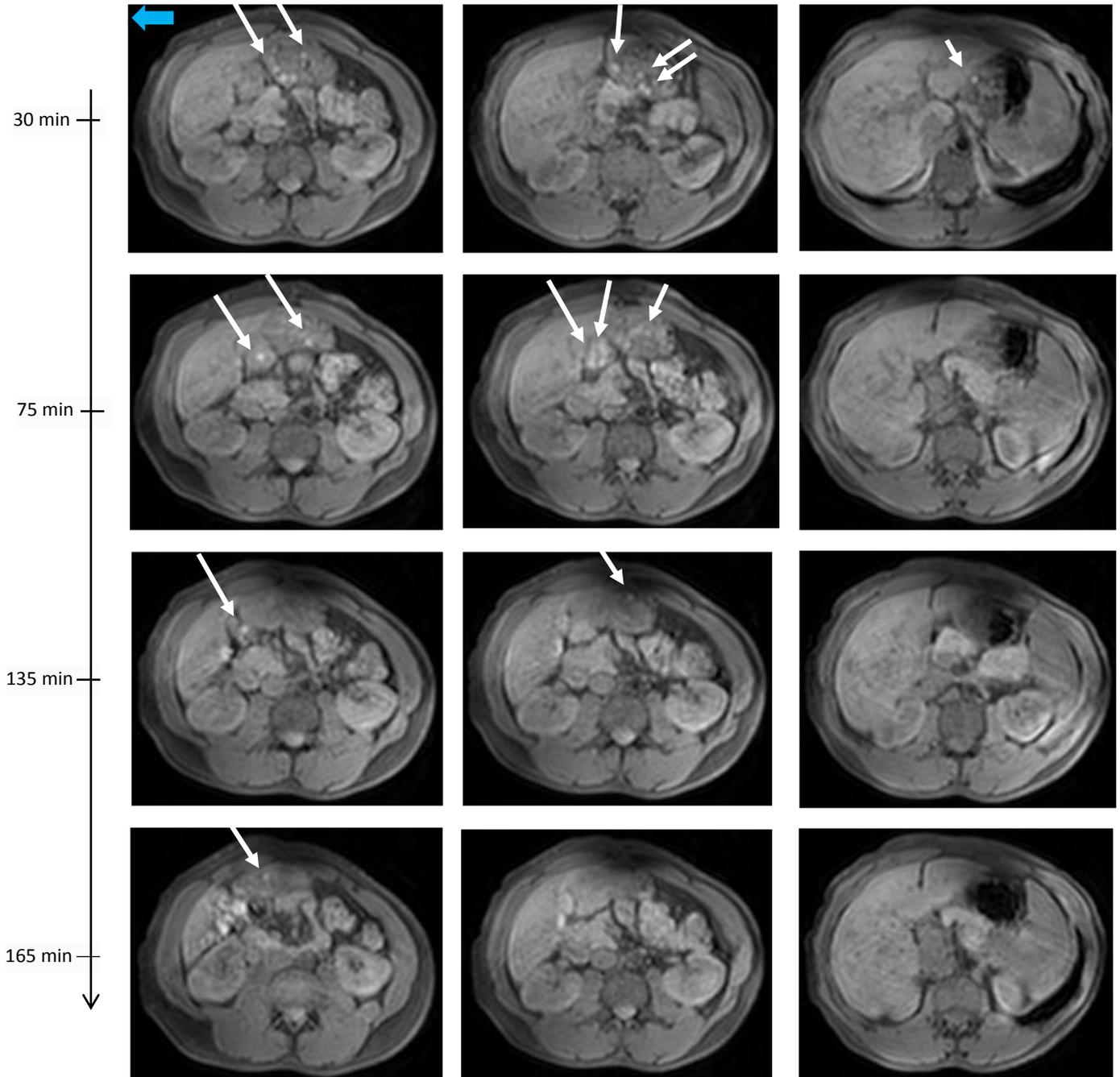


Figure 68 - Examples of magnetic resonance imaging of one healthy adult who ingested 100 g of white-wheat-bread, 20 peas and 250 mL of water. The images were extracted from motility volume scans performed 30, 75, 135 and 165 min after the meal. Slices shown on the 2 left columns correspond to the lower-mid region of the stomach, those in the right column correspond to the mid-upper part of the stomach. The chyme appears in shades of grey with white dots that correspond to peas and is visible in all slices. The air appears in black and is visible on the right side of the stomach in the slices presented on the right column. White arrows ( $\downarrow$ ) point towards regions where peas are discernible. The thick blue arrow on the top left indicates the direction of gravity.

### 3.3. GASTRIC EMPTYING AND BLOOD GLUCOSE RESPONSES

All meals were consumed in a similar amount of time. Consumption times ( $\bar{x} \pm \text{SEM}$ ) for the water, tea and lemon juice meals were  $12 \pm 1$ ,  $13 \pm 1$  and  $13 \pm 1$  min, respectively.

#### 3.3.1. QUALITATIVE ANALYSIS OF INDIVIDUAL RESULTS

The time course of the volume of gastric contents and blood glucose response for each participant is presented in Figure 69.

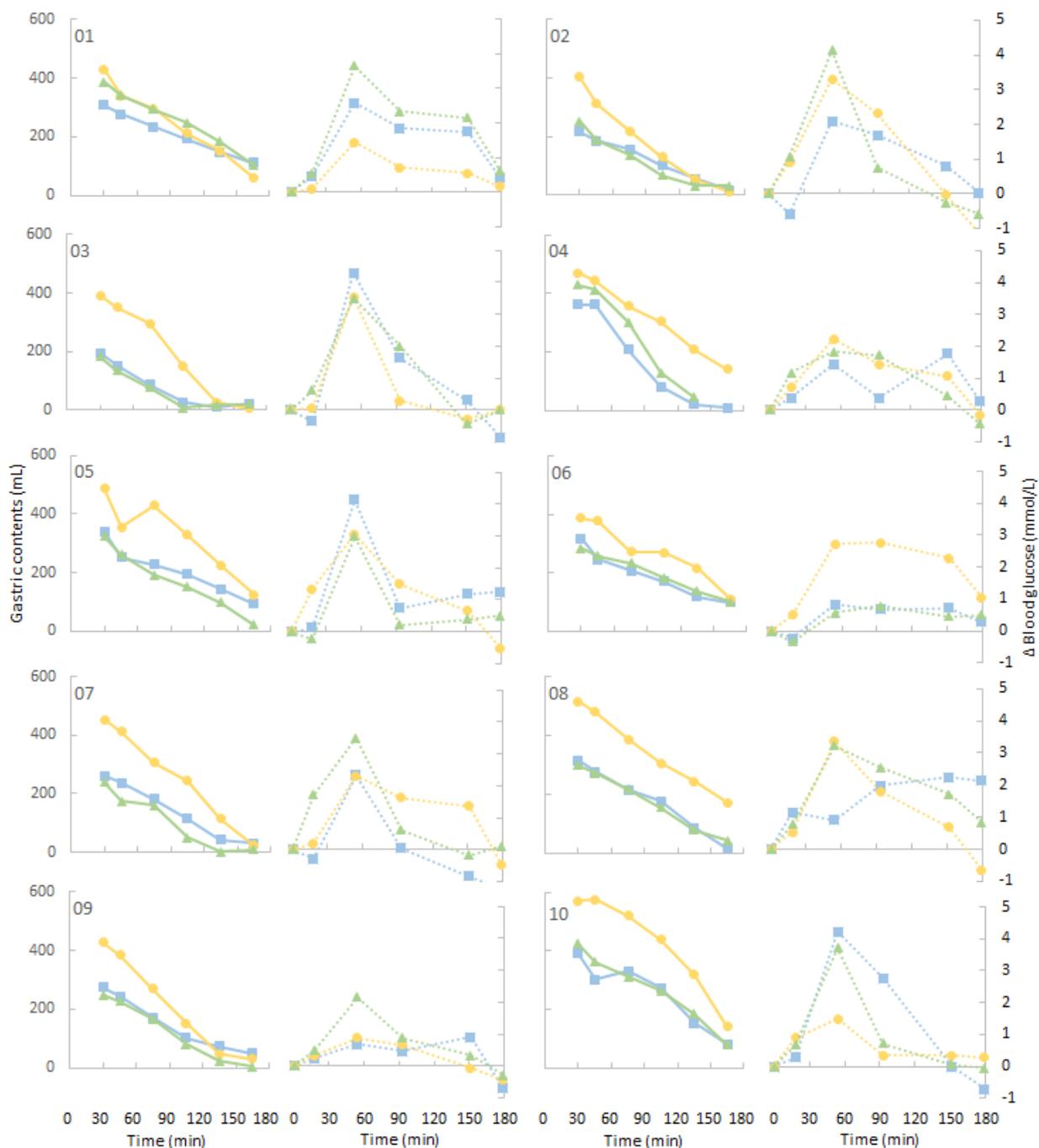


Figure 69- Case-by-case results. Variation of the volume of gastric contents (solid lines) and glucose response (dotted lines) for each participant after consuming equal amounts of starch from a white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●).

A summary of the trends observed for the lemon juice and tea meals in comparison with the water meal is presented in Figure 70. The numbers next to each symbol indicate how many subjects showed an increased (Upward arrows), similar (almost equal symbol), or decreased (downward arrows) gastric volume or glucose peak, in comparison with the water meal’s results. Interestingly, during the early postprandial phase (up to 45 min), the volume of the gastric contents following the lemon juice meal was higher than for the water meal with all subjects. As the digestion progressed, this initial difference in gastric volumes tended to vanish, with 7 participants showing gastric volumes similar to those observed with the water meal at 165 min. The evolution of the gastric volumes following the tea meal was very similar to those observed for the water meal for most subjects, as previously illustrated in Figure 69.

The highest glucose concentration was observed at approximately t= 60 min for after all meals. At the individual level, the highest glycaemia was alternatively obtained with the water (3 subjects), tea (4) or lemon juice (3) meals at this time-point (Figure 69). Thus, the consistently higher volume of gastric contents observed during the first 45 min with lemon juice did not seem to be directly correlated to these blood glucose concentrations.

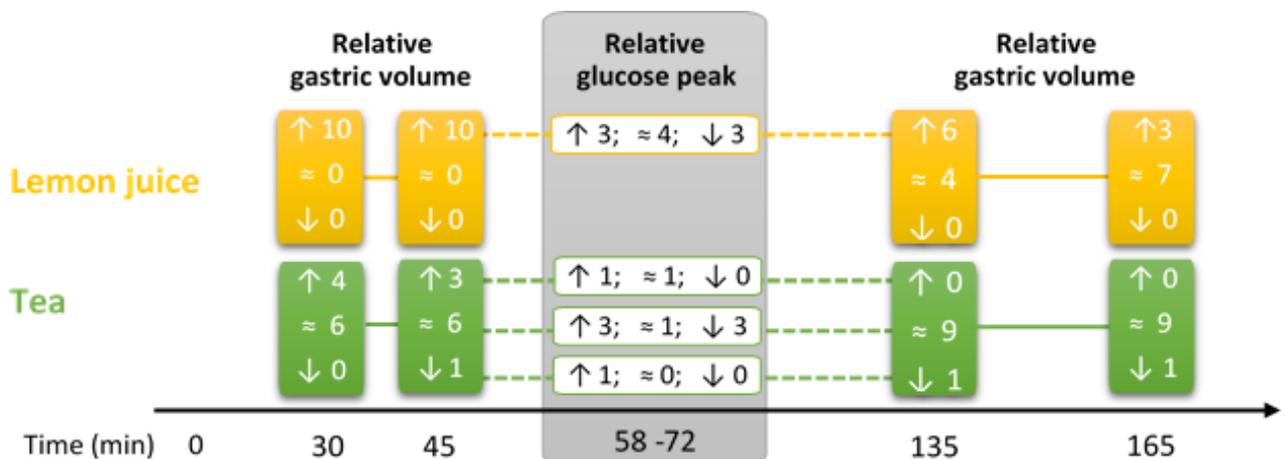


Figure 70 - Summary of the trends observed in the case-by-case analysis of the volume of gastric contents and glucose peak after consuming equal amounts of starch from a white-wheat-bread with either tea or lemon juice compared to water. “Upward arrows”, “downward arrows” and the “almost equal to” symbols indicate a higher, lower or similar result for a specific parameter (volume of gastric contents or peak blood glucose concentration) of the tea (green) or lemon juice (yellow) meals compared to the corresponding parameter for the water meal at a given time. The numbers next to each symbol indicate for how many subjects that was observed.

## 3.3.2. QUANTITATIVE ANALYSIS OF MEAN RESULTS

**VOLUMES OF THE GASTRIC CONTENT.** The volume of beverage was 250 mL. The volume of the bread serving (100g) was about 452 mL however, it is not easy to estimate the final volume that this food would have represented in the chime. About 3/4 of its weight is air, but because of its porous structure of it is compressed during mastication, and it also absorbs saliva (0.2-0.4 g /g of bread, so 20-40g in total). Globally it seems reasonable to expect its final volume to be between 100-150 mL, what would lead to a final meal volume between 350 – 400 mL. The time-course of the average gastric volume for each meal is presented in Figure 71. Overall, they were significantly higher with the L J meal until 135 min, and were not statistically different between the tea and water meals.

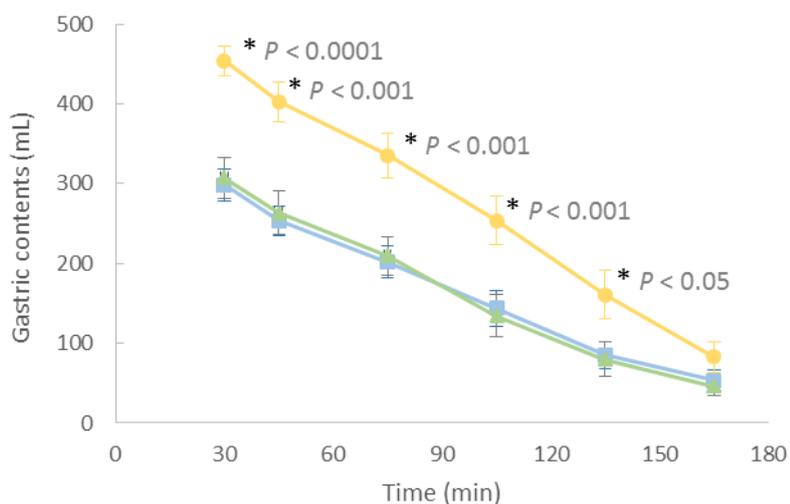


Figure 71- Postprandial volume of gastric contents. Mean ( $\pm$ SEM) volumes after consumption of three meals with equal volume containing equal amounts of starch from a white-wheat-bread and of water (■), tea (▲) or lemon juice (●). The \* symbol indicates significant differences (ANOVA followed by paired t-test) between the meal containing lemon juice and the other two meals. n = 10 healthy adults.

The postprandial volume of gastric contents 30 min after the start of the meal was about 50 % higher when bread was consumed with lemon juice compared with the water meal ( $454.0 \pm 18.6$  vs.  $298.4 \pm 19.5$  mL,  $\bar{x} \pm$  SEM). The overall gastric emptying rate (as estimated from the changes in the volume of gastric contents at t=30 and t=165) was about 50 % faster for the lemon juice meal ( $1.8 \pm 0.2$  for water vs.  $2.7 \pm 0.1$  mL/min for lemon juice,  $\bar{x} \pm$  SEM). Both of these differences were statistically significant with  $P < 0.00001$  and  $P < 0.01$ , respectively. Using the gastric volume measured at 30 min as reference, the half-gastric emptying time was similar for all meals (78 to 85 min). This information is summarized in Table 16.

**VOLUMES OF THE SOLID AND LIQUID PHASES.** The results of the preliminary assessment of the volumes of the solid and liquid phases within the stomach are presented in Figure 72. The volumes of the solid phase were very similar for the three test meals throughout digestion. For water and tea, the liquid phase appears to have behaved similarly, and it between 30-45 min there was a reduction in volume. For the lemon juice meal however, the volume of the liquid phase tended to be much higher than in the other meals throughout the monitored period.

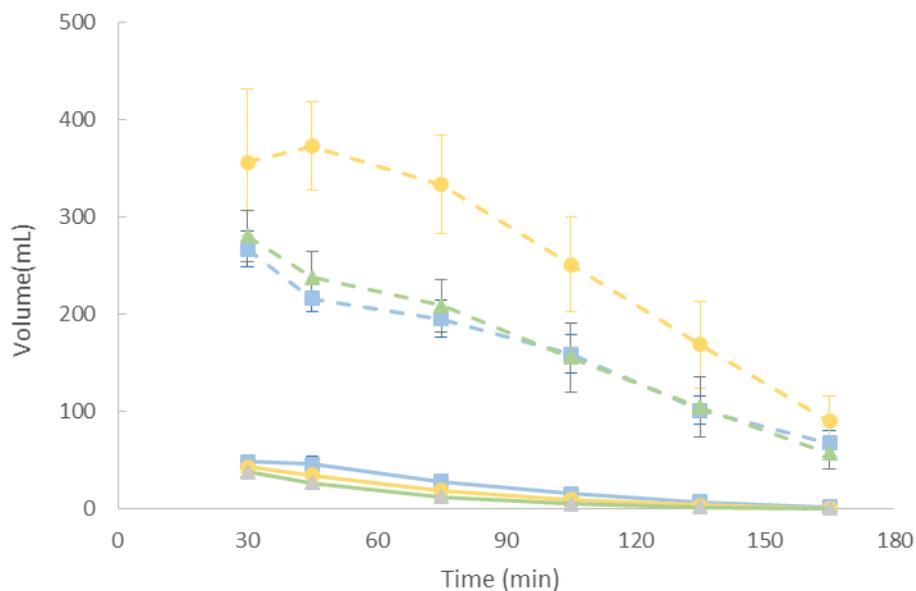


Figure 72- Estimated postprandial volume of the solid (full lines) and liquid (dashed lines) phases. Mean ( $\pm$ SEM, smaller than symbol size for the solid phase) volumes after consumption of three meals with equal volume containing equal amounts of starch from a white-wheat-bread and of water (■), tea (▲) or lemon juice (●).

**BLOOD GLUCOSE RESPONSES.** Postprandial blood glucose responses elicited by the water, tea and lemon juice meals are presented in Figure 73. There were no significant differences in baseline blood glucose concentrations between the three test-meals ( $P = 0.79$ ). Lemon juice tended to elicit a lower response but statistical significance was only found in comparison with the tea meal at  $t = 55$  min ( $P = 0.02$ ) (between lemon juice and water  $P = 0.12$ ). No significant

differences were found between water and tea. Mean AUC under the blood glucose curve (range 0-85 min and 0-165 min), and the equivalent changes as percentage of the corresponding results obtained with the water meal are presented in Table 16.

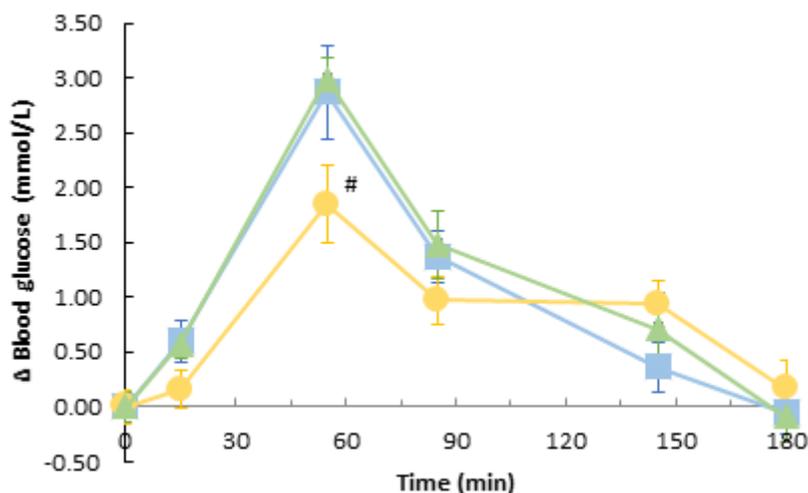


Figure 73 - Postprandial blood glucose responses. Mean ( $\pm$ SEM) incremental changes ( $\Delta$ ) in glucose concentrations in response to equal amounts of starch from a white-wheat-bread consumed with water (■), tea (▲) or lemon juice (●). The # symbol indicates significant differences were found (ANOVA followed by paired t-test) statistically significant difference between lemon juice and tea ( $P = 0.02$ ).  $n = 10$  healthy adults

No statistically significant differences were found between water and tea. The AUC for the lemon juice meal was significantly lower between 0 and 85 min ( $P = 0.03$  with water, and  $< 0.01$  with tea) with a reduction of about 40%. The AUC between 0 and 165 min tended to be 30% lower for the lemon juice meal but this was not statistically significant ( $P = 0.14$ ). We must add that the peak value and time-to-peak for the glycaemic response were not analysed because of a lack of measurements in the adequate time-windows. Indeed, we have previously shown, using the same test-meals, that the blood glucose peak is reached around 30-45 min for both water and tea (Freitas, et al., unpublished), *i.e.* a period during which we did no analyses of the glycaemia in the present study.

Table 16 - Gastric emptying and glucose responses for the water, tea and lemon juice meals. Initial volume of gastric contents, estimated gastric emptying rate (up to blood glucose peak and total) and half emptying time (T50%), postprandial areas under the curve (AUC) from 0 to 85 min and 0 to 165 min, for the three studied meals<sup>1</sup>

		<b>Water</b>	<b>Tea</b>	<b>Lemon juice</b>
<i>Volume of gastric contents at T=30 min</i>	mL	298 ± 20	307 ± 26	454* ± 19
	Change % <sup>2</sup>	-	3	52
<i>Total emptying rate</i>	mL/min	1.8 ± 0.2	2.0 ± 0.2	2.7* ± 0.1
	Change % <sup>2</sup>	-	12	50
<i>T50%</i>	min	84 ± 5	78 ± 5	85 ± 5
	Change % <sup>2</sup>	-	-7	1
<i>Glucose AUC (0-85 min)</i>	mmol · min/L	140 ± 18	150 ± 13	82* ± 11
	Change % <sup>2</sup>	-	7	-41
<i>Glucose AUC (0-165 min)</i>	mmol · min/L	198 ± 23	232 ± 31	159 ± 21
	Change % <sup>2</sup>	-	17	-31

<sup>1</sup>All values are  $\bar{x} \pm SEM$ . Values followed by an asterisk (\*) are significantly different from that obtained with water for the same row (ANOVA followed by paired t-test). Initial volume of gastric contents, total emptying rate and AUCs (0-85 min) for the lemon juice meal were significantly different than for the water meal ( $P < 0.00001$ ,  $< 0.01$  and  $= 0.03$ , respectively).  $n = 10$  healthy adults.

<sup>2</sup>Change as a percentage of the bread + water reference meal.

### 3.4. APPETITE PERCEPTIONS

The results of the participants' ratings for hunger, satiety, fullness, prospective food consumption, thirst and nausea are presented in Figure 74. There were no differences in baseline ratings. There was also no statistically significant effect of the three meals at any given time-point. Hunger, thirst and prospective food consumption scores lowered immediately after the meal, increasing steadily afterwards. The opposite was observed with the satiety and fullness ratings, which increased immediately after the meal and progressively declined after. As it can be observed in Figure 75, the relationship between hunger, satiety, fullness and prospective food consumption and the volume of gastric contents tended to be linear.

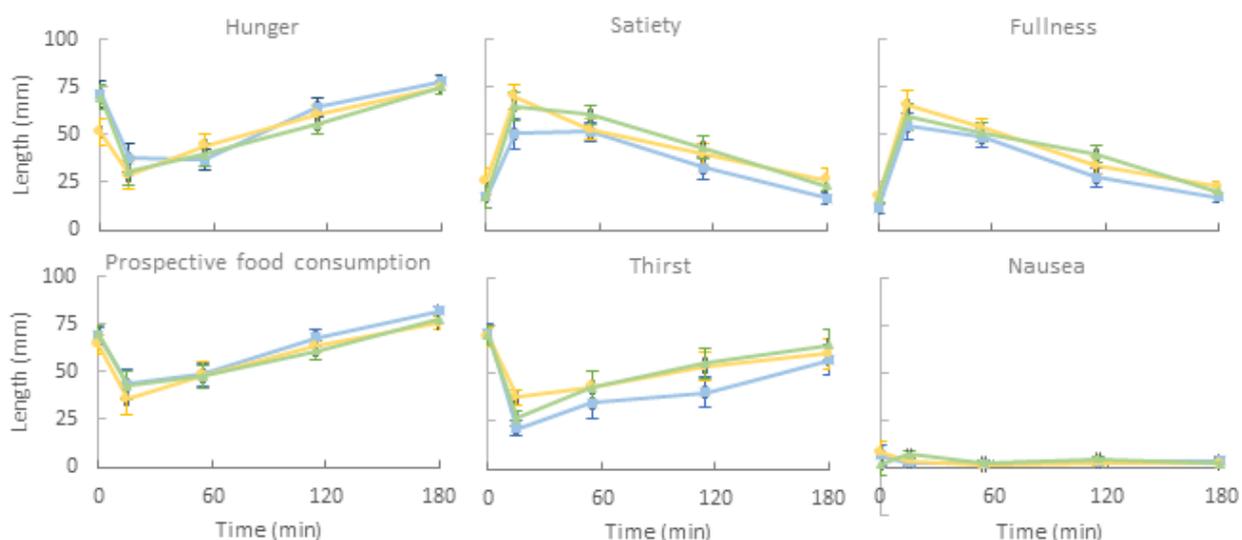


Figure 74 – Appetite perceptions. Mean ( $\pm$ SEM) incremental changes in ratings of hunger, satiety, fullness, prospective food consumption, thirst and nausea in response to equal amounts of starch from a white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●). n= 10 healthy adults

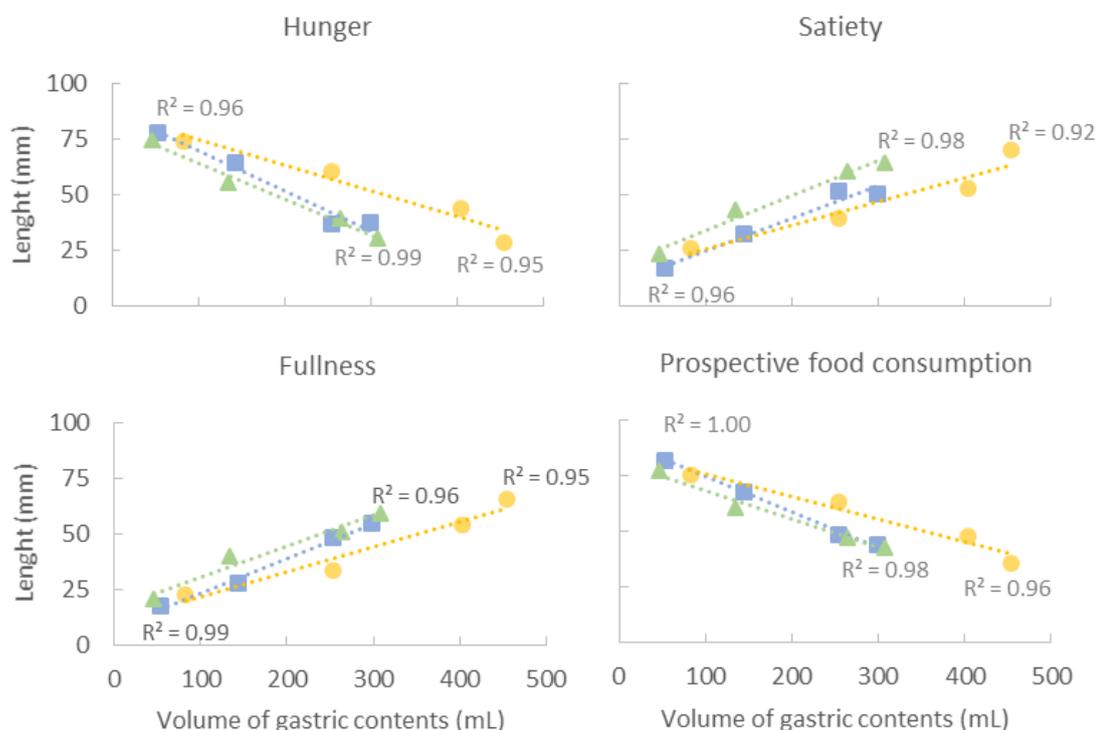


Figure 75 – Relationship between appetite perceptions plotted against volume of gastric contents for the sense for three meals composed of equal portions of white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●). Data were grouped by time point of measurement with a 5-10 min difference between time of VAS and of volume of gastric contents. The sense of hunger and prospective food consumption decreased linearly, the senses of satiety and fullness increased linearly. n= 10 healthy adults

#### 4. DISCUSSION

The water and tea meals yielded similar results both in terms of gastric emptying and blood glucose response. Marked differences were observed with the lemon juice meal, after which the volume of the gastric contents was higher, gastric emptying was faster and the blood glucose response lower. The reduced glycaemic response with the lemon juice meal is in very a good agreement with the more accurate measurements that we performed in the first part of this clinical study with the same meals, 17 participants, and more frequent blood glucose measurements (Freitas, et al., unpublished). In that part of the study, corresponding data showed a glucose peak lowered by 30 % ( $P < 0.05$ ), reached on average 35 min later ( $P < 0.01$ ), with the lemon juice meal in comparison with the water meal. Our present results therefore show that our previously observed effect of lemon juice did not disappear with the lying position of the subjects in the MRI.

In a first analysis of our data, one could deduce that a constantly higher volume of gastric contents (Figure 71) indicates a longer intragastric retention of the chime for the lemon juice meal, which would explain the lower glycaemic response, as previously suggested in another study on a bread meal supplemented with vinegar (H. Liljeberg & Björck, 1998). This straightforward interpretation is easy to conceive as it could directly relate our two main observations in the presence of lemon juice: higher initial gastric volumes and a reduced blood glucose response. However, this reasoning is not be sufficient to explain all our data, in particular the fact that the higher gastric volumes with lemon juice seemed to result from differences in the amount of the liquid phase (Figure 72). The higher citric acid content of this meal could have led to a combination of changes including an increase in salivary and/or gastric secretions and/or an initial lag phase of the liquid phase (Hunt & Knox, 1962, 1969, 1972) which could explain the higher liquid volume. In any case, it is worth noting that the rate of gastric emptying of the lemon juice meal was also significantly higher (by about 50%) leading to similar overall durations of gastric emptying whatever the meal, as shown by the very close gastric volumes at 165 min (Figure 71 and Figure 72). This contrasts with classically observed lag phases in gastric emptying kinetics, for which the initial delay is never recovered, hence leading to parallel gastric emptying patterns (Malagelada, Go, & Summerskill, 1979).

To better understand all of our observations, specifically the relationship between gastric volume patterns and the blood glucose response, it is necessary to discuss them with the available literature in these research fields.

#### 4.1. AN ANALYSIS OF THE GASTRIC EMPTYING PATTERNS

As shown in Figure 65 and Figure 67, none of the meals exhibited homogeneous intragastric distributions, and it is known that inhomogeneous intragastric chimes impact gastric emptying patterns (Marciani, et al., 2013; Siegel, et al., 1988). Siegel and colleagues used scintigraphy to study the gastric emptying patterns of the solid and liquid components of a meal somewhat similar to that in our study. In their study, the meal was composed of an egg sandwich (solid) and water (liquid). The egg and water were labeled differently and monitoring was initiated immediately after the meal was consumed. They observed that emptying of water started straightaway and at a fast rate, while the solid phase had an initial 31-min-long lag phase. After 30 min, at least 90% of the solid phase was still in the stomach, while only about 50% of the liquid phase remained (Siegel, et al., 1988). Although we had no measurements of the volume changes during the first 30 min, considering the similarities between the composition of our meals and those in Siegel's article, the inhomogeneity observed (Figure 65), and the differences between the solid and liquid volumes at 30 min (Figure 72), it is likely that our meals behaved in a similar manner. During this period, a faster emptying of the liquid phases of the water and tea meals than the lemon juice meal, due to different acidity levels, could explain the difference between the first meal volumes (about 30% lower for the water and tea meals). The pH of the lemon juice was about 2.2 whereas that of the other beverages was neutral. Thus, the higher acidity of the lemon juice meal is likely to have initially impaired the emptying of the liquid phase (Hunt & Knox, 1962, 1969, 1972). This effect, together with the reported higher salivary flow during consumption of acidic foods (Watanabe & Dawes, 1988) may explain the higher volume at 30 min.

Pioneer gastric emptying studies were conducted by the team of Hunt who observed that solutions of citric acid were emptied from the stomach slower than water due to a feedback mechanism activated by acidity receptors in the duodenum (Hunt & Knox, 1972). As a matter of course, there are other factors that regulate gastric emptying rate and the subsequent work of his team (Hunt, Smith, & Jiang, 1985) along with that of other research groups (Calbet & MacLean, 1997; Kwiatek, et al., 2009) has pointed out the major importance of caloric density. They have observed that the stomach adapts to the physical and chemical properties of the meals to maintain a constant rate of calorie delivery to the small bowel (Calbet & MacLean, 1997; Hunt, Smith, & Jiang, 1985; Kwiatek, et al., 2009). This also seems to have been the case here. Indeed assuming equal lag phases for the solid fraction of all meals, the differences in volumes derived from variations in the liquid phase. Therefore, at 30 min, the caloric density of the chime for the lemon juice meal, would be approximately 30% lower than for water (Table

17), and the gastric emptying rate seems to have been adjusted accordingly as it was significantly higher for this meal (Table 16).

In other words, if the caloric density of the chime at 30 min is used to estimate the gastric emptying rate in terms of kcal/min (still assuming a common lag phase for all meals' solid fractions), very similar results are obtained (from 1.7 to 1.9 kcal/min) (Table 17).

Table 17 – Estimated caloric density of the meal at 30 min and gastric emptying rate as a function of caloric density.

	<b>Water</b>	<b>Tea</b>	<b>Lemon Juice</b>
Caloric density of the chime at 30 min (kcal) <sup>1</sup>	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
Emptying rate (kcal/min) <sup>2</sup>	1.7	1.9	1.8

<sup>1</sup>Estimated mean (± SEM) considering a 30 min lag phase n= 10.

<sup>2</sup>Estimated according to caloric density at 30 min and the total gastric emptying rate (Table 16).

Remarkably, a recent MRI study has even reported that grapefruit juice (pH=3.3) and a glucose solution (pH=7.8) with equal caloric density were emptied from the stomach at the same rate (Grimm, et al., 2018). The effect of caloric density seems to surpass that of meal acidity, what support the assumptions and interpretations presented above.

#### 4.2. BLOOD GLUCOSE RESPONSE

According to the above-described literature and associated interpretation of our results, it is therefore likely that the delivery of nutrients to the small intestine occurred at approximately the same rate, hence discarding a gastric emptying based explanation for the differences in the glycaemic response. Numerous studies have reported the effect of lower pH meals on attenuating the glycaemic response in healthy adults, but only three of them assessed gastric emptying and blood glucose concentrations concurrently (Brighenti, et al., 1995; H. Liljeberg & Björck, 1996, 1998). In two of these, no differences were observed in the gastric emptying rates despite confirmation of a lower glycaemic response the for the lower pH meals (Brighenti, et al., 1995; H. Liljeberg & Björck, 1996). In the third study, the gastric emptying was evaluated indirectly by monitoring blood paracetamol concentrations. In the presence of lemon juice, the glycaemic response was lowered by 36%, while the area under the blood paracetamol curve was lowered by 20%. (H. Liljeberg & Björck, 1998). In this study the paracetamol was in the bread loaf, so this opens the question as to whether the lower paracetamol concentrations actually

derived from a delayed emptying rate or were the consequence of a slower digestion of the food and consequently slower release of the marker.

We may therefore support a mechanism related with the premature acid-induced inhibition of salivary amylase, which we have previously put forward, and that can be recapitulated as follows. It is expected that the lemon juice meal causes a premature acidification of the gastric contents, resulting in an early inactivation of salivary  $\alpha$ -amylase. This is the only amylolytic enzyme that could be affected by meal acidity, since it is the only one present during gastric digestion. Therefore, nutrient delivery to the small intestine, although quantitatively equivalent in terms of caloric content, is likely to be qualitatively different because the extent to which nutrients have been digested is not the same. According to our previous *in vitro* studies, after 20-30 min of gastric digestion, as much as 70% of starch from the same bread and water meal can be released, with 45% thereof in the form of oligosaccharides. For the lemon juice meal, only about 40% of the starch was released, with approximately 10% thereof in the form of oligosaccharides at the gastric stage (Freitas & Le Feunteun, unpublished). A number of recent reviews have focused on the analysis of the factors leading to the attenuation of the glycemic response by low pH meals. Although the salivary hypothesis is hardly addressed (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014; Lim, Henry, & Haldar, 2016; Poutanen, Flander, & Katina, 2009), or not at all (Lim, Henry, & Haldar, 2016; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014), in our view, the acid-inhibition of starch amylolysis by salivary  $\alpha$ -amylase appears as the main contributor to those results.

#### 4.3. APPETITE PERCEPTIONS

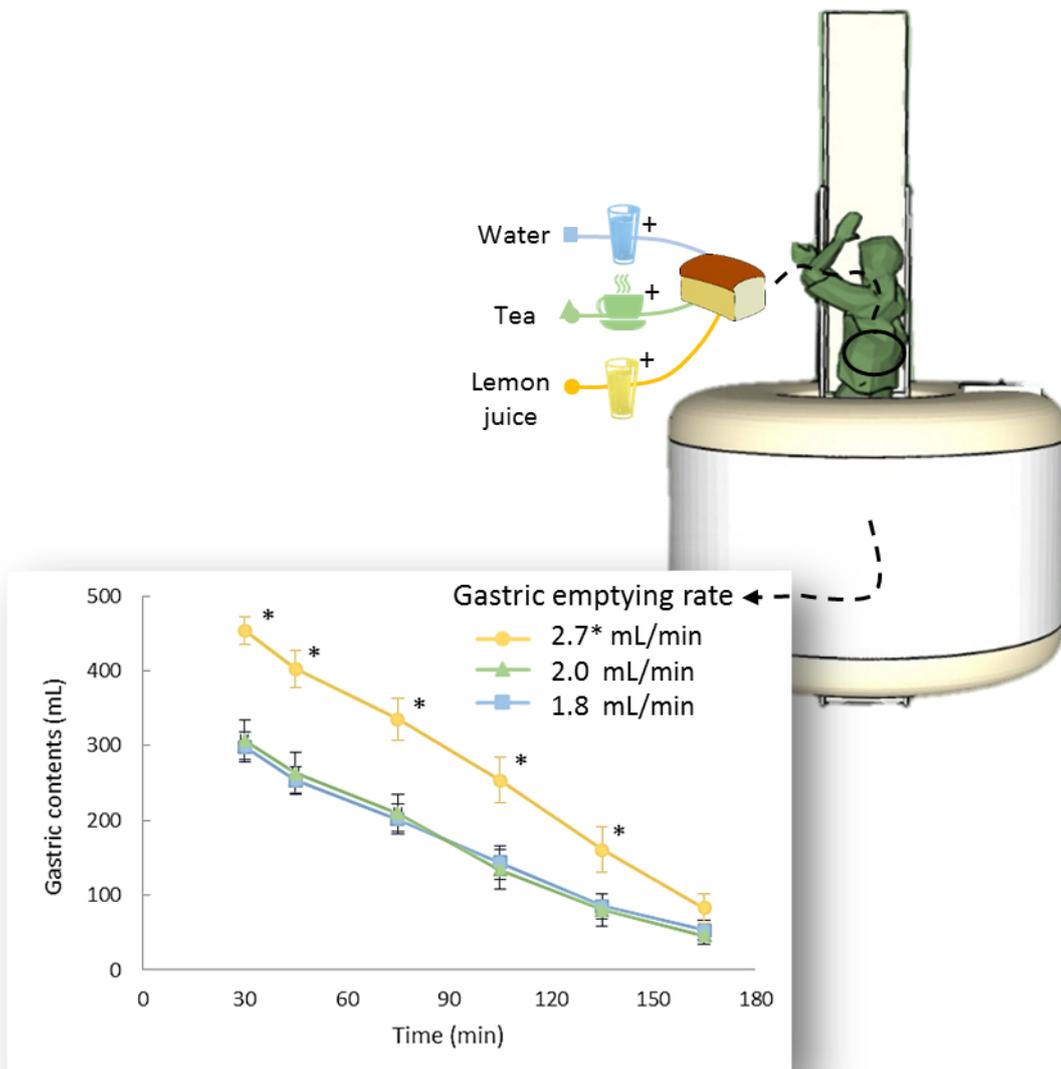
Despite differences in the blood glucose response we did not observe any effect of the test meals on satiety perceptions. It has been suggested that slower starch digestion leading to lower glucose concentrations could prolong satiety (Blaak, et al., 2012). Satiety perceptions were also evaluated in four of the aforementioned studies reporting lower glycaemic responses for lower pH meals. The results were contradictory, with 2 studies reporting an increase (Bo, et al., 2017; Elin Östman, Granfeldt, Persson, & Björck, 2005), and 2 others finding no effect (H. Liljeberg & Björck, 1996; H. G. Liljeberg, Lönner, & Björck, 1995). This study was not powered for this parameter and this can explain why we failed to observe an effect, but in any case, research on the effect of the glycaemic response *per se* on appetite and satiety has been inconclusive (Blaak, et al., 2012). As explained by Wolever and colleagues, it is difficult to conceive test meals which elicit distinct glycaemic responses and are controlled for all the possible factors which could affect satiety, and so results are often confounded by lack of control of variables which could lead to an increased satiety, other than the glycaemic response (Thomas MS Wolever, Leung,

Vuksan, & Jenkins, 2009). To better understand this, Wolever and colleagues have compared the effect of different glycaemic responses to the exact same meal on satiety perceptions and found no relationship. This is in agreement with our results.

## CONCLUSION

We have studied the gastric phase of digestion, blood glucose response and perceptions of satiety following the consumption of equal portions of bread with water, tea or lemon juice at breakfast. No effect was observed on satiety perceptions. The glycaemic response was similar between the water and tea meals, but in the presence of lemon juice the peak blood glucose concentration tended to be lower. Gastric volumes were similar following consumption of the water and tea meals. In the presence of lemon juice, the overall gastric volume, but also the gastric emptying rate were higher. The higher volume seemed to be explained by a higher volume of the liquid part of the chyme, for which emptying could have been delayed in comparison with the other meals because of the higher acidity. Nevertheless, the higher gastric emptying rate compensated for these differences in volume and appears to have ensured similar rates of nutrient delivery to the small intestine. In light of these results, we support the idea that the reduction of the glycaemic response due to meal acidity derives from the inhibition of salivary  $\alpha$ -amylase, and not from a reduction or delay of gastric emptying.

**KEY MESSAGES**



- We have studied gastric digestion following the consumption of equal portions of bread with water, tea or lemon juice.
- Gastric volumes were similar following consumption of the water and tea meals.
- In the presence of lemon juice, the overall volume of gastric contents was higher, probably due to a higher volume of the liquid part.
- The gastric emptying rate was significantly higher with lemon juice, what appears to have ensured similar rates of nutrient delivery to the small intestine.
- The hypothesis that the reduction of the glycaemic response due to meal acidity derives from the inhibition of salivary  $\alpha$ -amylase, and not from a reduction or delay of gastric emptying is supported.

IV  
GENERAL CONCLUSIONS  
AND  
PERSPECTIVES



## GENERAL CONCLUSIONS

The results obtained have been discussed in detail in each of the previous chapters. In this section, only a short overall analysis will be presented, focusing on the key findings and the relationships between them.

### 1. THE CONTRIBUTION OF SALIVARY AMYLASE TO STARCH DIGESTION

A total of 6 starch-rich foods were studied: 3 white wheat breads, and 3 types of pasta (wheat pearl shaped pasta, wheat spaghetti, and gluten-free spaghetti). All of these foods were digested in an *in vitro* dynamic system, comprising an oral phase with real saliva and a gastric phase with acidification kinetics that replicated *in vivo* observations. In some cases an intestinal phase was also included.

Considering all the experiments conducted with bread (Figure 76), we have studied 3 gastric acidification kinetics in which the pH of the *in vitro* chime was reduced from 6 to 2 in either: (1) 30 min, (2) 60 min or (3) 90 min. For all breads, the quantity of released starch was significant at the end of the oral phase, despite its short duration (2.5 min), with a mean close to 20% of the total starch content. Moreover, regardless of the pH reduction kinetics, the same exponential pattern of starch release was observed during the gastric phase, with a plateau in the range 70-85% reached within the first 15-20 min of digestion.

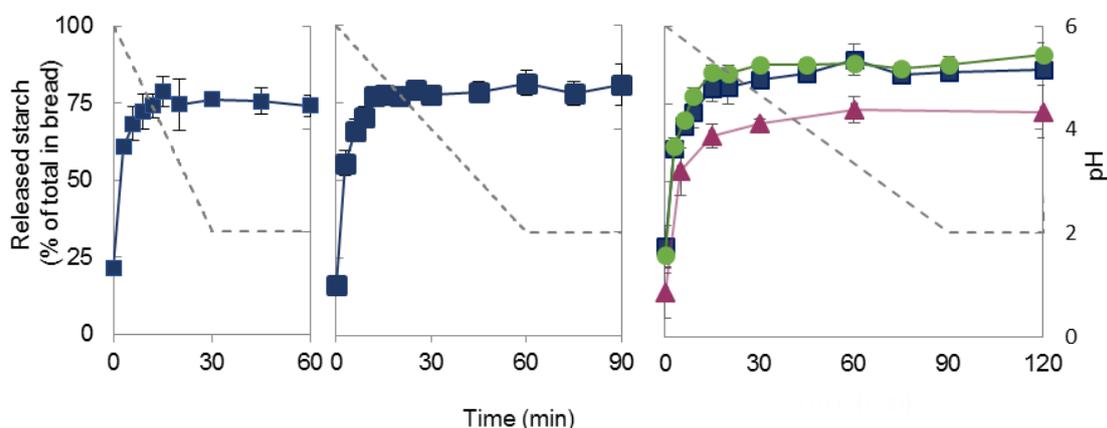


Figure 76 - Dynamic *in vitro* digestion of bread. Released starch (full symbols) and pH of the chime (dashed lines) during the course of oro-gastric digestions of three breads. Different symbols illustrate the results obtained with different breads. Two baguettes (■ and ●) and one sandwich bread were studied (▲). Average  $\pm$  SD, 3 rpt.

When the analyses were conducted to determine the proportion of starch that had been hydrolysed into oligosaccharides, we observed that this also followed an exponential evolution, with a plateau reached a few minutes later than for starch release (Figure 77). More importantly,

the quantity of released oligosaccharides at the plateau appeared to be remarkably high, as it corresponded to 60-65% of the released starch.

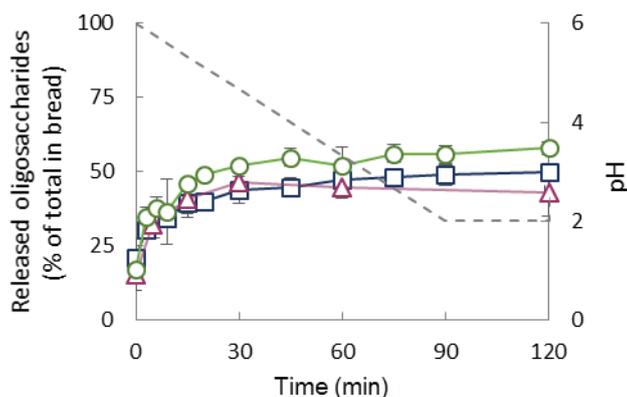


Figure 77 - Dynamic *in vitro* digestion of bread. Released oligosaccharides (open symbols) and pH of the chyme (dashed line) during the course of oro-gastric digestions of three breads. Different symbols illustrate the results obtained with different breads. Two baguettes (□ and ○) and one sandwich bread were studied (△). Average  $\pm$  SD, 3 rpt.

With pasta, which is commonly associated with lower glycaemic responses than bread, starch digestion progressed at a slower pace. The starch release plateau was reached after 45-50 min of digestion and was, on average, about 2.5 times lower than with bread (at about 30%). In all cases, 60-70% of the released starch had been hydrolysed into oligosaccharides by HSA.

As most bread starch had already been released during the gastric phase, when a subsequent intestinal phase was used, the further increases in released starch and oligosaccharides were only of about 5-8% and 15-20%, respectively. Conversely, as only a fraction of pasta starch had been released during gastric digestion, most of its release and hydrolysis was performed during the intestinal phase.

Overall, the combined action of pancreatic amylase and proteases appeared to be very efficient in resuming the hydrolysis into oligosaccharides of the large starch fractions that had been released during the gastric phase. However, the results of our *in vitro* studies demonstrated that the role of salivary  $\alpha$ -amylase should not be neglected. HSA indeed seems to play an important role in the hydrolysis of starch during the gastric phase, and more particularly for foods that are commonly associated with a higher glycaemic response, such as bread.

## 2. COMPLEMENTARITY BETWEEN ENZYMATIC ASSAYS, *IN VITRO* AND *IN VIVO* STUDIES

Based on our first results, we hypothesized that it was highly likely that factors influencing the amylolytic performance of salivary  $\alpha$ -amylase, other than the structural properties of a food, could translate into a modified glycaemic response. This is why we conducted preliminary experiments simulating the digestion of wheat bread, wheat pasta and gluten-free pasta with either lemon juice or water. As anticipated, the premature acidification of the chime by the lemon juice caused the complete interruption of enzymatic amylolysis after the oral phase, leading to a more pronounced effect with bread, for which the role of salivary amylase during gastric digestion is more preponderant, than with pasta.

Subsequent experiments aimed at obtaining a better understanding of these mechanisms and at investigating the impact of other food items on the activity of both salivary and pancreatic amylases. In addition to lemon juice, teas, coffees, wines, and vinegars were studied for their amylolytic inhibitory capacities using enzymatic tests. The reduction of the amylolytic activity of saliva by neutral pH foods increased in the following order: coffees (5-10%), green (20-45%) and black teas (30–70%). Similar levels of inhibition were found on the amylolytic activity of pancreatin for each product category. The effect of acidic foods (wines, vinegars and lemon juice) on the amylolytic activity of saliva was also studied at their native pH, and all of them exhibited a quasi-total inhibitory capacity (90-100%).

By repeating *in vitro* digestion experiments of bread in the presence of lemon juice we not only confirmed our preliminary findings, but also proved that comparable results could be obtained in control experiments with no salivary amylase. *In vitro* digestions of bread with the tea that had exhibited the strongest inhibitory effect in the enzymatic tests (a black tea) only showed a modest effect. For most of the experiment, starch was indeed digested at a similar rate as with water, and a small differentiation was only apparent during the intestinal phase with a proportion of released oligosaccharides about 20% lower.

Altogether, the results of our *in vitro* experiments provided a strong biochemical rationale for the development of dietary strategies that could be effective at improving the glycaemic response elicited by starch-rich foods. Providing that starch-rich foods are widely consumed, such strategies, based on pairing them with others that can inhibit amylolytic enzymes could be easy to adopt. Therefore, the next goal was to test these strategies in a clinical study, composed of two parts.

In both parts of the study, the volunteers consumed equal portions of bread with either water (control), black tea or lemon juice. The first part of the study was conducted in semi controlled conditions, as volunteers were free to follow their normal daily routines between two blood glucose measurements. The water and tea meals elicited similar glycaemic responses, but the lemon juice attenuated it significantly (Figure 78A). Both the  $AUC_{0-90min}$  and the peak blood glucose concentration were significantly lowered by about 30% ( $P < 0.05$ ) with lemon juice compared to water. Because of the semi-controlled nature of this study, it is suggested that pairing an acid drink with a starchy meal could be an effective strategy to reduce the glycaemic response to starch-rich foods in everyday-life.

Overall, our *in vitro* digestion experiments appeared to have successfully predicted the effect *in vivo*. The positive relationship between *in vivo* (Figure 78A) and *in vitro* (Figure 78B) observations therefore illustrates the potential of (semi-)dynamic digestion experiments as a complementary (or pre-screening) tool to clinical studies. It is however noteworthy that our enzymatic tests designed to estimate the inhibitory capacity of different beverages or condiments were only partially relevant. In fact, they provided useful tendencies for the acidic drinks that could be confirmed with lemon juice during both *in vitro* and *in vivo* digestions. However, the inhibitory effect estimated for black tea was of little consequences during *in vitro* digestions, and did not translated into measurable differences in humans. This discrepancy is probably due to the large

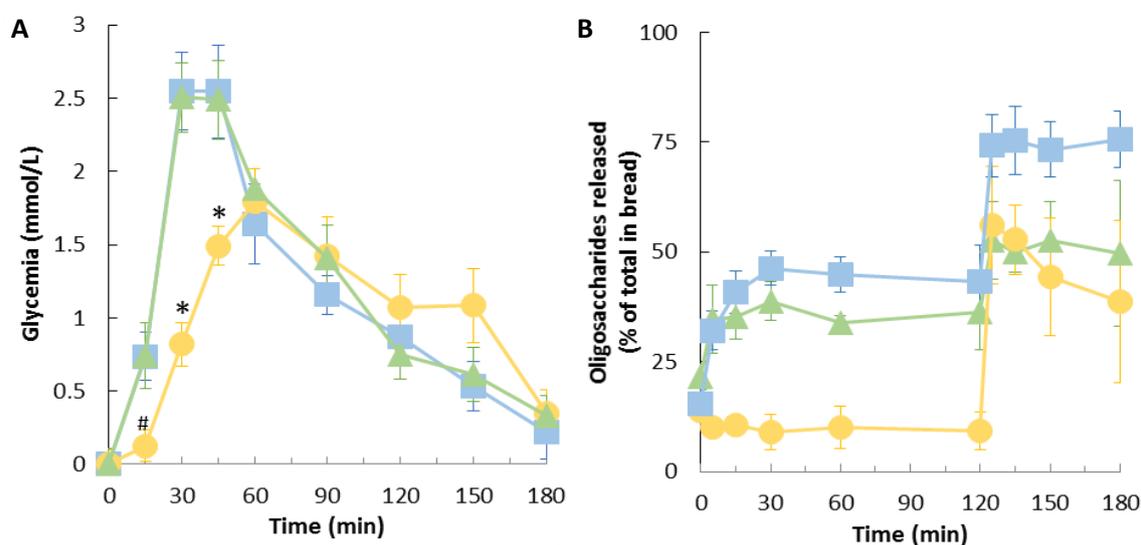


Figure 78 – Comparison of *in vivo* and *in vitro* results obtained with the same meals. Meals were composed of equal amounts of carbohydrate from a white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●). **(A)** Postprandial blood glucose responses. Mean ( $\pm$ SEM) incremental changes ( $\Delta$ ) in glucose concentrations. # indicates a statistically significant difference between lemon juice and water ( $P < 0.01$ ). \* indicates statistically significant differences between lemon juice and both water and tea at 30 min ( $P < 0.0001$  and  $0.001$ ) and at 45 min ( $P = 0.014$  and  $0.019$ ).  $n = 17$  healthy adults. **(B)** Released starch during the course of oro-gastro-intestinal digestions of the same meals *in vitro*. Average  $\pm$  SD, 3 rpt.

gap between the dilution ratios used during the enzymatic tests, during our *in vitro* digestion, and those achieved in humans. This might in fact be a good example showing the importance of thoroughly considering the relevance of a simplified method that aims at predicting outcomes in the *in vivo* reality.

### 3. ATTENUATION OF THE GLYCAEMIC RESPONSE IN THE PRESENCE OF LEMON JUICE – UNDERLYING MECHANISMS

The results obtained with lemon juice were consistent with numerous clinical studies investigating the effect of other acidic foods (namely vinegar, sourdough bread and pickled foods) on the glycaemic response to starch-rich foods. According to the review articles that analysed the possible underlying causes for the effects of vinegar, it seemed clear that the main mechanism was related to acidity, though the actual effect of acidity was not pin pointed. The effect on amylolytic enzymes, particularly salivary amylase, was rarely mentioned, and the question of the potential impact of other types of acidic foods was never raised. Indeed, it appears that the results obtained with different acidic foods had not necessarily been related with each other. According to the study of the literature, we had initially hypothesized that they could share a common mechanism (premature amylase inhibition) and that the same effect could be obtained with other acidic foods. Nevertheless, a another candidate explanation, still not fully accepted nor rejected so far, is that acidity could delay gastric emptying .

The second part of the clinical study was designed to investigate this possibility, using the same meals as in Part 1, and a MRI monitoring of the gastric phase. The meal was also supplemented with 20 peas, which the subjects were asked to swallow without chewing at the end of the meal, to evaluate how good the gastric mixing is. A comparison of the glycaemic responses obtained for each meal in Part 1 and Part 2 is presented in Figure 79. Due to constraints imposed by the MRI protocols in Part 2, the time-points of blood glucose measurements were slightly different, what explains the small variation in peak times. However, as it can be observed, the glycaemic responses elicited by the water, tea and lemon juice meals were similar in both parts of the study. This shows that the periods that the subjects spent lying down for the MRI scans did not seem to influence their glycaemic response, and that the results of the MRI exams can be put in relation with the glycaemic responses observed in the first part of the study.

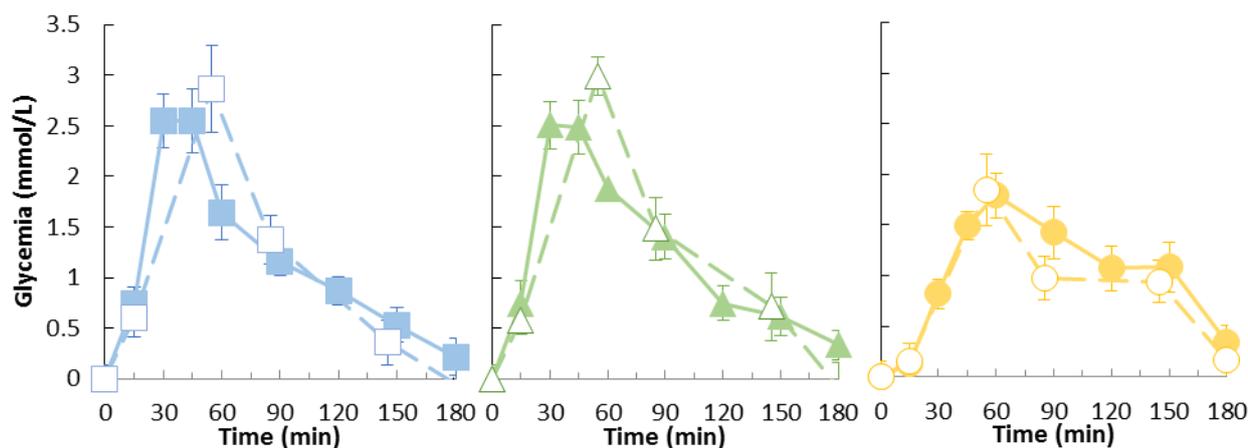


Figure 79 – Comparison of the glycemic responses in the two branches of the clinical study. Glycaemia response to equal amounts of carbohydrate from a white-wheat-bread consumed with either water (■), tea (▲) or lemon juice (●) in Part 1 (full symbols, full lines) and Part 2 (open symbols, dashed lines) of the clinical study.

The MRI exams comprised anatomic (high spatial but low temporal resolution) and motility (low spatial but high temporal resolution) scans. Due to time constraints, only the anatomic scans were quantitatively analysed, so far, to determine the gastric volume at each time point. In the presence of tea and water the results were similar. With lemon juice, the gastric volume was overall higher. According to our interpretation, this appeared to be a consequence of a longer initial retention of the liquid phase because of the high acidity of lemon juice. Indeed, the gastric emptying rate (significantly higher with lemon juice) appeared to have been adjusted to ensure a similar nutrient delivery rate to the small intestine as for the other meals. Therefore, our findings do not really support the hypothesis that low pH meals attenuate the glycaemic response to starch-rich meals because of a delayed or decreased gastric emptying rate. They are more in line with the idea that this effect on blood glucose concentrations is, at least in part, a consequence of a premature inhibition of salivary  $\alpha$ -amylase due to early acidification of the chyme during gastric digestion.

In conclusion, the present work has highlighted the role of salivary  $\alpha$ -amylase in the digestion of starch-rich foods, and offered novel insights into the development of strategies aiming at improving the glycaemic response elicited by starch-rich diets. A simple strategy based on matching starch-rich foods with acidic beverages could be easily integrated in dietary advice and adopted by the population.

## PERSPECTIVES

### 1. RE-EVALUATION OF THE PROCESS OF STARCH DIGESTION

#### 1.1. CONTRIBUTION OF EACH DIGESTIVE STAGE

Where is starch actually digested in our digestive system? The results of our *in vitro* digestion experiments contradict the traditional idea that almost all starch in foods is digested in the small intestine by pancreatic amylase. They clearly show that a considerable fraction of starch is digested by salivary  $\alpha$ -amylase in the stomach, particularly for foods such as bread, which are linked with high postprandial glycaemia. Evidence from our *in vitro* studies should be enough to question current views of this process and at least reformulate the way it is described to highlight the potential contribution of the gastric phase. Additionally, the results of our *in vivo* studies with lemon juice are also strong indicators of the importance of salivary  $\alpha$ -amylase. Our findings would now need to be confirmed, ideally with studies in which this enzyme would be selectively inhibited by other means than pH. Maybe part of the answer can already be found in the literature? An interesting example is the reported correlation between the number of chews with the glycaemic response to rice (Tan, et al., 2016). Another one was provided by Read N. W. and co-workers in 1986, who found that swallowing without chewing reduced the glycaemic response to diced starch-rich foods (corn, potato and rice) but not to a low-starch food (apple). The authors have pointed out that one of the reasons for the higher glycaemic response in the chewing situation could be an enhancement of salivation, which would increase digestion of food in the mouth and stomach. There are however other possible explanations. The absence of effect with apple may, indeed, also result from its lower glycaemic index and/or from differences in particle sizes (Read, et al., 1986). It would thus be interesting to design a similar experiment in which rich-starch foods are compared with lower-starch foods of similar glycaemic responses. And to account for the possible influence of the particle size, it could be appropriate to serve the foods for the non-chewing day chopped to a level that replicates the food particle size in a food bolus.

#### 1.2. IS THERE A RELATIONSHIP BETWEEN SALIVARY AMYLASE AND OVERALL HEALTH?

There is currently a debate about the relationship between salivary  $\alpha$ -amylase activity in saliva and health. In one study, taken as an example, it was found that people with a higher salivary amylase activity had a lower postprandial blood glucose response to starch, but not to glucose compared to others with a low activity (Mandel & Breslin, 2012). This could also be related with the findings of a recent systematic review showing that there is a higher prevalence of xerostomia (subjective complaint of dry mouth), and possibly of subjects with hyposalivation, in diabetes mellitus (DM) patients in relation to non-DM population (López-Pintor, et al., 2016).

However, in other studies, no relationship was found between the salivary amylase levels and the postprandial glycaemic response (Tan, et al., 2016).

The results obtained by the team of Mandel were the opposite of the hypothesis initially formulated by the authors (Mandel & Breslin, 2012). Indeed, it can be counterintuitive, especially considering our results. Their findings appeared to be linked not necessarily with a higher starch digestion rate, but with an increased plasma insulin concentration observed at the beginning of the session in individuals with higher salivary amylase activity. But what is the relationship between the amylolytic activity of saliva and blood insulin concentration? The specific process by which a higher salivary amylase concentration stimulates early insulin concentrations and affects glucose homeostasis is not clear. However, this early insulin release could be part of anticipatory metabolic responses that prepare the digestive system to digest food, as well as absorb and metabolize nutrients. There were a total of 14 overall healthy participants in this study, selected for their salivary amylase activity. In part 1 of our clinical study there were 17 participants, who were overall healthy, but no considerations were made regarding the concentration of  $\alpha$ -amylase in saliva. It would be interesting to do the opposite experiment and now determine the amylolytic activity of their saliva and see whether there will be any correlation with their postprandial glycaemia. This can be done in the future as all these participants provided one sample of saliva (which was not yet analysed due to time constraints).

### 1.3. IS THERE ANY RELEVANCE OF THE TESTED STRATEGIES FOR PUBLIC HEALTH?

The results of the first part of our clinical studies were encouraging. Could this effect be sustained in the longer term? That is, would there be any benefit for the metabolism of glucose in increasing the consumption of low pH products with starch-rich meals in healthy individuals? What about those with impaired glucose tolerance or those suffering from type-2 diabetes? This could be evaluated doing longer studies and comparing the concentrations of Hemoglobin A1C before and after the intervention. Could this strategy be transferred to, and useful in, clinical settings or as part of dietary advice for the overall population? It could be possible to obtain some answers from epidemiological studies examining the relationship between the consumption of low pH foods, proportion of starch in the diet and overall glycaemic control.

## 2. IMPROVEMENT OF *IN VITRO* PROTOCOLS

Considering the positive relationship between the results of our *in vitro* digestion experiments and those of the clinical study (Figure 78), it would be interesting to quantitatively compare these results and investigate the possibility of using our *in vitro* protocol to estimate the glycaemic response *in vivo*, as already done by other research groups. In order to validate our *in vitro* protocol in this way, it would be important to test it with more foods, for which the mean

postprandial glycaemic response is known. This could be a useful tool in the preparatory phase of clinical trials to easily screen large numbers of foods. This could allow to reduce the number of samples in clinical studies, thereby reducing the constraints for the volunteers.

Nevertheless, it is clear that there are limitations to *in vitro* systems and that further improvements could still be made. In general, it is important to note that current *in vitro* protocols that omit the oral phase and/or call for immediate acidification during the gastric phase are not sensitive to the contribution of salivary amylase, and would thus be unsuitable to identify inhibitory mechanisms related to acidity such as those studied here. Our protocol can also be improved, two elements that could bring it closer to physiological reality are the simulation of gastric emptying and of the absorption of digestion products. The results of our MRI study could provide a starting point for information on gastric emptying. The time-evolution of the gastric volumes could indeed be used to estimate the flow rate to apply during *in vitro* digestions of bread. Additionally, there is an ongoing project in our lab to improve the dynamic digestive system by installing a dialysis membrane, to simulate nutrient absorption, in the compartment that simulates intestinal digestion. This has already been done with the TIM model (the dynamic *in vitro* digestion system from the TNO, The Netherlands), for example. It would allow for continuous removal of the final products of starch digestion to mimic the rapid glucose absorption in the small intestine. Thereby eliminating potentially inhibitory effects of hydrolysis products on enzymatic activity and enabling the analysis of “post absorptive samples”, which would be a closer representation of what is absorbed by the blood stream.

### 3. COMPLETE THE ANALYSIS OF THE MRI DATA

#### 3.1. DETERMINE GASTRIC VOLUMES AT BASELINE

Because image analysis is time-consuming, it has been decided to consider only postprandial anatomic volume scans at a first stage. However, for a complete analysis, it is necessary and important to analyse also baseline scans to ensure that there were no significant differences between the gastric volumes of participants of participants at baseline.

#### 3.2. COMPLETE THE ANALYSIS OF INTRAGASTRIC CONTENTS

As we have mentioned before, we did not use any reference material during MRI exams to correct the possible variations of the MRI signal between the study sessions. Nevertheless, it is clear that there were different levels of contrast within the stomach in our images (as it can be observed in Figure 80A). Image texture analyses of these exams could be carried out enabling a subsequent quantitative determination of the volumes of differently contrasted regions (Figure 80B). A preliminary analysis of the different contrasted areas showed promising results. However only 19 studies over a total of 30 have been exploited so far, as the others contained

significant differences in terms of the average intensity of the images. Therefore, further work is needed to understand the origin of these differences and properly corrected them.

### 3.3. QUANTITATIVE ANALYSIS OF THE MOTILITY SCANS

So far, only a qualitative and indirect analysis of the motility scans was conducted, regarding the position of the peas at different digestion times. However, it would be interesting to perform a more thorough and quantitative analysis. Because of the different contrast of the peas, it should at least be possible to determine the proportion of signal derived from the peas present in each slice, and therefore in each region of the stomach. Moreover, although the spatial resolution of these scans was poor, a delineation of the gastric volume seems feasible in these images. It should thus be possible to make volume reconstructions of the gastric contents to study the gastric motility (6 gastric volumes were scanned during a breath-hold), whenever a gastric contraction fell within the observed time window.

Additionally, further analysis of these scans could theoretically provide a more accurate result in terms of gastric emptying. The gastric volume results from the volume of the meal, the flux of gastric secretions, and the flux of gastric emptying. If one could use the motility scans to estimate the volume of gastric contents that is emptied through the pylorus as well as their

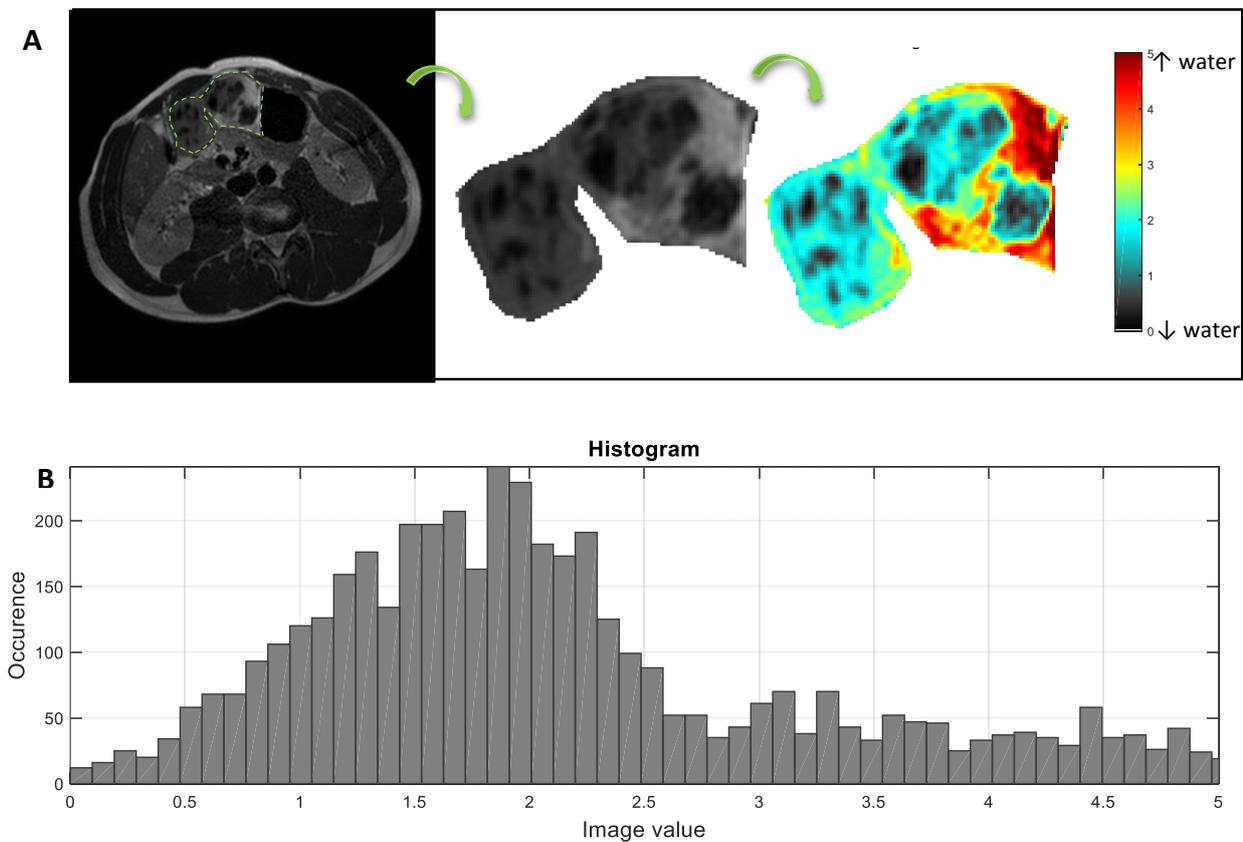


Figure 80 – Analysis of the intragastric chime. (A) Illustration of the differences in contrast observed in the chime. (B) Graphic representation of the occurrence of each level of contrast observed in (A).

frequency (using the motility scans acquired under free breathing conditions, which last 1 min 25 s), it could be possible to calculate the flux of secretions.

#### 4. FUTURE MRI STUDIES

MRI could be useful in so many experiments related to digestion! It would thus be presumptuous to say what could or should be done with this technique. With regards to our work, we may simply highlight that peas could work as a suitable probes for up to 2.5 – 3 hours. Future studies could therefore benefit from these findings and use them as markers of the gastric mixing.

Also, our initial preliminary tests have showed that the high resolutions achievable by MRI can enable to discern pasta spheres with a diameter of about 3 mm. It would certainly be interesting to capitalize on these preliminary works, and try to monitor the evolution of particle structure within the stomach. It is also noteworthy that it is possible to monitor *in vitro* digestion experiments with MRI. Doing so with the same MRI acquisition sequences as we used *in vivo*, would allow to correlate the digestion kinetics with the signal obtained by MRI. A more complete investigation of the evolution of the food relaxation times during the course of *in vitro* digestions could even be conducted. Ultimately, such data could be used to retrieve more complete information on the rate of nutrient hydrolysis and release within the stomach.



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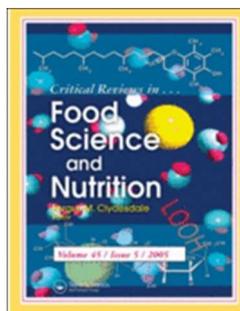
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# ANNEX

CORRELATION BETWEEN *IN VITRO* AND *IN VIVO* DATA ON  
FOOD DIGESTION. WHAT CAN WE PREDICT WITH STATIC  
*IN VITRO* DIGESTION MODELS  
*Review article (submitted version)*





**Correlation between in vitro and in vivo data on food digestion. What can we predict with static in vitro digestion models?**

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3 **Correlation between *in vitro* and *in vivo* data on food digestion. What can we**  
4 **predict with static *in vitro* digestion models?**  
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**Abstract**

During the last decade, there has been a growing interest in understanding the fate of food during digestion in the gastrointestinal tract in order to strengthen the possible effects of food on human health. Ideally, food digestion should be studied *in vivo* on humans but this is not always ethically and financially possible. Therefore simple static *in vitro* digestion models mimicking the gastrointestinal tract have been proposed as alternatives to *in vivo* experiments. On one hand, these models are extremely popular and widely used by the scientific community but, on the other hand, they are quite basic compares to the complexity of the digestive tract and it is essential before running experiments to know the type of information they can provide and perfectly understand their limitations. The objective of this article is to review a range of applications of *in vitro* digestion models, the parameters that were assessed and the physiological relevance of the data generated when compared to *in vivo* data. This is the result of a cooperative international effort made by some of the scientists involved in Infogest, an international network on food digestion.

## Introduction

There has been a growing interest from the scientific community in getting more information on the effect of food on human health. One strategy to reduce knowledge gaps is to focus on the fate of food during digestion in the gastrointestinal tract.

The fate of food in the GIT can be studied using several methods or models including static and dynamic *in vitro* models, animals and humans. Static *in vitro* digestion methods are particularly popular because they are easy to use, cheap and do not require specific equipment. However, a huge number of protocols differing in the experimental conditions (pH and duration of the different steps, amount of digestive enzymes and bile etc) have been proposed making the comparison of results between studies impossible. Recently, an international consensus was reached within the COST Action Infogest (<http://www.cost-infogest.eu/>) and a protocol published (Minekus et al., 2014) that has since been widely used internationally. Another major drawback of static *in vitro* digestion models is that they are oversimplified and do not take into account the dynamic aspects of the digestive process. Therefore, these models have been used to compare the digestion of related foods under the same conditions (Dupont et al., 2010b), to study the digestion of pure compounds (Benede et al., 2014a; Benede et al., 2014b; Dupont et al., 2010a) or to unravel the interactions between constituents at the molecular level. For instance, using static digestion models, Mandalari et al. demonstrated that interactions were occurring between one of the main milk protein, i.e.  $\beta$ -lactoglobulin, and phosphatidylcholine released by the stomach wall, protecting the protein from the action of pepsin during the gastric phase (Mandalari et al., 2009).

However, one can question the limits of these static *in vitro* digestion models to predict other parameters and limit the use of animal or human models. The objective of this opinion paper is to review a range of applications of *in vitro* digestion models, the parameters that were assessed and the physiological relevance of the data generated when compared to *in vivo* data. This is the result of a cooperative international effort made by some of the Infogest participants.

### 1 Estimation of the digestion of starch

Starch is the most important digestible polysaccharide in human nutrition usually accounting for 20-50% of the total energy intake. It is made up of two types of glucose polymers, amylose (15-20%) and amylopectin (80-85%), and is predominantly derived from plant seeds such as wheat, maize, rice, oats and rye (Caballero et al., 2012). Given its composition and the predominant role played in the human diet, starch is a major source of glucose that appears at relatively high concentrations in blood circulation during digestion (Butterworth et al., 2011).

Starch digestion is initiated in the oral cavity by salivary  $\alpha$ -amylase which starts hydrolyzing the glycosidic bonds of amylose and amylopectin (Bornhorst and Singh, 2012). Once the food bolus is swollen, starch hydrolysis by salivary  $\alpha$ -amylase can continue in the stomach until the pH lowers sufficiently to inactivate the enzyme (Bornhorst and Singh, 2012; Gropper and Smith, 2013). As no amylase is secreted into the stomach, the last stage of starch digestion occurs in the small intestine where pancreatic  $\alpha$ -amylase produces maltose, maltotriose and  $\alpha$ -limit dextrins. These intermediate products are finally hydrolyzed into glucose by specific "brush border enzymes" at the gut wall. Glucose is the final product of starch digestion and is very efficiently absorbed into the bloodstream (Gropper and Smith, 2013). The digestive process of starch containing meals can hence easily be followed *in vivo* by monitoring the rise and subsequent decrease of blood glucose concentrations. Such measurements allow the determination of the postprandial blood glucose response (GR), which

1  
2  
3 is defined as the change in blood glucose concentration in the 2h period that follows the start of a  
4 meal (ISO, 2010). This type of data is at the origin of a number of metrics used to classify the *in vivo*  
5 digestion of starch and other carbohydrate sources, the most popular of which is the Glycaemic  
6 Index (GI) (Dona et al., 2010). GI is a kinetic parameter first proposed in 1981 (Jenkins et al., 1981). At  
7 that time, nutritional recommendations for diabetes were based on food chemical composition only,  
8 but concerns that this was not fully representative of physiological effects induced by foods had  
9 started being raised. Jenkins et al. (1981) proposed the GI concept as a physiology related metric that  
10 could supplement such recommendations. Since then the popularity of GI increased as experts  
11 stressed the relevance of low GI diets to the prevention and management of diabetes and other  
12 health conditions including coronary heart disease and possibly obesity (Augustin et al., 2015).  
13 Nowadays, GI is defined as the incremental area under the blood GR curve (IAUC) after consumption  
14 of a portion of food containing 50 g (or in some cases 25 g) of available carbohydrates expressed as a  
15 percentage of the IAUC elicited by a portion of a reference food (white bread or glucose solution)  
16 with equivalent amount of available carbohydrate (ISO, 2010). GI is not considered to be a  
17 characteristic of the human being but rather a property of the food item itself, specifically, its ability  
18 to raise blood glucose (ISO, 2010).  
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23 The expensive (Dona et al., 2010) and time-consuming nature (Brand-Miller and Holt, 2004) of the *in*  
24 *in vivo* experiments poses obstacles to the GI use, and, along with other factors, these considerations  
25 have fueled research on *in vitro* assays to predict GI values or an equivalent metric (Brand-Miller and  
26 Holt, 2004; Dona et al., 2010). Indeed, shortly after proposing the GI concept, Jenkins and coworkers  
27 showed that there was a high correlation ( $r > 0.86$ ) between GIs of different foods and their *in vitro*  
28 digestion kinetics, as estimated with a mixture of human saliva and jejunal juice in dialysis bags  
29 (**Figure 1**) (Jenkins et al., 1982). Since then, several research teams have contributed to the  
30 development of *in vitro* static assays with the goal of accurately predicting the glycaemic response of  
31 foods. Such assays generally consist in mimicking the different phases of human digestion with  
32 commercially available enzymes. Most protocols comprise a simulated gastric phase followed by a  
33 small intestinal phase during which samples are collected to analyze the extent of carbohydrate  
34 digestion (Bjorck et al., 1994; Englyst et al., 1992; Gibson et al., 2011; Goñi et al., 1997; Monro et al.,  
35 2010). In certain cases, gastro-intestinal digestion can be preceded by an *in vivo* chewing phase  
36 (Bjorck et al., 1994; Monro et al., 2010) or an *in vitro* treatment that often replicates mechanical but  
37 not enzymatic oral conditions (Englyst et al., 1992; Gibson et al., 2011). An extensive review of the *in*  
38 *in vitro* protocols available to study starch digestion is outside the scope of the present review but can  
39 be found elsewhere (Dona et al., 2010; Woolnough et al., 2008). Here we will focus on a limited  
40 number of highly cited methodologies and their correlations with *in vivo* data.  
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45 One of the pioneer *in vitro* approaches to classify food carbohydrates in a way that reflected their  
46 digestion in the gut was performed by Englyst and coworkers (Englyst et al., 1992). The main  
47 innovative character of their study was the collection of samples at two distinct time-points of the  
48 intestinal phase of digestion (20 min and 120 min) to distinguish rapidly and slowly digestible starch  
49 fractions, a characteristic of starchy food that is still extensively used nowadays. Indeed, subsequent  
50 research carried out by the same team showed a significant correlation between the proportions of  
51 rapidly digestible starch of 39 food items and the corresponding GI values obtained from the *in vivo*  
52 literature ( $r = 0.76$ ) (Englyst et al., 1996). The reliability of this method as an indicator of the  
53 glycaemic response (GR) to foods was repeatedly confirmed (Araya et al., 2002; Ells et al., 2005; Seal  
54 et al., 2003). In the same period, the team of Goñi et al. (1997) was among the firsts to resort to a  
55 static *in vitro* digestion protocol, during which they monitored the time-course of *in vitro* starch  
56 hydrolysis of a series of starch containing foods. They concluded that their 90 min measurement had  
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3 the best correlation with *in vivo* GI values ( $r = 0.91$ ) and derived an equation to allow GI values to be  
4 predicted from this *in vitro* approach (Goñi et al., 1997). Other researchers have remarked that  
5 although it seems to overestimate the GI, this methodology is sensible enough to reproduce the  
6 overall trends observed *in vivo* (Ferrer-Mairal et al., 2012).  
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9 Although some researchers have found a proportional relationship between the quantity of ingested  
10 food and the glycaemic response (Englyst et al., 1999), most of *in vitro* protocols are standardized for  
11 fixed amounts of carbohydrate or food. These fixed portions are particularly advantageous for  
12 research purposes, yet they have an experimental character and differ from the food quantities that  
13 are usually consumed. In order to guide consumer choices based on food or diet effects, it is arguable  
14 that one should communicate the glycaemic response in grams per serving of food (Monro et al.,  
15 2010). This has raised questions on the ability of *in vitro* assays to predict glycaemic responses to the  
16 varying quantities and compositions of foods that are part of the human diet (Monro et al., 2010), *i.e.*  
17 GR rather than GI. As a matter of course, *in vitro* experiments overlook the fact that glycaemic  
18 responses to foods depend on the balance between blood glucose loading and disposal. As a matter  
19 of course, typical blood GRs elicited by carbohydrate-rich foods or meals comprise an initial increase  
20 of glucose concentration (loading) and is followed by a decrease to its initial level (disposal) which  
21 contrasts with the plateau obtained at the end of *in vitro* digestion experiments. With the aim of  
22 filling this gap, Monro et al. (2010) have proposed to estimate glucose disposal rates from *in vivo*  
23 blood GR data obtained by (Venn et al., 2006) for different glycaemic loads, and to subtract the so-  
24 estimated cumulative glucose disposal from the cumulated glucose release that is measured *in vitro*.  
25 This enables the construction of digestion curves that mimic *in vivo* blood GR curves for a better  
26 prediction of the *in vivo* glycaemic impact of foods (Monro et al., 2010). The correlation of the results  
27 obtained with this “*in vitro* model-assisted” approach with corresponding *in vivo* data on more than  
28 25 food items proved to be very good both when *in vivo* data were experimentally obtained as part  
29 of the study ( $r^2 = 0.88$ , **Figure 2**) (Monro et al., 2010) or collected from the literature ( $r^2 = 0.90$ )  
30 (Monro and Mishra, 2010).  
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36 Given the increasing evidences of health benefits associated with a low GI diet, the International  
37 Carbohydrate Quality Consortium has very recently reached a scientific consensus defining “an  
38 urgent need to communicate information on GI [...] to the general public and health professionals,  
39 through channels such as national dietary guidelines, food composition tables and food labels”  
40 (Augustin et al., 2015). In parallel, Monro and coworkers concluded that “valid measurement of  
41 glycaemic impact of foods may be obtained *in vitro*” (Monro et al., 2010) and can be applicable for  
42 “consumer and industry use” (Monro and Mishra, 2010). Although some researchers have reported  
43 discrepancies between *in vivo* and *in vitro* results (Berti et al., 2004; Brand-Miller and Holt, 2004;  
44 Casiraghi et al., 1992), overall, *in vitro* protocols do appear to be reliable indicators of *in vivo* glucose  
45 response (Araya et al., 2002; Ells et al., 2005; Englyst et al., 1996; Ferrer-Mairal et al., 2012; Goñi et  
46 al., 1997; Jenkins et al., 1982; Monro and Mishra, 2010; Monro et al., 2010; Seal et al., 2003).  
47 Employing *in vitro* digestion methods for characterizing the glycaemic impact of foods and labelling  
48 purposes is a question that remains to be debated in a larger scientific community. In any case,  
49 considering the practicality of *in vitro* assays, and their highly significant correlations with *in vivo*  
50 data, it certainly seems wise to continue optimizing these techniques and take advantage of them in  
51 research and early stages of product development.  
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## 55 **2 Assessing protein digestion**

### 56 ***Assessment of protein digestibility***

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3 Evaluating the quality of a dietary protein consists in determining how extensively this protein is  
4 digested and absorbed and its amino acids used by the human body. The most frequently used score  
5 to express the quality of a protein is the PDCAAS (Protein Digestibility-Corrected AA Score) which  
6 takes both the digestibility and the amino acid composition of the protein into account. Although the  
7 determination of the amino acid content of a protein is simple nowadays, an accurate and precise  
8 determination of its digestibility is still difficult. Digestibility of a protein is a key parameter of its  
9 bioavailability (Fuller and Tome, 2005), because it provides some information about the extent of  
10 digestion and absorption and the ability of the amino acids to be used. Digestibility (proportion of a  
11 protein absorbed from the digestive tract into the bloodstream) has been shown to significantly  
12 fluctuate according to the diet, especially between developed and developing countries (Gilani et al.,  
13 2005). Protein digestibility is measured indirectly by the quantification of residual nitrogen at the  
14 extremity of the digestive tract. Nevertheless, this residual nitrogen includes the non-digested  
15 dietary nitrogen but also the endogenous nitrogen. Thus, the quantification of the total nitrogen  
16 (exogenous + endogenous) corresponds to the apparent digestibility whereas the estimation of only  
17 the exogenous nitrogen corresponds to the true digestibility (Fuller and Tome, 2005). Determination  
18 of the true digestibility is more complicated to perform and requires the labelling of the dietary  
19 proteins or of the host (to label endogenous proteins). Assessment of ileal digestibility is now  
20 recognized as more relevant than determination of fecal digestibility (Darragh and Hodgkinson, 2000;  
21 Fuller and Tome, 2005; Moughan, 2003) that can be modulated by the metabolic activity of the  
22 intestinal microbiota.  
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27 Several groups have tried to determine protein digestibility through *in vitro* approaches. One of the  
28 advantages in using *in vitro* models to assess digestibility is that endogenous secretions are very  
29 limited in these models so that the data generated should be comparable to the true digestibility. In  
30 a pioneer work, Saunders et al. showed a good correlation between *in vitro* digestibility determined  
31 on alfalfa protein concentrates with a pepsin-pancreatin or a pepsin-trypsin method and *in vivo* data  
32 collected in a rat trial ( $r^2=0.914$ ) (Saunders et al., 1973). Satisfactory results were also obtained on  
33 soybean, lupine and rapeseed meal proteins. Indeed, true digestibility assessed on these proteins on  
34 rats showed a significant correlation with the degree of hydrolysis ( $r^2=0.663$ ,  $p<0.001$ ) and nitrogen  
35 digestibility ( $r^2=0.776$ ,  $p<0.001$ ) determined *in vitro* (Rozaan et al., 1997). Finally, Kitabatake and  
36 Kinekawa found the same behavior of native and heat-denatured milk whey protein  $\beta$ -lactoglobulin  
37 *in vitro* and in the stomach of rats (Kitabatake and Kinekawa, 1998). The protein was shown to be  
38 fully resistant to proteolysis when native but extensively hydrolyzed when heat-denatured.  
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42 However, using an *in vitro* models, other authors found the same ranking between the *in vitro*  
43 digestibility of different protein sources and *in vivo* but the values between the 2 approaches  
44 remained significantly different (Cho and Kim, 2011). These differences might be explained by an  
45 inadequate selection of the parameters of the *in vitro* digestion model used in this experiment  
46 making it physiologically irrelevant.  
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#### 50 ***In vitro/in vivo comparison of milk protein digestion***

51 One of the major goals of the Infogest COST Action was the harmonization of *in vitro* digestion  
52 experiments. Towards this end, the consensus static protocol by Minekus et al (Minekus et al., 2014),  
53 based on physiological *in vivo* data was developed. The success of the harmonization was  
54 experimentally tested in several inter-laboratory trials, using skim milk powder (SMP) as standardized  
55 food matrix. Thus, these efforts not only show the comparability of different digestion protocols, but  
56 also serve as a basis for gathering information about milk protein digestion. The participants were  
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3 asked to digest the SMP first with their in-house method and second with the consensus protocol.  
4 Protein decomposition was analyzed at different levels of hydrolysis, from proteins, to peptides,  
5 down to free amino acids. Gel electrophoresis, performed with samples after the gastric- and the  
6 intestinal phase of *in vitro* digestion, showed a highly heterogeneous pattern, when the participants  
7 applied their in-house methods (data not shown). In contrast to that, the protein patterns of the  
8 samples digested with the harmonized protocol were much more homogeneous (**Figure 3**). Although,  
9 in two of these samples, protein hydrolysis was decreased after the gastric phase (**Figure 3**, lines 6,  
10 7), compared to the five other samples (**Figure 3**, lines 1-5). This difference was explained by a lower  
11 pepsin activity, originating from problems with pepsin activity measurement prior to *in vitro*  
12 digestion. However, at the end of the digestion, the protein patterns were very similar and no intact  
13 milk proteins were detectable on gels with the harmonized protocol (**Figure 3**, intestinal phase),  
14 whereas some of the samples digested with the in-house methods still had intact  $\beta$ -lactoglobulin  
15 present (data not shown). Analysis of peptides by mass spectrometry showed that the in-house  
16 protocols in general had a lower hydrolysis rate after the gastric phase compared to the harmonized  
17 model. Moreover, at the end of the digestion, the peptide patterns were very similar in all the  
18 samples digested with the harmonized protocol. The specific digestion patterns for the most  
19 abundant milk proteins were visualized by an amino acid counting method, where for the frequency  
20 of each amino acid, identified within a specific milk peptide, a color code was attributed and aligned  
21 within the protein sequence e.g.  $\beta$ -casein (**Figure 4**). This method reveals that the different milk  
22 proteins are not randomly but very specifically hydrolyzed during the digestion phases. Considering  
23 the five most abundant milk proteins, ( $\beta$ -,  $\alpha$ s1-,  $\alpha$ s2-,  $\kappa$ -casein, and  $\beta$ -lactoglobulin), and using their  
24 characteristic digestion data, a Spearman correlation was calculated and the improvement in sample  
25 comparability could be confirmed at the peptide level as well in the harmonized protocol (Egger et  
26 al., 2016), compared to the in-house methods.

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32 The major conclusion of this work was that the harmonized protocol indeed improved the  
33 comparability of experimental results. However, if the *in vitro* process indeed was comparable to the  
34 *in vivo* situation, was still an open question. To answer this second and important question, an *in vivo*  
35 pig trial was performed, feeding pigs the exact same SMP that was previously digested with the *in*  
36 *vitro* models. The pigs were fed three times, 6-, 3-, and 1.5h prior to sacrifice, respectively with the  
37 SMP; the samples were collected at four different zones of the digestive tract. A gastric (stomach)-,  
38 duodenal-, upper intestinal- (Int 1), and lower intestinal- (Int2) sample, was collected in a total of  
39 eight pigs. Protein degradation, generation of peptides and free amino acids were analyzed with the  
40 same methods as for the previous inter-laboratory *in vitro* studies. The similarity of the different  
41 investigated digestion models was assessed in a partial least square analysis (PLS), considering the  
42 peptide analysis. An average was calculated for each sampling zone (**Figure 5**, pig- stom, duodenum,  
43 Int1, and Int2). The progression in pig *in vivo* digestion was clearly visible as indicated with the arrow.  
44 The samples from the gastric phase of the harmonized protocol all cluster close to the pig stomach  
45 sample (**Figure 5**, light blue) and the samples from the harmonized intestinal phase cluster below the  
46 pig Int 2 average (**Figure 5**, dark blue), indicating that they are possibly to some extent further  
47 digested than the pig Int 2 sample. The samples from the gastric- or intestinal- phase of the in-house  
48 protocols are spread in two groups in the lower part of the PLS analysis, showing that their results  
49 are on one hand very variable between each other and more importantly, different from the *in vivo*  
50 samples. In conclusion, it can be stated, that by setting the harmonized protocol in operation, two  
51 major goals were achieved. First, the comparability of experimental results between labs and  
52 countries was improved and even more importantly, the two endpoints (gastric and intestinal phase)  
53 indeed reflect the *in vivo* pig situation considering the digestion of proteins. Additional work is  
54 needed to compare other nutrients in a similar way.

### ***Comparison of bioactive sequences found in vitro and in vivo***

The role of protein digestion in health is undeniable, not only from the nutritional point of view but due to the benefit of released bioactive peptides on health. In this context, it is generally observed that particular stable regions from milk proteins to gastrointestinal hydrolysis are precursors of sequences described as bioactive peptides. This raises the question of the physiological significance of these regions that, in some cases, have deserved to be considered as “food hormones” (Teschemacher et al., 1997). It is also worth considering that the peptide active form is sometimes released only after digestion. An example is fragment 169-175 from  $\beta$ -casein KVLVPVQ, that possessed a low *in vitro* ACE-inhibitory activity, but produced a significant antihypertensive effect after its oral administration to spontaneously hypertensive rats. This sequence was shown to lose the Gln at C-terminal end during simulated gastrointestinal digestion, giving rise to KVLVPV, which is presumably the *in vivo* active form (Maeno et al., 1996).

Some studies have been selected to determine whether bioactive sequences identified after *in vitro* digestion are illustrative of those found *in vivo*. In these studies, opioid, antihypertensive and caseinophosphopeptides (CPPs) appear as the best represented cases. Through the reviewed years, the level of evidence and technical advance has increased from finding immunoreactive materials to peptide sequencing.

Bovine  $\beta$ -casomorphins are opiate-like acting fragments from bovine  $\beta$ -casein comprising the 60-66 ( $\beta$ -casomorphin-7) or C-terminally shortened fragments thereof and were originally isolated from an enzymatic casein digest (Brantl et al., 1979). They show the common N-terminal amino acid sequence YFPF and behave preferentially as  $\mu$ -type opioid agonists (Brantl et al., 1981). In adult volunteers after ingestion of milk,  $\beta$ -casomorphin-7 and reduced amounts  $\beta$ -casomorphin-4 or -6 immunoreactive materials occurred in the duodenal section of the small intestine. The main component of the immunoreactive material was larger than  $\beta$ -casomorphin-7 and was identified as its precursor (Svedberg et al., 1985). Indeed, this fragment was later isolated from the duodenal chyme of mini-pigs after feeding with the milk protein casein and was referred to as  $\beta$ -casomorphin-11 which presented four additional amino acids at the C-terminal side (Meisel, 1986). The low amount of this peptide in the duodenum was attributed to rapid brush border degradation and/or high absorption rate of the fragments. More recently, in duodenum of mini-pigs fed gelled skim milk, two additional precursors of  $\beta$ -casomorphin-7,  $\beta$ -casein f(58-72) and  $\beta$ -casein f(59-67), were found (Barbe et al., 2014). In human jejunum, upon casein ingestion,  $\beta$ -casomorphin-7, numerous precursors of this peptide, and the short form,  $\beta$ -casomorphin-5, were reported (Boutrou et al., 2013).

Jinsmaa and Yoshikawa examined the conditions necessary for the release of  $\beta$ -casomorphins during *in vitro* gastrointestinal digestion (Jinsmaa and Yoshikawa, 1999). Pepsin cleaves the Leu<sup>58</sup>-Val<sup>59</sup> peptide bond and leucine aminopeptidase removes the Val<sup>59</sup>, rendering the Tyr at the N-terminus. For the carboxyl terminus, pancreatic elastase was found responsible for the hydrolysis of the peptide bond Ile<sup>66</sup>-His<sup>67</sup> giving rise to  $\beta$ -casomorphin-7 (YFPFGPI). This means that the inclusion of pancreatic enzymes other than trypsin and chymotrypsin in the *in vitro* protocol will determine the generation the specific and physiological cleavages releasing  $\beta$ -casomorphin-7 from its precursors.  $\beta$ -casomorphin-7 has been found upon sequential digestion of infant formulas with pepsin and Corolase PP® under conditions simulating infant digestion, with gastric pH 3.5 (Hernandez-Ledesma et al., 2004). De Noni, by hydrolysing  $\beta$ -casein under similar conditions, has also reported its occurrence; the highest amount being recovered when the peptic attack with pepsin was conducted at pH 2.0 (De Noni, 2008). Some precursors included in the  $\beta$ -casein 57-68 region were found when cheese or milk were digested with pepsin and pancreatin (Hernandez-Ledesma, Quiros et al. 2007;

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3 Sanchez-Rivera, Diezhandino et al. 2014) or with human gastrointestinal secretions (Qureshi,  
4 Vegarud et al. 2013). Interestingly, an additional hydrolysis step with brush border peptidases  
5 released  $\beta$ -casomorphin-7 (Picariello et al., 2015).  
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7 Neocasomorphin-6, f(114-119) from  $\beta$ -casein (YPVEPF) was first identified in a gastrointestinal *in*  
8 *vitro* digest (Jinsmaa and Yoshikawa, 1999) and more recently, it has been reported in human  
9 jejunum after milk ingestion (Boutrou et al., 2013). Likewise, this sequence appeared in duodenal  
10 effluents of mini-pigs after ingestion of liquid heated milk (Barbe et al., 2014). Neocasomorphin has  
11 been also identified after *in vitro* digestion of milk and cheese (Sanchez-Rivera et al., 2014), and  
12 under both adult and infant conditions employing physiologically relevant settings (Dupont et al.,  
13 2010a; Hernandez-Ledesma et al., 2007). In contrast, other authors have reported the release of a  
14 precursor,  $\beta$ -casein f(114-124), (Picariello et al., 2010).  
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17 Antihypertensive peptides are probably the most numerous bioactive compounds of proteinaceous  
18 nature, milk being the main source of peptides with this activity. The complete correspondence  
19 between *in vitro* activity (inhibition of the angiotensin converting enzyme, ACE) and *in vivo* effect  
20 (blood pressure lowering) has not been demonstrated, and it has been attributed to peptide  
21 degradation during gastrointestinal digestion or to a poor bioavailability. Hence, the presence of  
22 active sequences in digestion effluents marks the first step to know their bioaccessibility and  
23 potential absorption (Martinez-Maqueda et al., 2012).  
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26 **Table 1** shows some antihypertensive peptide sequences that have been reported along the different  
27 parts of the intestinal tract in *in vivo* studies, after consumption of bovine milk or casein in the case  
28 of adults, and human milk or infant formula in the case of newborns  
29

30 The sequences KVLPIQ and VVPYPQR from human milk, that correspond to the Ile- and Val-replaced  
31 antihypertensive peptides  $\beta$ -casein f(169-175), KVLVPVQ and f(177-183), AVYPYQR, respectively,  
32 have been identified in the stomach of newborns but also in the undigested milk, which suggested  
33 that proteolysis begins during lactation or in the time before feeding (Dallas et al., 2014). In contrast,  
34 many unique peptides absent in maternal milk have been found in gastric digests, which suggests  
35 that extensive proteolysis also occurs in the term infant stomach. The  $\beta$ -casein sequence 133-138,  
36 LHLPLP, was reported in the jejunum of volunteers ingesting a commercial casein (Boutrou et al.,  
37 2013). Precursors of this sequence, f(132-140) and f(130-140), are found in the duodenum of pigs  
38 upon ingestion of heated milk and acid gels prepared from milk (Barbe et al., 2014). This sequence  
39 belongs to a highly conserved  $\beta$ -casein region in different mammals, resistant to digestion due to the  
40 abundance of Pro residues. Numerous related peptides have been found after simulated  
41 gastrointestinal digestion of human (Hernandez-Ledesma et al., 2007) and bovine milk proteins  
42 (Benede et al., 2014b; Qureshi et al., 2013; Sanchez-Rivera et al., 2014). The inclusion of the brush  
43 border hydrolases on the digestion model favored the release of different forms with Pro<sup>138</sup> at the C-  
44 terminus (Picariello et al., 2015). From this sequence LHLPLP, various fragments HLPLP, HLPL, LPLP,  
45 HLP, LPL and PLP have shown antihypertensive activity (Quiros et al., 2007; Sanchez-Rivera et al.,  
46 2016).  
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50 In the case of  $\alpha_{s1}$ -casein f(143-149), AYFYPEL was found in stomach and duodenum of humans after  
51 milk or yogurt ingestion (Chabance et al., 1998), while in duodenum the reported form was f(144-  
52 149), YFYPEL. These peptides have been identified as antihypertensive in a casein hydrolysate  
53 (Contreras et al., 2009; Sanchez-Rivera et al., 2014) but recently, its ability to interact with opioid  
54 receptors and exert an agonist activity has been described (Fernandez-Tome et al., 2016). Different  
55 peptide forms from this region have been found after gastric digestion of milk under dynamic  
56 conditions (Sanchez-Rivera et al., 2015) and after *in vitro* digestion of milk (Dupont et al., 2010b),  
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3 cheese (Sanchez-Rivera, Diezhandino et al. 2014) and infant formulas (Hernández-Ledesma et al.,  
4 2007).

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6 Bovine caseins are also a source of caseino-phosphopeptides (CPPs) that have been proposed to  
7 prevent the precipitation of metal ions at alkaline pH in the distal small intestine (Meisel and  
8 FitzGerald, 2003). Interestingly, the phosphorylated regions are relatively resistant to hydrolysis  
9 during gastrointestinal digestion. Boutrou et al. showed that in addition to phosphorylation, the  
10 presence of bound minerals also prevents CPP from hydrolysis (Boutrou et al., 2010). The analysis of  
11 intraluminal digests of Fe- $\beta$ -casein f(1-25) in vascularized rat loop model evidenced that the  
12 fragment, which contains the four phosphoserines, f(15-25), is resistant to proteinases. In the human  
13 stomach, phosphorylated fragments from  $\beta$ -casein 29-41, 33-44, 30-41, 6-17 and 29-40 were  
14 identified after milk ingestion. In human duodenum, related forms from these sequences were found  
15 (Chabance et al., 1998). In pig duodenum, CPPs from  $\beta$ -casein together with monophosphorylated  
16 sequences from  $\alpha_{s1}$ - and  $\alpha_{s2}$ -casein have been reported. However, other phosphorylated regions  
17 exhibiting the cluster sequence S(P)S(P)S(P)EE could not be identified under the analysis conditions  
18 due to the low ionization capacity of these multiple phosphorylated forms (Barbe et al., 2014). In  
19 human jejunum and ileum, related sequences have been found, which implies that phosphopeptides  
20 can survive the prolonged intestinal passage *in vivo* (Boutrou et al., 2013; Meisel et al., 2003).

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22 Phosphopeptide formation and resistance has been followed during simulated gastrointestinal  
23 digestion. The use of sequential hydrolysis with pepsin and pancreatin has been shown to release  
24 phosphorylated sequences previously reported *in vivo*, also in infant formula employing suitable  
25 conditions (Miquel et al., 2005). Moreover, isolation of the CPP fraction by selective precipitation or  
26 TiO<sub>2</sub> chromatography allowed to identify several sequences containing the phosphorylated cluster  
27 (Miquel et al., 2006; Picariello et al., 2010). Adt et al. compared the number of sites of  
28 phosphorylation of CPPs in non-digested and digested Beaufort cheese samples, and found a  
29 decrease in the number of polyphosphorylated peptides (Adt et al., 2011). Still, an important part of  
30 the identified peptides contained the characteristic cluster sequence S(P)S(P)S(P)EE. The fact that the  
31 analysis was performed on a selective precipitate highlights the importance of this step on the  
32 thorough identification of all possible forms.

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34 Altogether, these results denote that *in vitro* digestion under different conditions is able to release  
35 certain gastrointestinal resistant peptides found in human digests. In addition the above examples  
36 highlight that not only the enzymes but their concentration, as well as, ion composition and pH of the  
37 digestive juices greatly affect the observed results and highlight the importance of employing  
38 parameters deduced from human determinations to obtain physiologically relevant results.

#### 39 ***What happens when proteins are cross-linked at the interface of an emulsion?***

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41 Recently, there were extensive studies carried out on how the digestion of interfacial protein in  
42 emulsion could be controlled by targeted enzymatic modification of the adsorbed protein layer  
43 (Juvonen et al., 2015; Macierzanka et al., 2011; Macierzanka et al., 2012). The main hypothesis was  
44 that a change in the pattern of proteolysis might alter colloidal phase behaviour of the emulsion in  
45 the stomach and the small intestine, which ultimately might influence both satiety and food intake.  
46 Triglyceride oil-in-water emulsions were produced with the milk protein sodium caseinate (NaCN),  
47 which was then modified after emulsification by enzymatic crosslinking with microbial  
48 transglutaminase (TG). The enzyme was selected because of its common use in food and  
49 pharmaceutical applications to strengthen or otherwise modify protein networks (Dickinson, 1997;  
50 Fontana et al., 2008). The work on the effect of the TG crosslinking was divided into three parts: (i)  
51 development of the emulsification and the crosslinking conditions (Macierzanka et al., 2011), (ii) *in*  
52 *vitro* static digestion studies, including an interfacial characterisation of changes in the adsorbed  
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3 protein layer during the digestion (Macierzanka et al., 2012), and (iii) *in vivo* human studies on  
4 postprandial responses (Juvonen et al., 2015).

5 The *in vitro* studies (Macierzanka et al., 2012) focused on the effect of enzymatic structuring of NaCN  
6 stabilised emulsion on the pattern of simulated gastro-duodenal digestion of the protein, the  
7 interactions with physiologically relevant surfactants (phosphatidylcholine, PC; bile salts, BS), and the  
8 resulting microstructural transformations of the emulsion as it passes through different stages of *in*  
9 *vitro* digestion. By modifying the structure of the NaCN interfacial layer in emulsions, the aim was to  
10 alter the pattern and/or the rate at which the protein was digested under *in vitro* conditions  
11 mimicking physicochemical conditions of the human upper GIT. Significant differences were found  
12 between the rates of digestion of NaCN crosslinked in emulsion (adsorbed protein) and in solution.  
13 The crosslinking of interfacial protein delayed proteolysis and prevent the emulsion from  
14 destabilising under simulated gastric conditions. In emulsion, the digestion of a population of  
15 polypeptides of  $M_r$  ca. 50–100 kDa was significantly retarded through the gastric digestion. The  
16 persistent interfacial polypeptides maintained the original emulsion droplet size and prevented the  
17 system from phase separating. In contrast, a rapid pepsinolysis of adsorbed but non-crosslinked  
18 NaCN and its displacement by PC led to emulsion destabilisation. The results suggested that the  
19 changes in protein accessibility and structure induced by adsorption to the oil–water interface and  
20 TG crosslinking might render the protein less susceptible to hydrolysis in the stomach. After passing  
21 from the gastric to the duodenal conditions, the interfacial properties of emulsion droplets were  
22 found to be almost exclusively governed by the BS. The interfacial BS also had a dominant role in  
23 determining diffusion of emulsion droplets through the small intestine mucus.

24 In order to determine whether the TG-induced structuring of the interfacial protein layer in emulsion  
25 plays a role in controlling postprandial physiological responses, a human clinical trial was carried out  
26 using isoenergetic and isovolumic emulsions stabilised with either sodium caseinate (NaCN-E) or TG-  
27 crosslinked sodium caseinate (TG-NaCN-E) (Juvonen et al., 2015). Blood samples were collected from  
28 the participants at baseline and during the 6-h period postprandially for the determination of serum  
29 TAG and plasma non-essential fatty acids (NEFA), cholecystokinin (CCK), glucagon-like peptide 1 (GLP-  
30 1), glucose and insulin responses. It was found that the TG structuring of the emulsion interface  
31 affected early postprandial metabolic and hormonal responses as reflected by the different  
32 postprandial glucose, insulin and CCK profiles. However, the crosslinking did not have a significant  
33 effect on the gastric emptying (GE) rate or the overall postprandial lipid digestion after the ingestion  
34 of the test emulsions. The *in vitro* study (Macierzanka et al., 2012) showed that the microstructures  
35 of NaCN-E and TG-NaCN-E produced with 1% (w/w) NaCN can be very similar to each other,  
36 producing evenly sized oil droplets that are stable to coalescence throughout the course of the *in*  
37 *vitro* digestion. This could suggest that the GE rate would not be significantly affected by the  
38 crosslinking of the interfacial protein. Indeed, the *in vivo* Magnetic Resonance Imaging showed  
39 comparable phase separation for the two emulsions in the stomach, which was probably the reason  
40 for the similar GE rates observed (Juvonen et al., 2015). Glucose and insulin blood profiles were  
41 lower after consuming TG-NaCN-E than NaCN-E, which suggested that protein was released from the  
42 TG-NaCN-E with a lower rate during the early postprandial phase when compared to the NaCN-E.  
43 Plasma glucose concentration decreased significantly more after the ingestion of TG-NaCN-E than  
44 after the ingestion of its non-crosslinked counterpart. As the GE rates were comparable between the  
45 test emulsions, the difference observed in the glucose and insulin profiles most probably resulted  
46 from the different release and/or absorption rates of the nutrients during the small intestinal phase  
47 due to the different initial digestion behaviours of the test emulsions. The postprandial secretion of  
48 CCK and GLP-1 was stimulated after consuming the two emulsions, but only the CCK profile differed  
49 significantly between the emulsions, showing a stronger increase after the ingestion of TG-NaCN-E  
50 than NaCN-E. Those results suggest that even a subtle structural modification of the interfacial  
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3 protein layer of fat droplets in emulsion can alter the early postprandial profiles of glucose, insulin  
4 and CCK through decreased protein digestion without affecting significantly on GE or overall lipid  
5 digestion.  
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### 7 ***Effect of processing on protein digestion – the example of meat***

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9 The effect of cooking on meat protein digestion has been investigated using both *in vitro* and *in vivo*  
10 approaches (Bax et al., 2013a; Bax et al., 2013b). Although, meat pieces and cooking conditions were  
11 not the same, some interesting conclusions can be drawn from the comparison of the obtained  
12 results. The *in vitro* approach consisted of a static digestion miming both gastric and intestinal  
13 digestion. For both compartments the kinetics was described and interpreted using a modelling  
14 approach allowing the evaluation of the maximal rate of digestion, the time at which it was observed,  
15 and the maximal degradation. *In vivo*, true ileal digestibility of proteins was measured using <sup>15</sup>N  
16 labeled meat in cannulated mini-pigs, and digestion rate was evaluated from the kinetics of amino  
17 acids appearance in blood. The main data of the 2 studies are presented in **Table 2**.  
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21 Whatever the type of meat (beef, pork or poultry), the muscle composition is essentially driven by  
22 contractile proteins (actin and myosin) and sarcoplasmic proteins (myoglobin), that are well  
23 conserved across species. The cooking temperatures were also slightly different, but for both meats  
24 it can be equated to medium and well-done meat. The comparison of the *in vitro* and *in vivo* data  
25 thus makes sense. Both approaches showed a decrease in the digestion rate with the more drastic  
26 cooking conditions. This is particularly true for pepsin digestion which is probably, with gastric  
27 emptying, one of the main determinants of protein digestion rate. The *in vitro* static model used was  
28 not adapted to measure intestinal protein digestibility. For that, instead of measuring OD increase,  
29 the measurement of non-digested proteins would have been required. Furthermore, in order to  
30 better mimic the *in vivo* intestinal digestion, *in vitro* intestinal digestion would have been conducted  
31 on samples that underwent different pepsin digestion duration. Nevertheless, in agreement with the  
32 true ileal digestibility observed *in vivo*, the maximal degradation observed *in vitro* after digestion with  
33 pancreatic enzymes was not different according the cooking conditions. The effect of meat cooking  
34 on peptides release from proteins during digestion has been investigated using both *in vitro* and *in*  
35 *vivo* approaches (Sayd et al., 2016). For example, in the case of myoglobin, a total of 34 peptides  
36 were identified *in vitro* and 14 *in vivo*, with 4 with a same amino acid sequence. Interestingly, the  
37 protein presented the same area not degraded when using both approaches (**Figure 6**).  
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39 From this comparison, it can be concluded that the *in vitro* model properly ranks solid foods  
40 according to their digestion rate. It should be noticed that this good correlation was observed with  
41 minced meat. As the chewing efficiency greatly affects the *in vivo* meat protein digestion rate  
42 (probably through modification in gastric emptying), the *in vitro* digestion rate should however be  
43 considered as 'potential'. Regarding the overall digestion efficacy, the *in vitro* static approach proves  
44 to be a useful tool to compare the effects of food processing, however it does not provide a  
45 digestibility measurement as such.  
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### 50 **3 Bioaccessibility & bioavailability of micronutrients and phytochemicals**

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52 Simulated in-vitro digestion has, in addition to macronutrients, also been carried out with a large  
53 number of micronutrients and phytochemicals, i.e. compounds that may have beneficial health  
54 effects but which are not strictly essential. Micronutrients comprise minerals, trace elements and  
55 vitamins, while the group of phytochemicals or secondary plant compounds is much larger, including  
56 e.g. phytosterols, carotenoids, polyphenols, glucosinolates, triterpenes, and many more. In vitro  
57 digestion has been employed for many micronutrients and phytochemicals, including trace elements  
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3 (iron, zinc), minerals (calcium, magnesium), carotenoids, a range of polyphenols, phytosterols  
4 (Moran-Valero et al., 2012), vitamins including B6, B12, E, D, (Etcheverry et al., 2012), and other  
5 dietary constituents such as cholesterol (Bohn et al., 2007). In the following section, we will focus on  
6 the most prominently examined compounds for which sufficient data is available. For this purpose,  
7 the focus will rest on iron as a trace element, carotenoids as vitamin A precursors and the most  
8 prevalent lipo-soluble phytochemicals, and polyphenols as the most abundant water-soluble  
9 phytochemicals. In addition, their dietary intake has been related to the prevention of micronutrient  
10 deficiencies (vitamin A, iron), and chronic disease prevention (carotenoids, polyphenols).

### 11 ***Carotenoids – micronutrients and most abundant lipo-soluble phytochemicals***

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14 Carotenoids as lipophilic constituents differ in their digestion behavior from water-soluble  
15 compounds as they require incorporation into mixed micelles, consisting of bile acids, partially  
16 digested lipids (mono-, diglycerides), phospholipids, and other lipophilic/amphipathic constituents.  
17 Thus, bioaccessibility is equivalent to the amount of carotenoids that are incorporated into mixed  
18 micelles in the aqueous phase following oro-gastro-intestinal digestion. For assessment of  
19 bioaccessibility, centrifugation and filtration (through 0.2  $\mu\text{m}$  or even 0.02  $\mu\text{m}$  filters) is typically  
20 carried out (Corte-Real et al., 2014), to remove carotenoids trapped in oil or present in the  
21 precipitate. Dialysis (through a semi-permeable cellulose membrane) may also be carried out but has  
22 shown to produce lower results (Veda et al., 2006). As carotenoids are typically consumed in form of  
23 fruits and vegetables, *in vitro* studies have focused on these food items, which are low in starch, and  
24 thus the oral digestion phase has often been left out. It is likewise assumed that carotenoids are  
25 mostly absorbed in the small intestine, and that colonic metabolism and uptake is negligible, though  
26 very little data is available on this topic (Alminger et al., 2014; Bohn et al., 2015). Additional  
27 endpoints that have been determined *in vitro* include total recovery (Failla et al., 2008), accounting  
28 for the presence of non-degraded but not absorbable carotenoids, and carotenoid retention after  
29 colonic fermentation (Goni et al., 2006; Kaulmann et al., 2015). Often, digesta have further been  
30 investigated for cellular uptake, i.e. coupling *in vitro* digestion with cellular uptake or transport  
31 models of the small intestinal epithelium, typically based on Caco-2 cell models (Biehler et al., 2011a;  
32 Corte-Real et al., 2016), reflecting intestinal enterocytes, taking into account cellular uptake,  
33 intracellular transport, and further sequestration, as well as potential metabolism/cleavage.

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As incorporation into micelles is paramount and depends on the presence of bile salts, pancreatic  
lipase, and lipids during digestion (Bohn, 2008; Borel, 2003), it is important that *in vitro* methods take  
these considerations into account, i.e. supplying sufficiently high enzyme and bile salt concentrations  
(Biehler and Bohn, 2010). Even though additional factors influence the bioavailability of carotenoids,  
e.g. cellular uptake, transport in the enterocyte, secretion to chylomicrons, re-distribution of  
carotenoids in lipoproteins (Borel, 2012; Reboul and Borel, 2011), it is believed that bioaccessibility  
constitutes the most influential factor. Thus, determination of bioaccessibility *in vitro* should  
reasonably well reflect bioavailability *in vivo*.

Bioavailability of carotenoids *in vivo* is typically determined by analyzing the most recently absorbed  
carotenoids that appear in the plasma, i.e. in the triacyl-glycerol rich lipoprotein (TRL) fraction, rich in  
chylomicrons. Mostly, the area-under-time-curve (AUC, for 10-12 hours) above baseline (before test  
meal intake) is determined as a marker for bioavailability, which can be translated into fractional  
absorption (Bohn et al., 2013; O'Neill and Thurnham, 1998).

Only a few studies have attempted to compare results from *in vitro* to *in vivo* studies, with generally  
promising conclusions, i.e. a reasonable correlation between the two. Reboul et al. (2006) compared  
fractional incorporation into micelles of alpha-and beta-carotene as well as of lutein and lycopene

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3 from different test meals following *in vitro* digestion to earlier conducted *in vivo* incorporation into  
4 micelles from similar test meals, obtained by the same group (Tyssandier et al., 2003). In general, a  
5 high correlation ( $R=0.90$ ,  $P=0.038$ ) was encountered, suggesting that the bioaccessibility results *in*  
6 *vitro* in tendency reflect *in vivo* bioaccessibility, pointing out that indeed intestinal solubility is a  
7 crucial parameter for carotenoid bioavailability (**Figure 7**). Furthermore, the authors compared their  
8 own bioaccessibility results for lycopene, beta-carotene and lutein with bioavailability results (TRL-  
9 AUC values) from other authors, employing similar test meals, finding a high overall correlation of  
10  $R=0.98$  ( $P<0.001$ , **Figure 8**), with a better correlation for beta-carotene ( $R=0.998$ ) compared to  
11 lycopene ( $R=0.54$ ), perhaps due to the lower availability of lycopene in general. However, it should  
12 also be noted that up to 8-fold differences occurred between bioavailability and bioaccessibility, and  
13 that individual values can significantly deviate from an average correlation. These differences may be  
14 due to factors related to absorption and further transport, which can greatly vary between  
15 individuals. Similarly, in a study by Alminger et al. (2012), bioaccessibility of lycopene and beta-  
16 carotene from soups containing tomato, broccoli and carrots, was compared to human bioavailability  
17 (determined via plasma concentrations after several weeks of consumption), and found generally  
18 comparable results for beta-carotene ( $R=0.93$ ) and 5-cis lycopene ( $R=0.87$ ) for various types of soups.  
19 Interestingly, cellular uptake equally well correlated with *in vivo* data. Recent studies have shown  
20 that inter-individual absorption of lutein (Borel et al., 2014), lycopene (Borel et al., 2015b) and beta-  
21 carotene (Borel et al., 2015a) depended also on the presence of single nucleotide polymorphisms  
22 (SNPs), related e.g. to carotenoid transporters or cleavage enzymes (Borel, 2012), which are not  
23 accounted for by *in vitro* studies.

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28 As not all of the bioaccessible fraction may be taken up and further transported and absorbed by  
29 intestinal lining, bioaccessibility, at least for the better soluble xanthophylls, may tend to  
30 overestimate bioavailability, as also seen in dynamic models (Van Loo-Bouwman et al., 2014; Van  
31 Loo-Bouwman et al., 2010). For example, Unlu et al. (2005) reported bioavailability of lutein from  
32 spinach/mixed salad (when served with sufficient amounts of fat) of 28% while *in vitro*  
33 bioaccessibility from another type of spinach was reported as 58% (Biehler et al., 2011b). For  
34 lycopene from tomato juice, human bioavailability was calculated as 3.1% in one trial (Bohn et al.,  
35 2013) while bioaccessibility was in a similar range (2.9% et al., Corte-Real and Bohn, unpublished),  
36 though again, based on different juices.

### 37 38 39 40 41 **Polyphenols - water soluble phytochemicals**

42 Unlike carotenoids, most polyphenols (with few exceptions such as resveratrol and curcumin), are  
43 reasonably well water-soluble. Thus, their bioaccessibility does not rely on micellization, but rather  
44 on release from the matrix and their staying in solution in the aqueous phase, as some polyphenols  
45 may be complexed with proteins (digestion enzymes) or minerals, and may precipitate (Bohn, 2014).

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48 Upon matrix release, polyphenols are partly taken up by the epithelium in the small and/or large  
49 intestine. In order to be absorbed, it appears (apart for some anthocyanins) that glucosides must be  
50 cleaved to liberate the respective aglycons (by e.g. cytosolic beta-glucosidase, phlorizin-hydrolase of  
51 the brush-border, or, to a lesser extent, by the low pH in the stomach). These aglycons can then be  
52 taken up via passive diffusion or via transporters. Many compounds are however re-excreted into the  
53 gut via transporters, or are glucuronidated/sulfated, and can be further metabolized in other tissues  
54 (e.g. the liver). Thus, compared to their native compounds, they may undergo substantial  
55 metabolism, depending largely on the polyphenol type.

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3 Following *in vitro* digestion, bioaccessibility is either determined following centrifugation (Mandalari  
4 et al., 2013) centrifugation/filtration (Kaulmann et al., 2015), or, alternatively, following diffusion  
5 through semipermeable membranes (dialysis), with e.g. 10,000-12,000 Da cut-offs (Bouayed et al.,  
6 2012; Bouayed et al., 2011; Gil-Izquierdo et al., 2002), simulating that only soluble, small molecules  
7 can be taken up by the epithelium. The differences between these methods may be considerable,  
8 and recoveries appear lowest for dialysis (Bouayed et al., 2012). In contrast, *in vivo* bioavailability of  
9 polyphenols may either be compared by their (or their metabolites) urinary excretion (for water  
10 soluble polyphenols such as isoflavones, (Bohn et al., 2013), shown to vary between 0.3-43%  
11 (Manach et al., 2005), by their plasma appearance (Guo et al., 2014) over time (AUC), or via the  
12 faecal balance method (reflecting absorption) when colonic metabolism can be neglected, such as in  
13 ileostomists (Erk et al., 2014). The AUC following a single dose application is supposed to best reflect  
14 bioavailability of polyphenols (Carbonell-Capella et al., 2014).

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17 Thus, factors effecting polyphenol matrix release are crucial and must be mimicked *in vitro*, possibly  
18 including mastication, mechanical movements during gastric phase, pH (that could aid in liberation  
19 via hydrolyzing reactions), influence of enzymes aiding in the degradation of the matrix and in  
20 polyphenol release. For polyphenols however, there are at least 3 major concerns regarding *in vitro*  
21 digestion that clearly limit the predictability of bioavailability. These are:

- 22 1. The absence of a colonic fermentation step for most *in vitro* models, where polyphenols are  
23 heavily metabolized. Many polyphenols reach the colon, where the microbiota can lead to  
24 additional reactions, including ring fission, deglycosylation, hydrolysis, deglucuronidation,  
25 and demethylation (Alminger et al., 2014; Bohn et al., 2015), resulting in products that may  
26 be absorbable in the colon.
- 27 2. Many polyphenols are bound in the non-extractable fraction (NEPP) that may not be  
28 released in the gastric phase/small intestine (Bohn, 2014), but perhaps in the colon, such as  
29 phenolic acids. A large proportion of polyphenols is covalently bound, and are not  
30 extractable by chemical means (Arranz et al., 2010; Perez-Jimenez et al., 2013) or released in  
31 the small intestine. This NEPP fraction may exceed the extractable fraction.
- 32 3. Bioavailability of polyphenols is altered by transporters in the gut, phase I/II metabolism  
33 reactions, and rapid turnover/excretion. For example, polyphenols may interact in a way that  
34 favours cellular uptake due to blocking certain efflux transporters into the gut lumen, or may  
35 override certain phase I/II metabolizing enzymes (Bohn, 2014), processes that are typically  
36 not studied *in vitro*, unless *in vitro* digestion is coupled to cellular models (Andre et al., 2015;  
37 Kaulmann et al., 2015), though detection problems may then become a concern.

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39 These factors impede estimating polyphenol availability by *in vitro* methods compared to e.g. less  
40 heavily metabolized compounds (e.g. carotenoids). Nevertheless, a few studies have compared their  
41 *in vitro* bioaccessibility with *in vivo* human trials. Brown et al. investigated the polyphenol  
42 composition of lingonberries following both *in vitro* (including faecal fermentation) and *in vivo*  
43 digestion in ileostomists (Brown et al., 2014). Certain differences in polyphenol composition were  
44 found between the ileal efflux and the bioaccessible fraction *in vitro*, and were explained by the  
45 absence of brush-border enzymes, e.g. phlorizin-hydrolase, but also cytosolic beta-glucosidase  
46 (cleaving sugar-moieties), from *in vitro* model. Nevertheless, general trends for *in vitro* and *in vivo*  
47 digested polyphenols were similar (bioaccessibility *in vitro* 28% and 49% *in vivo*). The results  
48 emphasize that the correlation between *in vitro* digestion with faecal fermentation and colonic  
49 bioaccessibility *in vivo* (ileostomist model) can be reasonable. Vetrani et al. compared an *in vitro*  
50 model including colonic digestion with human *in vivo* availability as determined via urinary excretion  
51 (Vetrani et al., 2016), based on over 70 subjects consuming a polyphenol rich diet over 8 weeks. A  
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3 significant though low correlation of  $R=0.280$  ( $P=0.04$ ) was found for 15 metabolites, suggesting that  
4 when combined with colonic fermentation, the model may predict polyphenol uptake and excretion  
5 to a certain extent.  
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7 For compounds that are unstable (but absorbable) during the upper stages of digestion, *in vitro*  
8 models may more accurately predict bioaccessibility. For example, anthocyanin (as parent  
9 compounds) urinary excretion has been reported to be below 5.1% (range 0.004 – 5.1%, (Kalt et al.,  
10 2014; Manach et al., 2005), while *in vitro* studies have suggested a bioaccessibility between zero in  
11 apples (Bouayed et al., 2011) to 4.6% from mulberries (Liang et al., 2012). For compounds that are  
12 absorbed to a high extent in the small intestine, such as isoflavones, it is also worth to compare *in*  
13 *vitro* with *in vivo* results. While most *in vitro* studies of soy isoflavonoids suggest a higher  
14 bioaccessibility of the glucosides (due to their higher water solubility), between 80-100% (**Table 3**),  
15 aglycons (more apolar, perhaps requiring micellarization) range lower in bioaccessibility, approx. 30-  
16 60%. However, *in vivo* differences between aglycons and glucosides appear negligible (Nielsen and  
17 Williamson, 2007; Zubik and Meydani, 2003). It is assumed that glucosides require cleavage into  
18 aglycons prior to uptake, reducing the initial advantage of higher solubility. However, bioaccessibility  
19 may well predict relative absorption between various isoflavone aglycons, such as daidzein vs.  
20 genistein (**Table 3**), as both *in vitro* and *in vivo* find higher potential uptake/absorption of the more  
21 polar daidzein vs. genistein, when measured by urine excretion or by fractional absorption (faecal  
22 balance method). When measured as AUC in plasma however (a more accurate measure of  
23 bioavailability, accounting for uptake, biodistribution and excretion), genistein showed higher  
24 bioavailability (Setchell et al., 2003), as daidzein more rapidly is distributed into other tissues (higher  
25 volume of distribution ( $V_D$ )). It is therefore important to clearly define “bioavailability” – noting that  
26 the latter is different from absorption or excretion.  
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31 As measuring all individual polyphenols may be less practical, some *in vitro* methods have  
32 investigated total polyphenol recovery following *in vitro* digestion by the Folin-Ciocalteu method  
33 (**Table 3**), though not being specific for polyphenols (also detecting e.g. reducing sugars). When  
34 compared to *in vivo* bioavailability (either estimated by urine appearance or by plasma AUC), results  
35 appear to be in the same range of availability, though it is hard to compare the various test meals  
36 and various measures of bioavailability. Unfortunately, the Folin-Ciocalteu method is not applicable  
37 *in vivo*, where too many other factors influence anti-oxidant capacity of plasma.  
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### 42 **Trace elements – example iron**

43 One of the first digestion methods that has latterly been transferred to many other applications was  
44 described by Miller et al. (1981), set up originally to study iron bioaccessibility. Unlike organic  
45 micronutrients (vitamins) and phytochemicals, minerals do not undergo significant metabolism  
46 during digestion. However, oxidation/reduction may take place that can influence mineral  
47 bioavailability, and especially divalent minerals may undergo complexation during digestion,  
48 impinging on their availability. In order to be available, minerals/trace elements are believed to  
49 require solubilization (except perhaps, in the form of nanoparticles), which depends on the pH,  
50 concentration of the mineral, and the presence of other complexing agents. For example,  $Fe^{2+}$  may  
51 be oxidized into the non-absorbable  $Fe^{3+}$ , or it may be complexed by organic acids such as phytic  
52 acid, oxalic acid or polyphenols, greatly reducing availability, similarly for other minerals (Bohn et al.,  
53 2004a; Bohn et al., 2004b; Hurrell, 2007). Higher pH generally limits the availability of divalent  
54 minerals/trace elements, as solubility decreases with higher pH ( $>7$ ), as insoluble oxides/hydroxides  
55 may form.  
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3 As for most minerals, iron is believed to be primarily absorbed in the upper intestine, though also  
4 colonic absorption, especially at lower pH (improved solubility), such as following fermentation of  
5 probiotic fiber, cannot be excluded (Scholz-Ahrens et al., 2007). An additional consideration is that  
6 for certain minerals there may be several non-exchangeable sources, termed “pools”, with different  
7 availability, such as for iron, zinc, and selenium. There are two main dietary sources of iron which do  
8 not mix during digestion, i.e. iron in hemoglobin in meat/fish and products (heme-iron), and non-  
9 heme iron from plant sources, which are absorbed in a different way, with heme-iron having a 5-10  
10 times higher bioavailability. This is because iron bound to heme is taken up as an entire peptide  
11 (possibly via endocytosis), while non-heme iron is absorbed in the intestine as  $Fe^{2+}$  via divalent metal  
12 ion transporter 1 (Fuqua et al., 2012), susceptible to oxidation and complexation. As iron, unlike  
13 other minerals, where bioavailability may be best determined via plasma measurements, is mostly  
14 incorporated into erythrocytes (hemoglobin), measuring iron in red blood cells, such as several  
15 weeks following the intake of isotopically labelled iron, is the gold standard for iron absorption  
16 studies. However serum appearance curves have shown to correlate well with erythrocyte  
17 incorporation (Conway et al., 2006). In vitro, many studies have either focused on the bioaccessible  
18 fraction as measured via dialysis, to exclude iron bound/complexed to macromolecules (proteins,  
19 high molecular weight polyphenols) and not being bioavailable (Miller et al., 1981), or following  
20 prolonged centrifugation and filtration (Kulkarni et al., 2007), resulting in generally higher values  
21 than dialysis. As heme-iron absorption cannot be studied *in vitro*, *in vitro* digestion does not allow to  
22 compare between heme-iron and non-heme iron availability.  
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27 Many studies have compared *in vitro* available iron with bioavailable iron in human trials. For  
28 example, iron availability has been studied from different iron salts with and without the addition of  
29 sodium EDTA (ethylene diamine tetra-acetic acid) from corn masa flour tortillas. A high correlation  
30 was found between *in vitro* bioaccessibility (after dialysis) and the amount recovered in erythrocytes  
31 ( $R=0.89$ ,  $P<0.001$ , **Figure 9**). However, bioavailability was higher (by a factor 2) than bioaccessibility  
32 (Walter et al., 2003). It appears that for other minerals, which are usually absorbed to a higher  
33 degree, such as Mg and Ca (absorption approx. 20-40%), bioaccessibility and bioavailability values  
34 show less discrepancy. However, comparable results were obtained when correlating *in vitro* results  
35 by Aragon et al. (2012) with those of Davidsson et al. (2002), contrasting *in vitro* dialysability of  
36 various iron salts with and without EDTA, and erythrocyte incorporation of stable iron labelled test  
37 meals with the same iron compounds ( $n=6$ ,  $R=0.80$ ). In another comparison, various concentrations  
38 of ascorbic acid were added to test meals to investigate the effect on iron absorption. When  
39 comparing results obtained from this study, i.e. dialyzable iron fraction (Aragón et al., 2012) with  
40 earlier results from a similar study *in vivo* (erythrocyte incorporation (Cook and Mosen, 1977), a  
41 similar high correlation was obtained ( $R=0.98$ ). In addition to iron salts, also different food items have  
42 been compared. Sandberg et al. (2005) presented data from various test meals, comparing *in vitro*  
43 solubility with human absorption studies (erythrocyte incorporation), and found a very high  
44 correlation ( $R=0.97$ ), though with solubility being approx. twice as high as iron absorption (**Figure 9**).  
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48 General limitations of the dialysis technique are that large molecules such as heme-iron or ferritin  
49 would be bioavailable, but not dialyzable, while some smaller compounds that are dialyzable such as  
50 phenolic complexes or complexes with organic acids can diffuse through the semipermeable  
51 membrane, but would generally not be bioavailable. As transporters may play important roles,  
52 especially between different mineral pools (e.g. heme iron and non-heme iron), predictability across  
53 those different forms is not possible by *in vitro* techniques. Some of these limitations may be  
54 overcome by coupling *in vitro* digestion with other techniques, i.e. based on cell culture. Correlations  
55 were high when comparing cellular uptake (a Caco-2 based model) following *in vitro* digestion and  
56 human bioavailability (erythrocyte uptake) following the addition of ascorbic acid or tannic acid (a  
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polyphenol) to a semi-synthetic meal (R=0.934, P= 0.012 and R=0.927, P=0.007, respectively), though iron uptake was (as mostly done for cellular studies) determined via ferritin expression (Yun et al., 2004).

In summary, while the tendency, that is the direction of bioavailability (higher, lower, equally well) appears to be generally predictable by methods incorporating *in vitro* digestion, this does mostly not predict the magnitude of bioavailability in humans, as too many processes, namely transport, phase I/II metabolism, colonic changes (especially for polyphenols), biodistribution and (renal) excretion affect bioavailability of micronutrients and phytochemicals in many different manners, which cannot be predicted by this method. A more accurate method is the coupling of *in vitro* digestion to cell models of the epithelium, such as Caco-2 cells, which may simulate, to a certain extent, transport and secretion to the basolateral side, as well as model some aspects of further metabolism. It should further be noted that though bioaccessibility often appears to be reasonably correlated with absorption (and urinary excretion), this is not necessarily true for bioavailability as determined via the AUC in plasma, which depends on further biodistribution. Generally, bioaccessibility tends to overestimate bioavailability, due to the involvement of limiting transporters *in vivo*. Despite these limitations, *in vitro* models have developed into very useful tools for hypothesis building and investigating a large number of potential influential factors governing digestion of micronutrients and phytochemicals.

#### 4 Lipid digestion

The use of *in vitro* models in the study of food materials during digestion invariably requires the progression of findings from simulation to human studies. Ideally, observations made as part of human trials should be able to be correlated with findings from *in vitro* analysis, thus demonstrating the physiological relevance of the model. A particular challenge with this approach, beyond ensuring that any model provides sufficient representation of human digestive physiology, is the identification and characterisation of complementary markers across both *in vitro* and *in vivo* analysis that can be used to establish particular correlations between simulated and human studies. The uptake of dietary lipids involves luminal hydrolysis of triglycerides by gastric and intestinal lipases followed by absorption across the epithelium and re-assimilation into triglycerides in the enterocytes. Biomarkers most commonly used to follow lipid digestion are fatty acid release (*in vitro*) and triglyceride appearance in blood, plasma-TAG (*in vivo*). Good *in vitro/in vivo* correlations are thus often impaired by the fact that *in vitro* data mostly reflect the intraluminal step of lipid digestion while *in vivo* data reflect the overall process of digestion and intestinal absorption without any information on rate limiting steps. A first challenge is therefore to find *in vitro* and *in vivo* data that reflect similar processes. Another challenge when creating *in vitro* models to assess lipolysis is ensuring accurate representation of both the biochemistry and biophysics of the gastrointestinal tract.

When considering replication of the biochemistry of lipolysis, good *in vivo-in vitro* correlations were obtained during the gastrointestinal lipolysis of complete test meals and O/W emulsions, using a two-step static *in vitro* digestion model. This model was initially developed based on *in vivo* data recorded during the digestion of a liquid test meal in healthy volunteers and the arbitrary choice of conditions existing in the stomach and small intestine at 50% gastric emptying (Carriere et al., 2000; Carrière et al., 2001). The mean pH values of 5.5 and 6.25 measured in gastric and duodenal contents, respectively, were chosen for the gastric and duodenal steps of *in vitro* digestion. Gastric lipase concentration was 17 µg/mL (20 U/mL on tributyrin; (Gargouri et al., 1986)) for the gastric step

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3 and the solution mimicking human gastric juice was prepared using either purified human gastric  
4 lipase (HGL), recombinant dog gastric lipase (rDGL) or a rabbit gastric extract (RGE) containing rabbit  
5 gastric lipase (RGL) because these enzymes have similar lipase activity and specificity. For the  
6 duodenal step, a 1.7-fold dilution of gastric mixture was applied, resulting in a gastric lipase  
7 concentration of 10 µg/mL, while pancreatic lipase and bile salt concentrations were set at 250  
8 µg/mL (2,000 U/mL on tributyrin; Carrière et al., 1993) and 4 mM, respectively, using either purified  
9 human pancreatic lipase (HPL) or porcine pancreatic extracts (PPE) as a source of pancreatic lipase,  
10 and either human or bovine bile (Sigma B3883) as a source of bile salts. The choice of incubation  
11 times (30 min for the gastric step + 60 min for the duodenal step) was also arbitrary.  
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14 Nevertheless, the choice of these conditions allowed the measurement of gastric (t=30min) and  
15 gastric + duodenal (t=90min) lipolysis levels (% of fatty acids released from triglycerides) that were in  
16 the same range as the lipolysis levels measured *in vivo* during the whole meal digestion in the  
17 stomach and duodenum (**Table 4**). In this later case, gastric lipolysis was deduced from the total  
18 output of lipolysis products passing through the pylorus, while gastric + duodenal lipolysis was  
19 deduced from the output of lipolysis products collected by continuous aspiration at the Angle of  
20 Treitz (junction between duodenum and jejunum). Both *in vivo* and *in vitro* studies revealed a faster  
21 lipolysis of the liquid test meal compared to the solid-liquid test meal (**Table 4**). This was attributed  
22 to the fact that triglycerides in the homogenized liquid test meal were finely emulsified with lecithin,  
23 thus providing a good and accessible substrate for lipases, while triglycerides in the solid-liquid meal  
24 had various origins and reduced accessibilities (butter, cooking oil, meat fat). Further experiments  
25 with the lipase inhibitor Orlistat confirmed that the two-step static *in vitro* digestion model was  
26 predictive of *in vivo* gastric and duodenal lipolysis levels, as well as of undigested fat excretion in  
27 feces when gastrointestinal lipolysis was impaired (Carrière et al., 2001).  
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31 The same two-step static *in vitro* digestion model was used to study the effects of various emulsifiers  
32 on the bioavailability of  $\alpha$ -linolenic acid (ALA) from flaxseed oil, in combination with an *in vivo* study  
33 of ALA lymphatic secretion in rats (Couedelo et al., 2015). *In vitro* data showed that the  
34 emulsification of flaxseed oil with soya lecithin led to a higher level of gastric lipolysis compared to  
35 the emulsification with sodium caseinate (**Figure 10; Table 4**). Similarly, duodenal lipolysis of flaxseed  
36 oil was also higher with lecithin than with caseinate (**Figure 10; Table 4**). The *in vivo* data obtained  
37 with the same emulsions and lymph collection in rats showed significant changes ( $p < 0.05$ ) in the  
38 kinetics and overall absorption (AUC) of ALA over the 6 hours post-feeding. The resulting lymphatic  
39 secretion of ALA was 3-fold higher with soya lecithin ( $C_{max} = 24 \pm 3$  mg/mL;  $AUC = 60 \pm 32$  mg/ml  $\times$  h)  
40 than with caseinate ( $C_{max} = 7 \pm 4$  mg/mL;  $AUC = 24 \pm 14$  mg/ml  $\times$  h). In addition, the synthesized  
41 chylomicrons were notably larger and more numerous with soya lecithin whereas they were smaller  
42 in the presence of caseinate. These results suggest that the intestinal bioavailability of ALA is  
43 increased by the emulsification of flaxseed oil with soya lecithin via an improved lipolysis, favouring  
44 the intestinal absorption of ALA and the secretion of many large chylomicrons in lymph. *In vitro*  
45 lipolysis rate of flaxseed emulsions therefore appears as predictive of intestinal absorption rate,  
46 probably because lipolysis is the limiting step in the overall bioavailability of fatty acids. Using the  
47 same two-step static *in vitro* digestion model, similar differences in the effects of lecithin and  
48 caseinate on lipolysis rates were observed with rapeseed oil and milk fat olein emulsions (**Table 4**;  
49 (Vors et al., 2012)).  
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55 Since lipid digestion is an interfacial process its speed is controlled by droplet surface area  
56 (Benzonana and Desnuelle, 1965) and the nature of the surfactants which can impair enzyme  
57 accessibility at the surface (Delorme et al., 2011), hence the structural transformations that an  
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3 emulsion might undergo as a result of gastrointestinal biophysical processes need to be considered  
4 during *in vitro* testing. This consideration is highlighted in a series of studies progressing from *in vitro*  
5 to *in vivo* undertaken on “gastric structured” emulsions (Day et al., 2014; Golding et al., 2011; Keogh  
6 et al., 2011; Steingoetter et al., 2015; Wooster et al., 2014). The study design was based on the  
7 hypothesis that the rate and possibly extent of lipid uptake could be controlled by manipulating the  
8 surface area of fat to which digestive lipases could adsorb (such that lower surface areas would  
9 present fewer binding sites, leading to a reduction in the relative rate of digestion). To test this  
10 hypothesis, model emulsions were formulated to undergo a number of structural transitions when  
11 exposed to the biophysical conditions in the stomach (noting that all emulsions were initially stable  
12 and had comparable particle size distributions and viscosities). Dependent on composition,  
13 emulsions could be tailored to remain stable, flocculate, coalesce, partially coalesce (due to the  
14 presence of solid fat within the droplets at 37 °C), and fully break upon prolonged exposure to gastric  
15 environment (**Figure 11**).

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19 The study design initially involved the use of static *in vitro* gastric and small intestinal models to  
20 assess microstructural changes and extent of lipid digestion. Incubation of emulsion samples within  
21 the *in vitro* models, coupled with confocal microscopy, provided evidence of how conditions of pH,  
22 presence of enzymes and bile, and the application of shear at physiological temperatures impacted  
23 on emulsion structure during simulated digestion (Golding et al., 2011). In this respect, the static *in*  
24 *vitro* model was able to demonstrate the responsiveness of emulsion structures to particular  
25 physiological variables, enabling high throughput determination of structure dynamics based on  
26 iterative formulation design (**Figure 11a**). The *in vitro* model also enabled the extent of lipolysis to be  
27 measured for these emulsions during simulated small intestinal digestion, using a pH-stat  
28 methodology to determine the rate of fatty acid synthesis arising from triglyceride hydrolysis. This  
29 approach was able to confirm that low surface area emulsions that had undergone coalescence,  
30 partial coalescence or breaking during gastric incubation were significantly more slowly hydrolyzed  
31 compared to structurally stable or flocculated emulsions that retained high surface area.

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35 Based on *in vitro* findings, selected emulsion systems were studied *in vivo* using conventional dietary  
36 interventions and studies using MRI (Golding et al., 2011; Keogh et al., 2011; Steingoetter et al.,  
37 2015). Upon consumption, the different emulsions were isocaloric, isoviscous and had the same  
38 initial droplet size distribution, but were again designed to undergo particular gastric structuring  
39 mechanisms based on earlier *in vitro* findings. Identification and development of complementary  
40 measurement techniques between *in vitro* and *in vivo* study methodologies was an important  
41 consideration in allowing correlations (of both structural dynamics and digestive properties) between  
42 the two approaches to be established. In the case of emulsions undergoing dynamic gastric  
43 structuring, information on gastric structure (MRI Imaging), emptying (Paracetamol absorption & MRI  
44 stomach volume) and triglyceride absorption (Plasma-TAG) were key biomarkers of interest.

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47 Variations in plasma-TAG onset time, peak and area under the curve provided information on the  
48 relative rate and extent of fat digestion taking place, which could in turn be correlated to rate of fatty  
49 acid release during *in vitro* digestion (**Figure 11b**). Intralipid was used as a (stable, high surface area)  
50 control emulsion for these studies, thereby providing initial comparison between *in vitro* and *in vivo*  
51 measurements. Comparison of a sample's plasma-TAG profile to Intralipid's could accordingly be used  
52 as an indicator of whether changes to *in vitro* fatty acid release arising from emulsion structuring  
53 were similarly represented by changes *in vivo*. In this respect, specific (and reproducible) *in vitro-in*  
54 *vivo* correlations were able to be established. Notably, partially coalescing emulsions, for which *in*  
55 *vitro* fatty acid release was found to be greatly diminished when compared to Intralipid, showed a  
56 markedly suppressed plasma-TAG response when compared to the same control. However, a poor *in*  
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3 in vitro - in vivo correlation was found when comparing emulsions that broke under gastric conditions.  
4 In vitro digestion experiments predicted a slow intestinal uptake, however in vivo plasma-TAG curves  
5 showed a delayed but fast intestinal lipid uptake. It was proposed (and later confirmed by MRI) that  
6 the main source of this discrepancy arose from delayed gastric fat emptying with concurrent re-  
7 dispersion during passage through the antrum-pylorus (Steingoetter et al., 2015). This highlights the  
8 widely held belief that a key limitation of static in vitro models is accurate representation of digestion  
9 biophysics (gastric mixing and emptying), such limitations have vastly been improved in the TNO  
10 intestinal model and IFR Model Gut dynamic digestion models (Minekus et al., 1999; Wickham and  
11 Faulks, 2007).

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14 In summary, when considering lipid emulsion digestion, detailed understanding of digestion  
15 biochemistry has allowed in vitro models to become a relatively good predictor of in vivo behavior.  
16 This is especially true when considering simple/stable emulsions where the extent of gastric and/or  
17 gastric + duodenal lipolysis measured in vitro were in the same range as the lipolysis levels measured  
18 in vivo. As for the digestion of emulsions whose structure changes as a result of interaction with the  
19 biophysics of the digestive environment, there are moderately good in vitro-in vivo correlations, but  
20 they can be hampered by a lack of representation of gastric mixing and emptying in static in vitro  
21 models.  
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## 24 25 26 **General conclusion**

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28 Food digestion is a dynamic process that is characterized by a flow of food in the different  
29 compartments of the gastrointestinal tract, the evolution of pH in the different compartments, the  
30 continuous production and release of digestive enzymes and bile. Therefore, simulating digestion  
31 with rather basic static in vitro digestion models appears difficult and questions arise whether these  
32 simple models can predict and/or correlate with in vivo experiments.  
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34 Nevertheless, this exhaustive review of the recent knowledge acquired on the ability of static in vitro  
35 digestion models to mimic the in vivo reality clearly shows that, in some cases, static models can be  
36 physiologically relevant. One of the best examples is the excellent correlation that has been observed  
37 by several groups between in vitro and in vivo data for assessing the digestion of starch. There are  
38 indeed strong evidences that in vitro protocols do appear to be reliable indicators of the glucose  
39 response in vivo and could even be of great interest for the design of new foods with specific  
40 carbohydrate digestion rates.  
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43 For protein digestion, in vitro models appear as potential predictor of true digestibility or, at least,  
44 are useful to rank different protein sources according to their digestibility. Similar patterns of  
45 bioactive peptides have been observed when comparing in vitro and in vivo models, thus in vitro  
46 models could be used to predict the release of bioactive sequences in the gastrointestinal tract.  
47 However, more work is needed to assess whether in vitro models could evaluate concentrations of  
48 bioactive sequences in the lumen.  
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50 For carotenoids, in vitro bioaccessibility reasonably predicts in vivo bioavailability, especially on the  
51 greater average. While absolute bioavailability is harder to predict, the relative availability, e.g. when  
52 comparing availability of carotenoids from 2 test meals, appears to be quite well predictable.  
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55 The case of polyphenols is rather different. If fecal fermentation is not included in the model for  
56 predicting the bioavailability of individual compounds, results do not appear meaningful, except for  
57 compounds mostly absorbed in the upper part of the intestine such as for certain flavonoids. When  
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3 estimating global bioavailability from a product, i.e. the sum of total phenolics, without focusing on  
4 individual compounds, the *in vitro* method appears to perform reasonably well, when compared to  
5 absorption and/or excretion. It also appears that colonic bioaccessibility *in vitro* vs. *in vivo* (in short-  
6 bowel subjects) is well correlated, though differences due to brush-border enzyme activities exist.  
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8 Furthermore, when *in vitro* digestion is coupled to faecal fermentation, reasonable correlations to  
9 the *in vivo* situation (uptake, excretion) may be reached, though more data is required in this  
10 respect.  
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12 Therefore, although *in vitro* static models are over-simplistic and, as such, do not reproduce all the  
13 dynamic aspects of the gastrointestinal tract, they are extremely useful to predict *in vivo* digestion in  
14 some cases detailed in this review, offering a first step in screening the digestibility of food and  
15 understanding the effect of processing conditions on the digestive fate of certain food constituents.  
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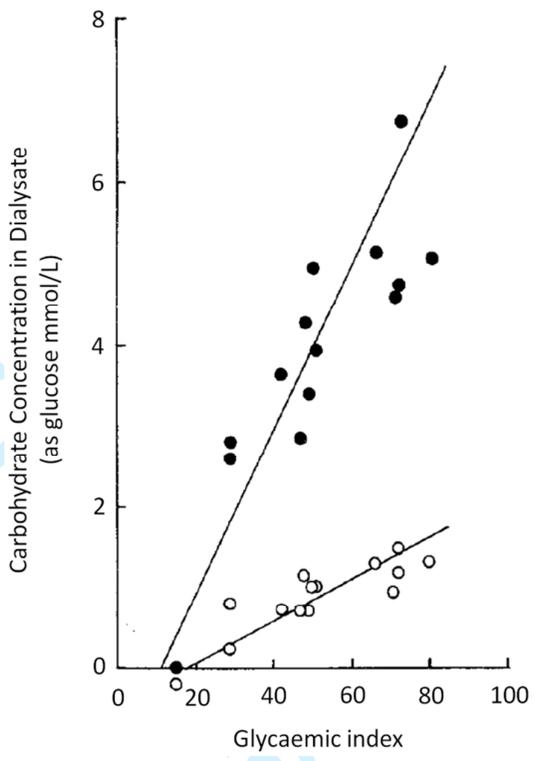


Figure 1

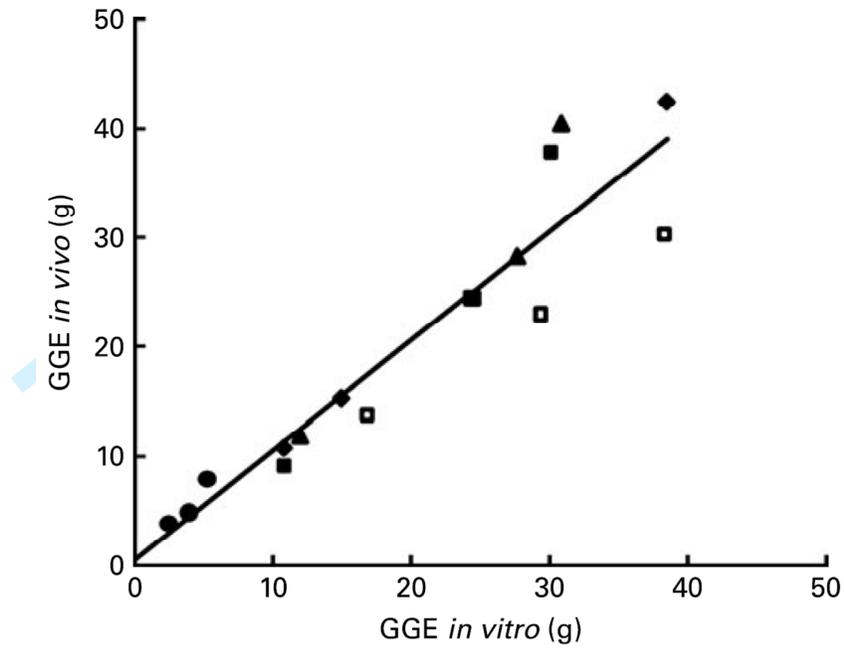


Figure 2

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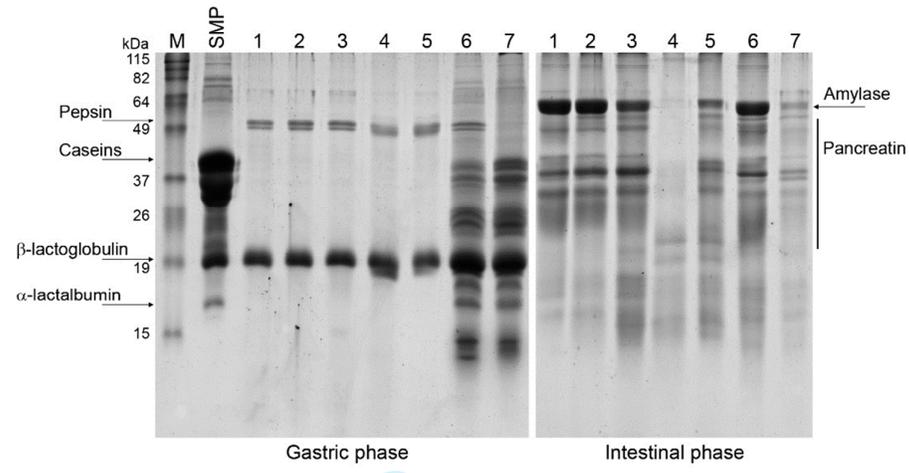


Figure 3

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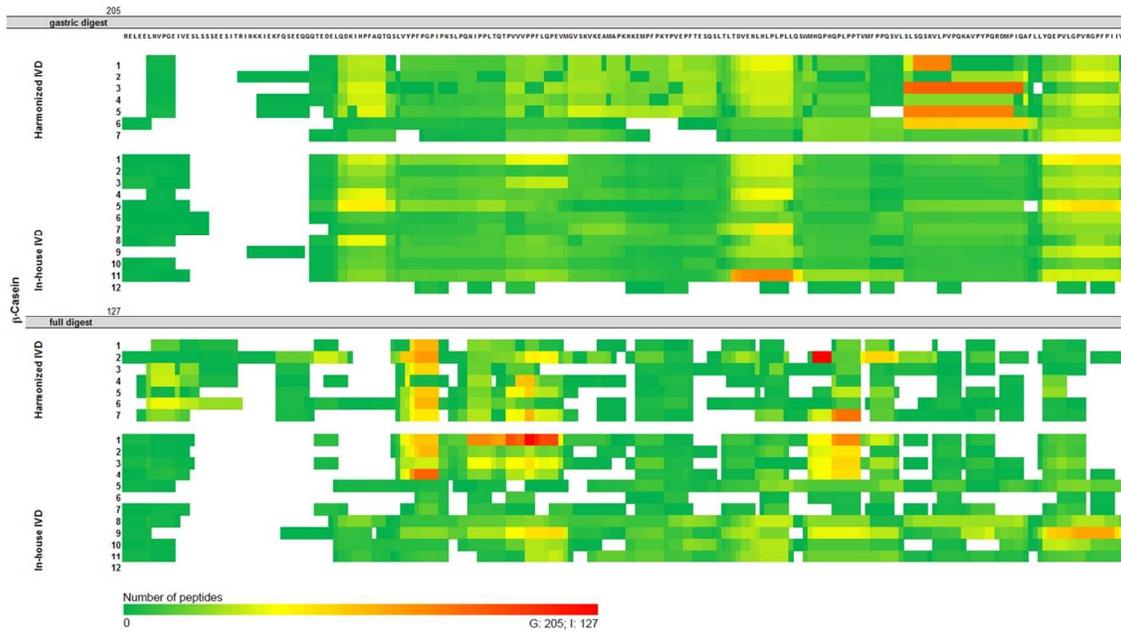


Figure 4

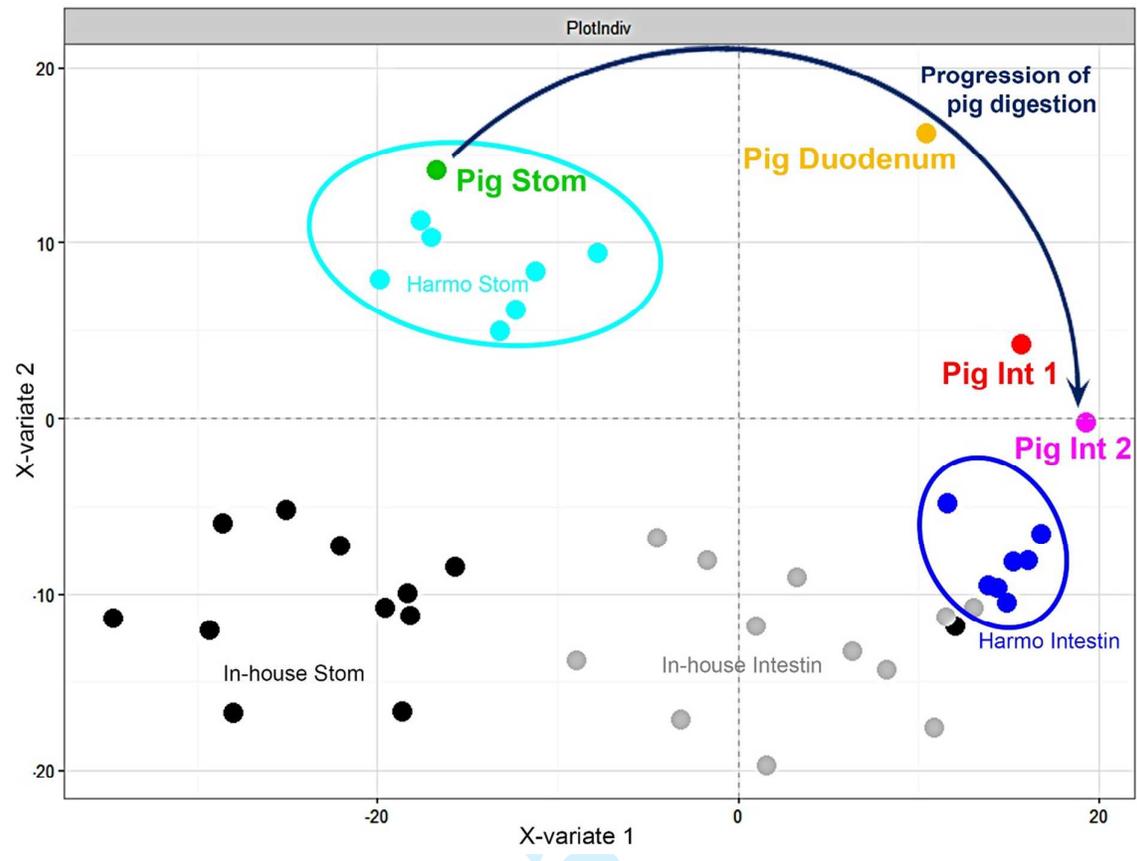
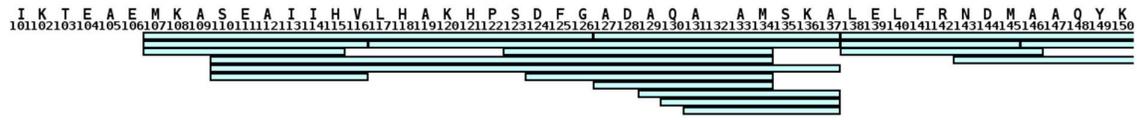
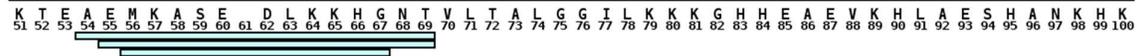
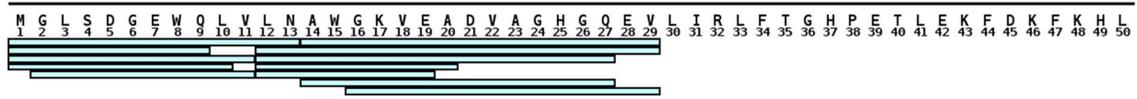


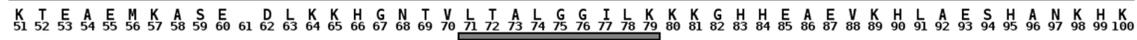
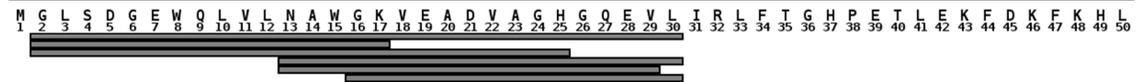
Figure 5

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in: 92 of 156 ~ 59%  
 Total: 92 of 156 ~ 59%



V L G F H G  
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in: 85 of 156 ~ 54%  
 Total: 85 of 156 ~ 54%



Figure 6

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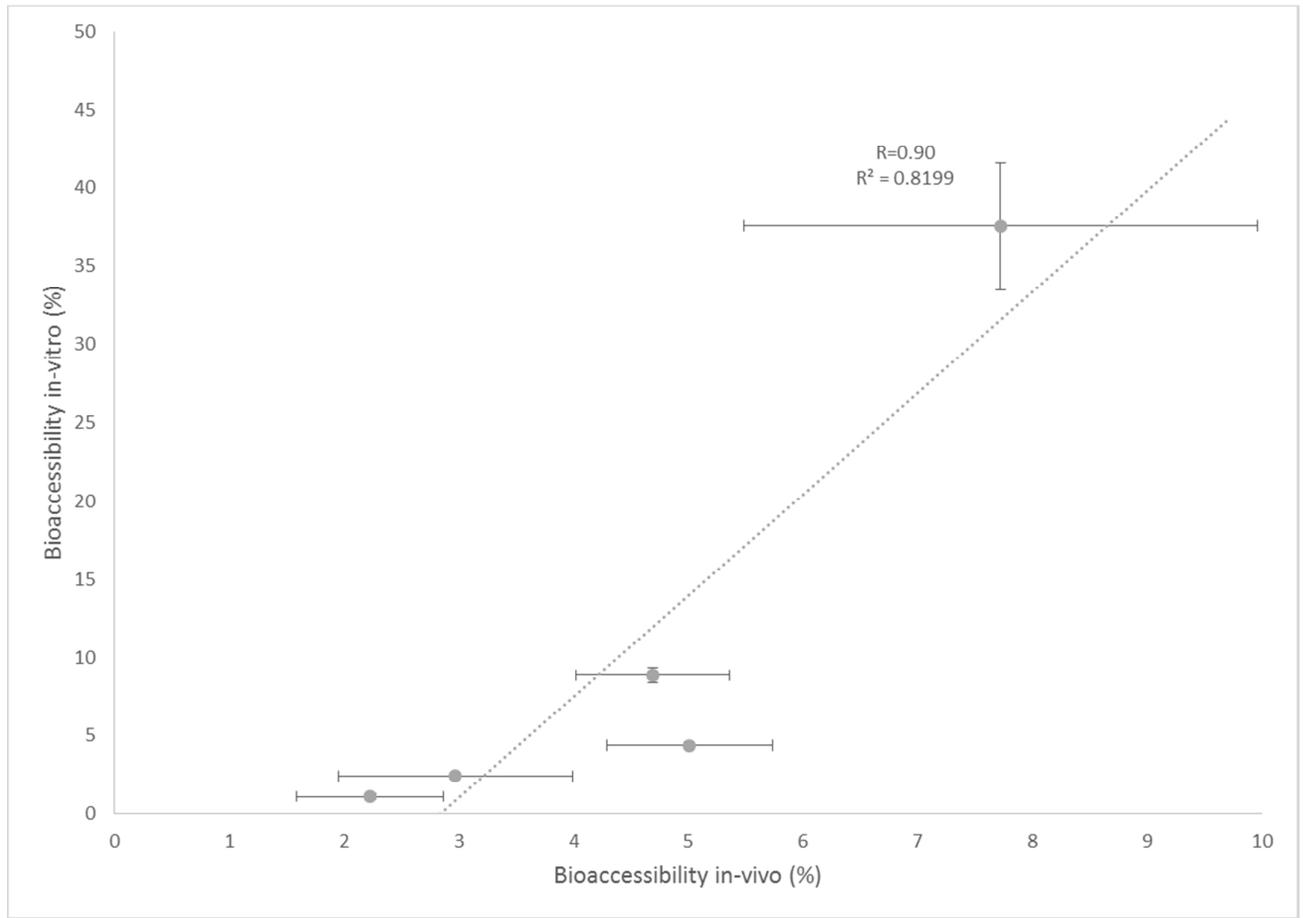


Figure 7

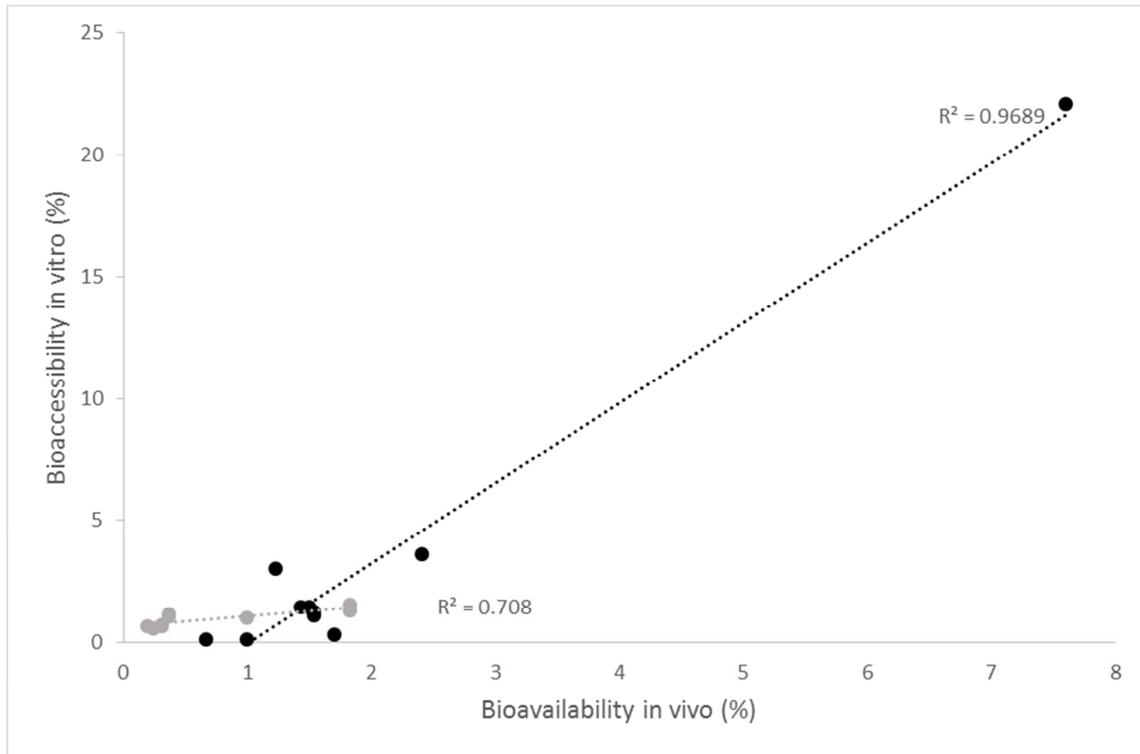


Figure 8

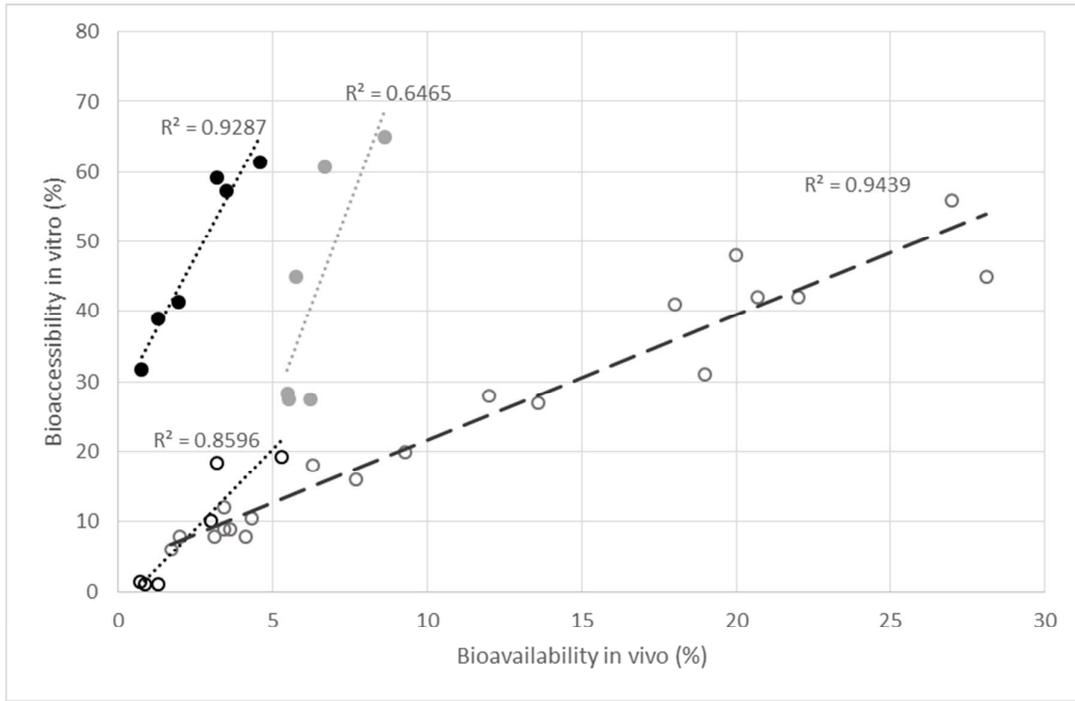


Figure 9

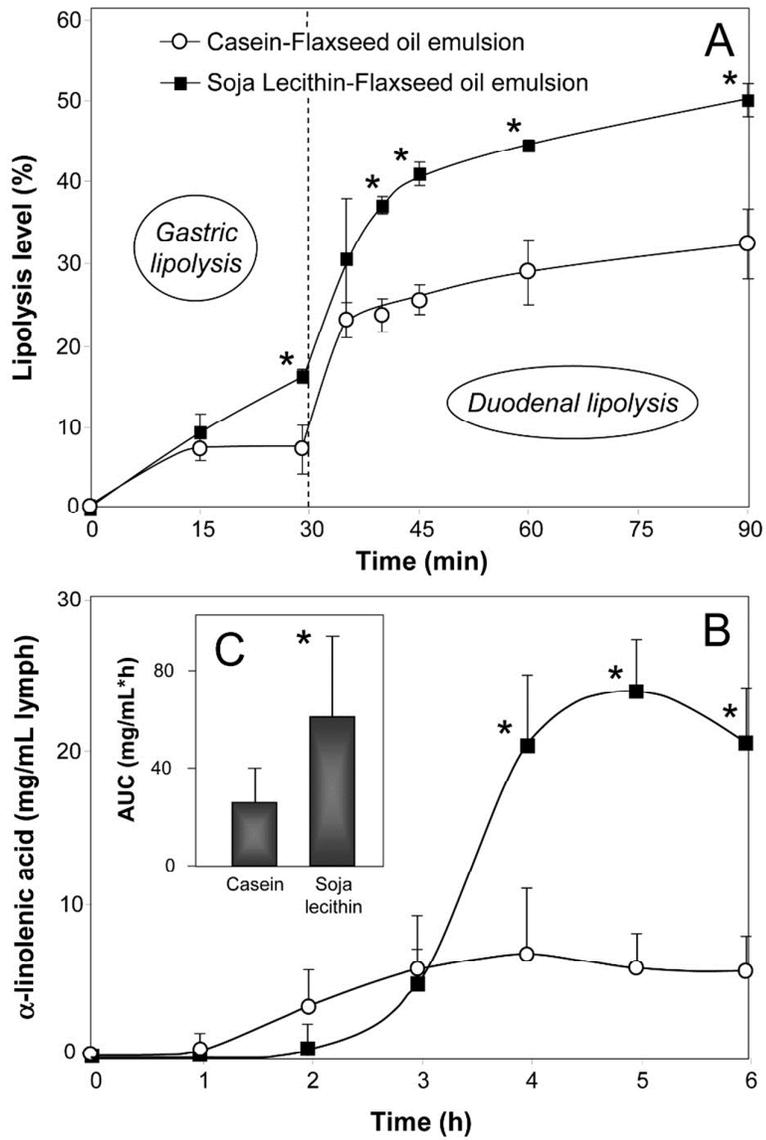


Figure 10

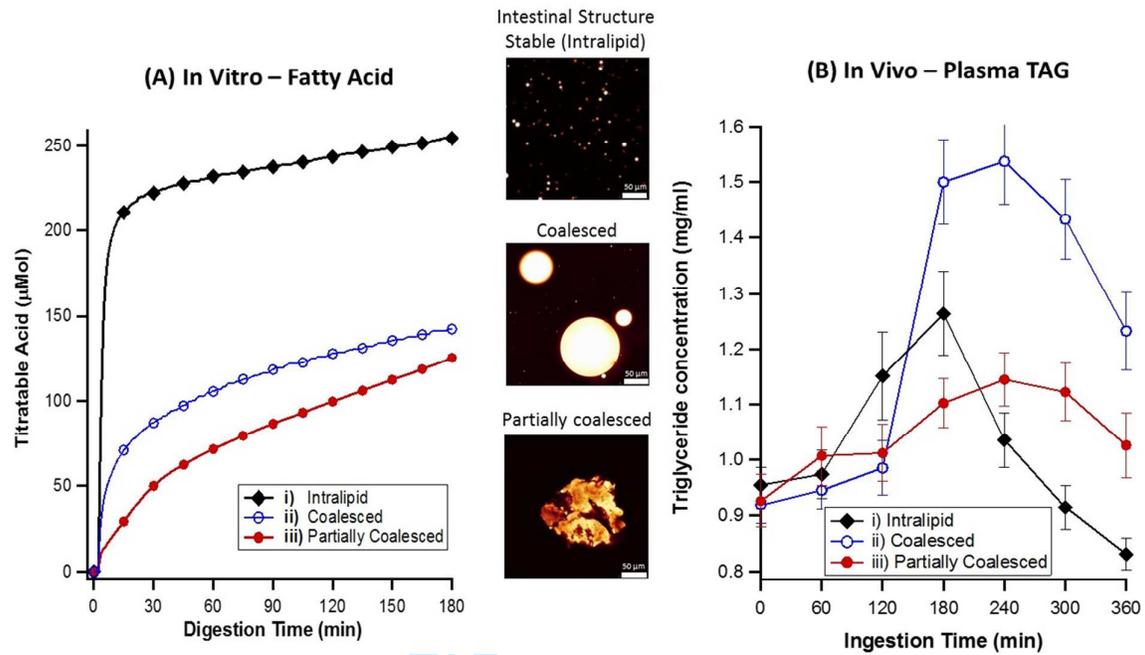


Figure 11

Table 1

Bioactive peptide (protein fragment) (reference)	Ingested food	Intestinal tract part	Species	Reference
KVLPVPQ ( $\beta$ -CN 169-175) (Maeno et al., 1996)	Human milk	Stomach	Human (newborn)	Dallas et al., 2014
AVPYPQR ( $\beta$ -CN 177-183) (Karaki et al., 1990)	Human milk	Stomach	Human (newborn)	Dallas et al., 2014
AYFYPEL ( $\alpha_{s1}$ -CN 143-149) (Contreras et al., 2009)	Milk or yogurt	Stomach and duodenum	Human	Chabance et al., 1998
YFYPEL ( $\alpha_{s1}$ -CN 144-149) (Sánchez-Rivera et al., 2014)	Milk	Duodenum	Human	Chabance et al., 1998
MKPWIQPK ( $\alpha_{s2}$ 190-197) (Maeno et al., 1996)	Milk	Duodenum	Pig	Barbé et al., 2014
FFVAPFPEVFGK ( $\alpha_{s1}$ -CN 23-34) (Karaki et al., 1990)	Milk	Duodenum	Pig	Barbé et al., 2014
YFPFGPIP (N ( $\beta$ -CN 60-68) (Saito et al., 2000)	Casein	Jejunum	Human	Boutrou et al., 2013
LHLPLP ( $\beta$ -CN 133-138) (Quirós et al., 2007)	Casein	Jejunum	Human	Boutrou et al., 2013
TPVVVPPFLQP ( $\beta$ -CN 80-90) (Abubakar et al., 1998)	Infant formula	Jejunum	Pig (newborn)	Bouzerzour et al., 2012

<sup>a</sup> Fragment comprising antihypertensive sequence

Table 2

	Meat		P
<i>In vitro</i>	Pork, 30 min at 70°C	Pork, 30 min at 140°C	
Pepsin digestion			
Maximal degradation (OD <sub>max</sub> )	0.106	0.122	***
Maximal rate of digestion ( $\Delta$ OD/h)	0.163	0.126	***
Half-life time (min)	31	45	***
Trypsin –chymotrypsin digestion			
Maximal degradation (OD <sub>max</sub> )	0.17	0.21	NS
Maximal rate of digestion ( $\Delta$ OD/h)	0.88	0.82	*
Half-life time (min)	9	13	NS
<i>In vivo</i>	Veal, 30 min at 75°C	Veal, 30 min at 95°C	
True ileal digestibility, %	95.6	95.3	NS
Maximal rate of IAA appearance in plasma ( $\Delta$ [IAA]/min)	22.8	13.0	**
Time of maximal plasma IAA concentration (min)	165	290	*

Table 3

Type of study	Polyphenols studied	Bioaccessibility/ Bioavailability (%)	Studied by	Reference
In vitro	Apple pp, FC*	55% mean bioaccessibility	GI digestion and dializability	(Bouayed et al., 2011)
In vitro	Apple polyphenols, HPLC	24.7% overall dializability, 49.2% overall bioaccessibility 31-56% intestinal recovery (0.5-21% dialysis) for chlorogenic acids	GI digestion and dializability	(Bouayed et al., 2012)
In vitro	Apple pp	Procyanidins: 55% Rutin: 47% Phloridzin: 45%	GI digestion and bioaccessibility	(Tenore et al., 2013)
In vitro	Cashew apple juice pp, FC	39% overall bioaccessibility	GI digestion and bioaccessibility	(de Lima et al., 2014)
In vitro	Isoflavones from bread	Daidzein: 59% Genistein: 33% Daidzein-genistein ratio: 1.8 Glycitein: 75% Glucosides: mostly >80%	GI digestion and bioaccessibility	(Walsh et al., 2003)
In vitro	Isoflavones from pretzels	Total isoflavones: 78-85% Aglycons (genistein, daidzein, glycitein): 40-60%	GI digestion and bioaccessibility	(Simmons et al., 2012)
In vivo	Apple pp in ileostomists,	<5.3% recovery for 5-caffeoylquinic acid, 4-p-coumaroylquinic acid, caffeic acid, (-)-epicatechin, phloretin, quercetin	AUC in plasma (8h)	(Kahle et al., 2011)
In vivo	Apple smoothie pp in ileostomists	63% recovery overall	Recovery in ileostomist fluids	(Hagl et al., 2011)
In vivo	Isoflavones	Daidzein: 29.5% (low dose) Genistein: 8.9% (low dose) Ratio daidzein: genistein: 3.3	Faecal balance based on stable isotope administration	(Setchell et al., 2003)
In vivo	Isoflavones from a soy-tomato juice, healthy subjects	49.3% recovery (after cleaving of glucuronides/sulfates) Daidzein: 70% Genistein: 15% Daidzein-genistein ratio: 4.7 Glycitein: 31%	Urinary excretion (24 h pool)	(Bohn et al., 2013)

\*pp= polyphenols, FC= Folin Ciocalteu method.

Table 4

<i>In vivo data / Digestion of test meals in healthy volunteers</i>	<i>Gastric lipolysis (%)<sup>a</sup></i>	<i>Lipolysis (%) at the angle of Treitz (gastric + duodenal lipolysis)<sup>b</sup></i>
Liquid test meal (Carrière et al., 1993)	10.0 ± 1.0	nd
Liquid test meal (Carrière et al., 2001)	24.4 ± 5.7	59.4 ± 5.6
Solid-Liquid test meal (Carrière et al., 2001)	9.3 ± 6.3	27.7 ± 6.8
Solid-Liquid test meal (Carrière et al., 2005)	7.3 ± 5.9	20.8 ± 11.2
<i>In vitro data / Digestion of test meals and O/W emulsions</i>	<i>Gastric lipolysis at t=30min (%)</i>	<i>Gastric + duodenal lipolysis at t=90min (%)</i>
Liquid test meal (Carrière et al., 2001)	7.4 ± 0.3	72.3 ± 0.9
Solid-Liquid test meal (Carrière et al., 2001)	3.0 ± 0.5	53.7 ± 0.3
Rapeseed oil/lecithin emulsion (Vors et al., 2012)	9.5 ± 2.0	43.2 ± 3.6
Rapeseed oil/caseinate emulsion (Vors et al., 2012)	4.0 ± 4.0	29.1 ± 2.2
Milk fat olein/lecithin emulsion (Vors et al., 2012)	6.2 ± 0.6	42.0 ± 2.3
Milk fat olein/caseinate emulsion (Vors et al., 2012)	4.8 ± 1.6	20.9 ± 2.6
Flaxseed oil/lecithin emulsion (Couedelo et al., 2015)	16.3 ± 0.8	50.3 ± 2.0
Flaxseed oil/caseinate emulsion (Couedelo et al., 2015)	7.3 ± 2.9	32.6 ± 4.3



**Titre :** Nouvelles perspectives à la digestion de l'amidon et à la réponse glycémique : des digestions *in vitro* à une étude chez l'Homme par imagerie par résonance magnétique (IRM)

**Mots clés :** Amylase salivaire, amylase pancréatique, pain, pâtes, jus de citron, thé, satiété.

**Résumé :** Nous passons plus des trois quarts de notre vie dans l'état postprandial. Pourtant, une plus grande attention a été accordée à l'étude du métabolisme à jeun qu'à l'impact de l'état postprandial sur la santé.

Il est prouvé scientifiquement qu'une alimentation optimale pour la santé passe par la prise en compte de l'impact glycémique des aliments au-delà de leur simple teneur en glucides. Un déterminant important de l'impact glycémique de notre alimentation est l'amidon, qui joue un rôle clé dans la nutrition humaine en fournissant jusqu'à 50% de l'apport énergétique total. S'il est établi que la cinétique de digestion des aliments riches en amidon est un élément essentiel de leur impact glycémique, les contributions de chaque étape digestive à ce processus restent un sujet de débats. Afin de mieux comprendre les facteurs qui peuvent

influencer la réponse glycémique aux aliments riches en amidon, et d'identifier de nouvelles stratégies pour atténuer leur impact glycémique, il est essentiel d'élargir notre compréhension du processus digestif de l'amidon.

Cette thèse visait à étudier la digestion de repas riches en amidon (pain et pâtes), à réévaluer la contribution l'amylase salivaire à l'aide de digestions semi-dynamiques *in vitro*, et à mener une étude chez l'Homme pour déterminer l'effet de boissons (jus de citron et thé) sur : la réponse glycémique au pain, l'apport *ad libitum*, et la digestion gastrique étudiée par imagerie par résonance magnétique (IRM). Nos résultats apportent une base scientifique à l'élaboration d'une stratégie simple et efficace pour réduire la réponse glycémique aux aliments riches en amidon dans des repas de tous les jours.

**Title:** Novel insights into starch digestion and the glycaemic response: from *in vitro* digestions to a human study using magnetic resonance imaging (MRI)

**Keywords:** Salivary amylase, pancreatic amylase, bread, pasta, lemon juice, tea, satiety.

**Abstract:** All of us spend over three quarters of our lives in the postprandial state. Still, more attention has been dedicated to the study of the fasting metabolism than to the impact of the postprandial state on health.

Scientific evidence supports that an optimum diet for health requires consideration of the glycaemic impact of foods in preference to consideration of carbohydrate content alone. An important determinant of the glycaemic impact of our diets is starch, which plays a key role in human nutrition, supplying up to 50% of the total energy intake. If it is clear that the digestion rate of starch-rich foods is an important determinant of their glycaemic impact, the contribution of each digestive stage to this process remains controversy. To better understand the factors that can influence the

glycaemic response to starch-rich foods, and to identify new strategies to attenuate the glycaemic impact of starch-rich diets, it is essential to expand our understanding of the digestive process of starch.

The aims of this PhD were to study the digestion of starch-rich meals (bread and pasta), to re-evaluate the contribution of salivary amylase using semi-dynamic *in vitro* digestions, and to conduct a human study to determine the effect of drinks (lemon juice and tea) on: the glycaemic response to bread, *ad libitum* intake, and gastric digestion assessed by magnetic resonance imaging (MRI). Our results provide scientific rationale for the development of a simple and effective strategy to reduce the glycaemic response to starch-rich foods in everyday-life meals.

