A standardised static *in vitro* digestion method suitable for food
An international consensus

Didier DUPONT

INRA Agrocampus Ouest – Milk and Egg Science & Technology
Rennes FRANCE
Gut = interface between food and human body
Digestion releases food components that can have a beneficial or a deleterious effect on human health

... but the mechanisms of food disintegration in the gastrointestinal tract remain unclear and the digestive process has been considered as a black box so far

By increasing our knowledge on food digestion, we will increase our knowledge on the effect of food on human health
However...

- During the last 10 years, an increase in the number of publications on food digestion, associating food scientists, nutritionists, and gut physiologists: a multidisciplinary new scientific community has been created.

- This community is scattered: many ongoing projects at the national level but no current research action on this topic in Europe and no network for exchanges.

- There is no scientific international congress on food digestion where scientists could have exchanges.

- There were no scientific journal dedicated to food digestion before the creation of «Food Digestion» and «Food & Function» (2010).

- There is a dramatic lack of harmonization between the in vitro digestion models used throughout Europe and a real need of validation of these models.

This is the perfect time for developing a trans-European network to improve dissemination of critical findings, develop truly multidisciplinary collaborations, and harmonise approaches between groups and...

COST IS THE BEST MECHANISM FOR THAT
Improving health properties of food by sharing our knowledge on the digestive process

COST Action FA1005

Dr. Didier DUPONT, Senior Scientist, INRA, France

June 2011 – May 2015
Objectives

• Compare the existing digestion models, harmonize the methodologies and propose guidelines for performing experiments
• Validate *in vitro* models towards *in vivo* data (animal and/or human)
• Identify the beneficial/deleterious components that are released in the gut during food digestion
• Determine the effect of the matrix structure on the bioavailability of food nutrients and bioactive molecules

But these goals can only be reached by...

• Gathering scientists from different disciplines (food science, nutrition, gastroenterology, immunology...) to share and improve our knowledge on food digestion
Chair
Didier Dupont - France

Vice-chair
Alan Mackie - UK

Dairy, Egg
Fruits & Vegetables
Meat, Cereals

Characterization of raw materials and processed food matrices for optimized nutrient bioaccessibility
WG1

In vitro, in vivo and in silico models of mammalian gastrointestinal digestion
WG2

Evaluation of the health effects
WG3

BFC identification
Stability during processing
Food multi-scale characterization

Digestion models harmonization
Comparison in vitro / in vivo
Digestion products identification
BFC absorption / bioavailability

Immunomodulatory properties
Regulation of appetite and satiety
Effect of BFC on human microbiota

B. Murray
UK

F Capozzi
Italy

A. Brodkorb
Ireland

I. Recio
Spain

A. Bordoni
Italy

Tor Lea
Norway

www.cost-infogest.eu
340 scientists - 130 institutes – 37 countries
Industry involvement

~ 40 European companies are involved in INFOGEST
The digestive process

Gastric phase = a very complex but crucial step for the whole digestion process

Storage, grinding and mixing in the stomach

Gastric emptying
Models for simulating digestion

**In vitro static models**

**In silico models**

\[ \Phi_{12} = k_{12_{\text{whey}}} \times (V_1 - m_{\text{caswpd}1} \times \alpha) + k_{12_{\text{aggr}}} \times m_{\text{caswpd}1} \times \alpha \]

**In vitro dynamic models**

**Human models**

**Animal models**
Static *in vitro* digestion models: pro’s & con’s

Main Reasons:
- Ethical
- Technical
- Financial

Advantages:
Standardisation of the experimental conditions
Good reproducibility and repeatability
Easy sampling, possibility to follow kinetics

Disadvantages:
You can’t mimic the complexity of the GI tract in a test tube!!!
Needs harmonization
**In vitro gastro-intestinal digestion**
Consensus INFOGEST protocol

**Oral phase**
Mix 1:1 with Simulated Salivary Fluid (SSF)
salivary amylase (75 U/mL)
2 min, pH 7

**Gastric Phase**
Mix 1:1 with Simulated Gastric Fluid (SGF)
Pepsin (2000 U/mL)
2h, pH 3

**Intestinal Phase**
Mix 1:1 with Simulated Intestinal Fluid (SIF)
Enzymes
  - Pancreatin (based on trypsin 100 U/mL) or
  - Pure enzymes
Bile (10mM)
2h, pH 7
The Infogest consensus *in vitro* digestion model

Consensus model based on available physiological data (*in vivo*)

**Oral Phase**
- Solid or liquid meal?
  - Liquid
  - Optional
  - Mix 1:1 with SSF + salivary amylase (75 U/mL), 2 min, pH 7
- Solid
  - Mince meal
  - Mix 1:1 with SSF + salivary amylase (75 U/mL), 2 min, pH 7

**Gastric Phase**
Mix 1:1 with SGF + pepsin (2,000 U/mL), 2h, pH 3
0.17 mM phospholipids (non-standard condition)

**Intestinal Phase**
Mix 1:1 with SIF + enzymes 2h, pH 7

**Individual enzymes**
- Trypsin (100 U/mL)
- Chymotrypsin (25 U/mL)
- Pancreatic lipase (2,000 U/mL)
- Collipase (2:1 molar ratio with lipase)
- Pancreatic amylase (200 U/mL)
- Bile (10 mM)

**Enzyme extract**
- Pancreatin (based on trypsin activity at 100 U/mL)
- Bile (10 mM)

**Sample collection and handling options**
- Snap-freeze in liquid nitrogen immediately
- Add protease inhibitor (e.g., 1 mM AEBSF, Roche)
- Freeze dry

OPEN ACCESS article
Calibration of the digestive enzymes, bile provided as supplementary material

29 authors

Minekus et al. 2014
Food Funct. 5, 1113-24
46 citations
Hot paper (0.1%)
Table 2  Preparation of stock solutions of simulated digestion fluids. The volumes are calculated for a final volume of 500 mL for each simulated fluid. We recommend to make up the stock solution with distilled water to 400 mL instead, i.e. 1.25× concentrate, for storage at −20 °C. In the Experimental section, these 1.25× concentrates are referred to as Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) electrolyte stock solutions. The addition of enzymes, bile salts, Ca\(^{2+}\) solution etc. and water will result in the correct electrolyte concentration in the final digestion mixture. CaCl\(_2\)(H\(_2\)O)\(_2\) is not added to the electrolyte stock solutions as precipitation may occur. Instead, it is added to the final mixture of simulated digestion fluid and food\(^a\).

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock conc.</th>
<th>Vol. of stock</th>
<th>Conc. in SSF</th>
<th>Vol. of stock</th>
<th>Conc. in SGF</th>
<th>Vol. of stock</th>
<th>Conc. in SIF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g L(^{-1})</td>
<td>mol L(^{-1})</td>
<td>mL</td>
<td>mmol L(^{-1})</td>
<td>mL</td>
<td>mmol L(^{-1})</td>
<td>mL</td>
</tr>
<tr>
<td>KCl</td>
<td>37.3</td>
<td>0.5</td>
<td>15.1</td>
<td>15.1</td>
<td>6.9</td>
<td>6.9</td>
<td>6.8</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>68</td>
<td>0.5</td>
<td>3.7</td>
<td>3.7</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>84</td>
<td>1</td>
<td>6.8</td>
<td>13.6</td>
<td>12.5</td>
<td>25</td>
<td>42.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>117</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>11.8</td>
<td>47.2</td>
<td>9.6</td>
</tr>
<tr>
<td>MgCl(_2)(H(_2)O)(_6)</td>
<td>30.5</td>
<td>0.15</td>
<td>0.5</td>
<td>0.15</td>
<td>0.4</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>(NH(_4))(_2)CO(_3)</td>
<td>48</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>0.5</td>
<td>0.5</td>
<td>—</td>
</tr>
</tbody>
</table>

For pH adjustment

<table>
<thead>
<tr>
<th>Stock conc.</th>
<th>Vol. of stock</th>
<th>Conc. in SSF</th>
<th>Vol. of stock</th>
<th>Conc. in SGF</th>
<th>Vol. of stock</th>
<th>Conc. in SIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol L(^{-1})</td>
<td>mL</td>
<td>mmol L(^{-1})</td>
<td>mL</td>
<td>mmol L(^{-1})</td>
<td>mL</td>
<td>mmol L(^{-1})</td>
</tr>
<tr>
<td>NaOH</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HCl</td>
<td>6</td>
<td>0.09</td>
<td>1.1</td>
<td>1.3</td>
<td>15.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

CaCl\(_2\)(H\(_2\)O)\(_2\) is not added to the simulated digestion fluids, see details in legend

<table>
<thead>
<tr>
<th>Stock conc.</th>
<th>Vol. of stock</th>
<th>Conc. in SSF</th>
<th>Vol. of stock</th>
<th>Conc. in SGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>g L(^{-1})</td>
<td>mol L(^{-1})</td>
<td>mmol L(^{-1})</td>
<td>mmol L(^{-1})</td>
<td>mmol L(^{-1})</td>
</tr>
<tr>
<td>CaCl(_2)(H(_2)O)(_2)</td>
<td>44.1</td>
<td>0.3</td>
<td>1.5 (0.75(^*))</td>
<td>0.15 (0.075(^*))</td>
</tr>
</tbody>
</table>

\(^a\) in brackets is the corresponding Ca\(^{2+}\) concentration in the final digestion mixture.
The oral phase

- Always include an oral phase (± enzymes)
- Ratio Food / Simulated Salivary Fluid (SSF): **50/50 w/v**
- Time of chew: **2 min**

Add 5 g food + 5 mL SSF
Add Human salivary alpha amylase 150 IU/ mL in the SSF
Add 0,5 µL of CaCl2 (588 g/ L) per mL SSF
The gastric phase

Ratio oral content / Simulated gastric fluid (SGF) : 50/50 w/v
Porcine pepsin: 2000 U/mL
Time of gastric digestion: 2 hours
pH of the reaction: 3

Why 2 hours?
Duration highly depends on the type of food/meal
  * Gastric emptying of a western type solid meal: 3-4h, of a liquid 0.5-1h
  * Addition of nutrients to a liquid meal increases the transit time
  * Strong inter and intra-individual variability

A time of 2h for gastric digestion represents the half emptying of a moderately nutritious and semi-solid meal

Why pH 3?
Fasted pH commonly found is around or below 2
pH increases to 5 and above because of the buffering capacity of the food/meal

pH 3 represents the mean value for a general meal exhibiting a gastric emptying half-time of 2 h
pH and duration of the gastric phase

Gardner et al. 2002
125g steak, 200g boiled potatoes, 200g fresh vegetables, 50g salad, 200mL dessert, 200 mL water

Dressman et al. 1990
6 oz hamburger, 2 slices bread, 2 oz potatoes, ketchup, mayonnaise

Tyssandier et al. 2003
Tomato puree, carrot puree or chopped spinach

Malagelada et al. 1976
Solid meal 400 mL 458 kcal pH 6
The intestinal phase

- Ratio Food (gastric content) / Simulated duodenal fluid (SDF): **50/50 w/v**
- Time of duodenal digestion: **2 hours**
- pH of the solution: **7**

20 mL gastric content
+ 3.0 µL of CaCl2 (H2O)2 (588 g/L, w/v)
+ Bile: (final concentration in total fluid 10 mM). There are two options for bile for the duodenal stage, which is to use either:
  - **Bile extract** (e.g. B8631-100G from Sigma) or
  - **Fresh porcine bile** (available from several InfoGest members including IFR (160 mM stock). The SDF the concentration is made up to 20mM.
+ fill up to a final volume of 40 mL with SDF to reach the same volume as the gastric digesta (20mL).

**At this point there are two options in how to proceed:**
1. Use pancreatin: sufficient pancreatin to provide 100 U/ml of trypsin (TAME Units). The proteolytic, lipolytic and amylolytic activity should be determined
2. Use individual enzymes
pH and duration of the intestinal phase

Why pH 7?

- pH measured in the duodenum is close to 6.5 (see below)
- In the small intestine, pH increases slightly over its length to a value of around 7.5 in the distal ileum

Tyssandier et al. 2003
Tomato puree, carrot puree or chopped spinach

Dressman et al. 1990
6 oz hamburger, 2 slices bread, 2 oz potatoes, ketchup, mayonnaise
Key points

The calibration of the digestive enzymes is crucial and not that easy to perform

International inter-laboratory assay (7 labs in Europe)

- **Pepsin reference**
  - From porcine gastric mucosa
  - P7012
  - Batch number Lot#SLBJ4999V

- **Pancreatin reference**
  - Porcin pancreatin
  - P7545
  - Batch number Lot#SLBJ7293V

- **2976 U/mg of pepsin powder**

- **7.0 trypsin U/mg of pancreatin powder**

Same batch of pepsin and pancreatin analyzed by trained people
Key points

Differences in digestive enzymes calibration leads to different peptidomes

Gastric phase

Intestinal phase

The extremely sensitive metabolomics approach clearly discriminates laboratory 6 and 7 as being outliers during gastric as well as intestinal phase
The consensus model can be learned with videos on YouTube

In vitro food digestion - COST action INFOGEST

Alpha Amylase Activity Assay for In Vitro Food Digestion

Static In Vitro Digestion Method for Food

In vitro food digestion - COST action INFOGEST

https://www.youtube.com
Conclusion

- The Infogest model has been applied successfully to several foods like milk, meat, pasta, bread... and it works! (46 citations in 1.5 year)

- The Infogest *in vitro* digestion model is now used all over the world, in Europe, USA, Australia, New Zealand, Argentina...

- Interlaboratory trials have been performed at the European level on the digestion of milk and meat

- People can easily learn how to run the model, calibrate the digestive enzymes and the bile with the open access publication and the videos available on YouTube

- Validation towards *in vivo* data is under investigation. Data on pigs will be available before the end of 2015
We are pleased to announce the next

5th International Conference on Food Digestion

in Rennes, France, April 2017