Influence of immobilisation of bacteria in a model cheese: spatial distribution and porosity of colonies and their consequences on metabolism and micro-environment.

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A multidisciplinary and multiscale approach, reinforced by two high-calibre facilities:

- **Dairy Platform**
- **Biological Resource Centre**

- **Structuration / destructuration mechanisms of food matrix:**
  
  *from structural characterisation to digestion*

- **Dairy processing and cheese making:**
  
  *toward sustainable dairy systems*

- **Microbial interaction:**
  
  *food matrix and host cell*
Outlines

- Immobilised bacteria in cheese and spatial distribution of colonies
- Non-destructive approaches

1. Microgradients of pH and redox around colonies
2. Porosity of colonies and physiological states
3. Consequences of spatial distribution on ripening
Why studying immobilised bacteria in cheese?
In all the cheeses, bacteria are immobilised:

⇒ They develop as colonies
⇒ Colonies are local concentration of enzymes
⇒ Bacterial colonies are the “hot spots” of ripening
In cheese... spatial distribution of colonies

1. Spatial distribution is random
2. Same final population
3. Spatial distribution of colonies depends on the inoculation level
4. Consequence: difference of interfacial area (exchange surface colonies/matrix)

**Experimental validation:** confocal microscopy: colonies of a **Green Fluorescent Protein**-producing *Lactococcus* strain

*Jeanson et al, AEM, 2011*
### In cheese... spatial distribution of colonies

<table>
<thead>
<tr>
<th>Initial population levels (cfu/g)</th>
<th>Distance between colonies (µm)</th>
<th>Colony diameter (µm) for a same final population</th>
<th>Interfacial area (cm²/cm³)</th>
<th>In cheese...</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^2$</td>
<td>2154</td>
<td>136</td>
<td>/</td>
<td>Indigenous flora of NSLAB</td>
</tr>
<tr>
<td>$10^4$</td>
<td>464</td>
<td>30</td>
<td>/</td>
<td>Adjunct cultures</td>
</tr>
<tr>
<td>$10^5$</td>
<td>123-215</td>
<td>10-12</td>
<td>1.3</td>
<td>Lactic starters</td>
</tr>
<tr>
<td>$10^6$</td>
<td>50-100</td>
<td>5-6</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>$10^7$</td>
<td>34</td>
<td>4</td>
<td>9.5</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Jeanson et al. (2011) and Kabanova et al. (2012)

X7
Among the main substrates of LAB:

**Lactose is soluble** but **caseins are mainly immobilised** in the network.

**If diffusion limitations occur** in cheese or in the colonies:

- **Microgradients** of concentration may appear in and around the colony (lactate, lactose, redox…)
- **Heterogeneity** at the microscopic scale may occur
Heterogeneity inside the colony

If caseins and big peptides **do not diffuse**
=> heterogeneity of physiological states between cells
=> **Colonies >400 µm in gelatine**
*Mckay et al. (1997)*

If caseins and big peptides **diffuse**
=> same physiological states for all cells

Heterogeneity outside the colony

\[ \Delta \] concentration of glucose in agar/gelatine
=> **always suggested for colonies with diameter 100-400 µm** *Pipe et al. (2008)*

\[ \Delta \] concentration of lactate
=> \[ \Delta \] pH
=> **Colonies diameter from 100 µm to few mm in gelatine**
*Wimpenny (1992); Malakar et al (2000)*

\[ \Delta \] Redox ??
No study

Substrates

End-products

*Jeanson et al., Review, submitted in Frontiers in Food Microbiology*
What are the questions in cheese?

1. Are there microgradients of pH around colonies in cheese?
2. Do molecules diffuse inside the colonies? Is there heterogeneity of physiological states?
3. Is ripening modulated by the spatial distribution of colonies?

For all these questions, what is the influence of the size of colonies?
Design for non-destructive approaches
A common design for all experiments...

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A model cheese</td>
<td>a non-fat ultrafiltered milk retentate coagulated into cheese</td>
</tr>
<tr>
<td>A known species</td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>Fluorescent dyes or molecules</td>
<td>fluorescent sensitive dyes (pH, redox) or fluorescent diffusing molecules</td>
</tr>
<tr>
<td>A non-destructive system</td>
<td>observation under confocal microscope</td>
</tr>
</tbody>
</table>
A common design for all experiments...

Gel cassette® = 40 or 1 mL

Gel cassette system® (Brocklehurst et al., 1995 from the Institute of Food Research, UK)

allows non-destructive observation under the confocal microscope

= homogeneous and repeatable cheese matrix

Model cheese

Imaging chamber = 1 mL
Do microgradients of pH and redox occur?
pH and redox microgradients?

SYTO 9: bacteria dye
C-SNARF-4F or resazurin: incorporated into the model cheese before coagulation
No microgradients of pH throughout 72 h of acidification, whatever the size of colonies.
Do substrates diffuse inside the colony?
Porosity of colonies?

- RITC-Dextrans from 4 to 155 kDa, and home-labeled milk proteins
- Spatio-temporal measurements of fluorescence intensity
Porosity of colonies to dextrans?

Diffusion way = Δ[C]

Dextrans (4 to 155 kDa, neutral and flexible)

The RITC-molecule did not reach the colony

The fluo intensity increases around and in the colony

Floury et al., IJFM, 2013
Porosity of colonies to dextrans?

**Diffusion way** = $\Delta [C]$  

Dextran molecules as big as 155 kDa diffuse inside the colony.  

*Dextrans (4 to 155 kDa, neutral and flexible)*

Increase of intensity in the colony  

Increase of intensity outside the colony

Floury et al., IJFM, 2013

Dextran molecules as big as 155 kDa diffuse inside the colony.
Lactoferrin (77 kDa, \(\oplus\) charged globular non flexible)

Lactoferrin does not diffuse inside the colony.

Floury et al., submitted in Frontiers in Food Microbiology
Porosity of colonies to proteins?

The $\alpha_{s1}$-casein (23.6 kDa, – charged and flexible)

$\alpha_{s1}$-casein does not diffuse inside the colony

$\Rightarrow$ does this generate heterogeneity of physiological states inside the colony?

Floury et al., submitted in Frontiers in Food Microbiology
Heterogeneity of physiological states?

However, in our conditions:

**No apparent heterogeneity** in the viability of cells between the periphery and the centre of the colony.

Thus, **peptides may diffuse** inside the colony.

Red: damaged cells
What are the consequences of spatial distribution on ripening?
Consequences of spatial distribution on ripening

Do different spatial distributions impact:

- Proteolysis: peptides, amino acids?
- Volatile compounds?
- Lactose, lactate, citrate?
Experimental design: two sets of cheeses

Different times of renneting

Inoculation $10^5$ cfu/mL

0h renneting

Big colony cheese

8h renneting

Small colony cheese

$10^8$ cfu/mL

Spatial distribution is the only parameter that differentiates the 2 sets of cheeses

Within 27 days

Le Boucher et al., in preparation
Consequences of spatial distribution on ripening Peptides

Total nr. of peptides

- β-casein: 485
- α_{31}-casein: 429
- α_{32}-casein: 250
- K-casein: 236

% of discriminant peptides

- β-casein: 38.6%
- α_{31}-casein: 17.0%
- α_{32}-casein: 40.4%
- K-casein: 45.3%

1370 identified peptides in total

More generally higher in small > big colonies cheeses at the beginning

Le Boucher et al., in preparation
Consequences of spatial distribution on ripening

Free amino acids

Total concentration of free amino acids

Le Boucher et al., in preparation
Consequences of spatial distribution on ripening

Volatile compounds

- 15 impacted volatile compounds
- For 10 out of 15: small > big colonies cheeses

**Diacetyl**

**Acetoain**

Le Boucher et al., in preparation
Untargeted metabolomics: an alternative and innovative approach

Untargeted metabolomics:
- MS-based techniques were used
- without a priori or previous knowledge
- identification and (semi-)quantification of soluble metabolites accessible to the analysis

Poster n°C10

- Water and acetonitrile extractions
- Positive and negative ionizations
- At 2, 13 and 27 days
Consequences of spatial distribution on ripening Metabolome of cheese

PCA at 2 days
Water extraction & positive ionization

26 metabolites identified
- Free amino acids
- Vitamin
- Organic acids
- Nucleotides
- Sugar

Same results for both extractions and both ionizations, at 2 and 13 days
Consequences of spatial distribution on ripening

Conclusions

• Quantitative but not qualitative differences
• Metabolites are more abundant in small colony cheeses than in big colony cheeses
• Differences were significant but moderate
**Take Home Messages**

Innovative approaches => understanding of ripening at the microscopic scale

1. No microgradients of pH whatever the size of colonies

2. Proteins seem not to diffuse

3. Metabolism of cells in *small colonies* are more active than in *big colonies* in cheese

- Big dextran up to 155 kDa diffuse

Microgradients of redox
MERCI

THANK YOU FOR YOUR ATTENTION

Please visit http://www6.rennes.inra.fr/stlo_eng

For the Metabolomics

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