Natural antimicrobials in sanitation…

… an alternative to enhance microbial food safety?

Florence Dubois-Brissonnet
Associate Professor

AgroParisTech – INRA
Unité Mixte de Recherche “Bioadhésion et Hygiène des matériaux”, Massy, France
Research unit « Bioadhesion and hygiene of materials »

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“Advanced Listeria monocytogenes Control Measures in RTE Meats and Poultry Products”
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General outlines

Introduction

Applications of natural antimicrobials:
- as preservatives in food products
- for surface decontamination
- in active-packaging

Hurdle-technology and applications

Conclusion
Introduction

Foodborne illness still has a huge health and economic impact

- 76 millions foodborne intoxications per year in USA (MMWR. 2002. 51:325-329)
- 11-13 millions per year in Canada (http://www.inspection.gc.ca)
- 67000 per year in France (estimation by the National French Food Safety Agency in 2004)

Consumers demand:

High quality, safe food products

- convenient and innovative
- natural flavor and taste
- extended shelf-life
- less salt, less acid and less chemicals

Innovative alternative technologies
Challenges

Available solutions to improve microbial safety and quality of food products?

**CHEMICALS**
- Biocides: chlorine, PAA, QACs, aldehydes...), etc...

**PROCESSES**
- Thermal processes
- Non-thermal processes: Irradiation, High Hydrostatic Pressure, etc...

**PACKAGING**
- Polymer films MAP, etc...
- Active-packaging

**NATURAL ANTIMICROBIALS**
- Bacteriocins, organic acids, enzymes, animal or plant extracts, etc...
Natural antimicrobials

Antimicrobials from micro-organisms
- Bacteriocins: nisin, pediocin…
- Organic acids: acetic, lactic acids…

Plant-derived antimicrobials
- Organic acids: citric, sorbic acids…
- Phyto-phenols, adhehydes, flavonoids, catechins, saponins …

Animal-derived antimicrobials
- Lacto-antimicrobials : lactoferrin, lactoperoxydase, lactoglobulins, lactolipids
- Ovo-antimicrobials : lysosyme, ovotransferrin, ovoglobulin

Others
- Sodium chloride
- Wood smoke

Natural doesn’t mean no toxic
I- Natural antimicrobials as food preservatives

Inhibition of microbial growth

Log N

Control
C1
C2 > C1
C3 > C2
C4 > C3
C5 > C4
C6 > C5

Time

Food matrix
Inhibitory effect of preservatives

Dilution method: bacterial growth is followed in presence of different concentrations of an antimicrobial.

Salmonella Typhimurium grown in presence of α-terpineol

Mineral Inhibitory Concentration

24h-MIC is between 4 and 4.5 mM
Inhibitory effect of preservatives

Dose-response curve modeling

For each antimicrobial concentration, modeling of growth response

\[ \text{OD} = f(\text{time}) \]

\[ \mu_{\text{max}} = f(C) \]

Growth inhibition of *Salmonella Typhimurium* by α-terpineol

Lambert-Pearson model (2000)

\[ \sqrt{\mu_{\text{max}}(c)} = \sqrt{\mu_{\text{max}}(c = 0)} \cdot g(c) \]

with

\[ g(c) = \exp \left[ - \frac{c}{\text{CMI}} \cdot \exp \left( \frac{\ln(\text{NIC}/\text{CMI})}{-e} \right) \right] \]

At 24h

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>OD</th>
<th>MIC</th>
<th>NIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>200</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>400</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>600</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>800</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\[ \text{MIC} = 4.2 \text{ mM} \pm 0.42 \]

\[ \text{NIC} = 2.3 \text{ mM} \pm 0.75 \]

\[ \mu_{\text{max}} \text{ growth rate} \]

\[ \ln(\text{OD}(t)) = \ln(\text{OD}_0) + A \cdot \exp \left( -\exp \left( \frac{\mu_{\text{max}} \cdot e}{A} \cdot (\text{lag} - t) + 1 \right) \right) \]
Inhibitory activity of phyto-phenols on *Salmonella Typhimurium* growth

- **Thymol**
  - Thyme
  - At 24h
    - NIC = 0.7 mM
    - MIC = 0.9 mM

- **Carvacrol**
  - Oregano
  - At 24h
    - NIC = 0.7 mM
    - MIC = 0.9 mM

- **α-Terpineol**
  - Rosemary
  - At 24h
    - NIC = 2.3 mM
    - MIC = 4.2 mM

- **Eugenol**
  - Clove
  - At 24h
    - NIC = 1.9 mM
    - MIC = 3.0 mM

- **Menthol**
  - Mint
  - At 24h
    - NIC = 0.9 mM
    - MIC = 3.4 mM

- **Geraniol**
  - Basil
  - At 24h
    - NIC = 1.1 mM
    - MIC = 3.0 mM

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Guillier *et al* (2007) JFP,70, 2243-2250
Inhibitory effect of preservatives

We have to be very careful with the MIC absolute values.

They depend on:
- the growth medium and conditions
- the inoculum size
- the incubation time
- the strain

They are only comparative.

Lee et al, 2009, JFP, 72, 1107-1111
Natural antimicrobials activity in food systems

**Bacillus cereus**
- Control
- Green tea extract (1% p/v)
- Rosemary extract (1% p/v)

**Staphylococcus aureus**
- Control
- Green tea extract (1% p/v)
- Rosemary extract (1% p/v)

Levels of natural preservatives required in food systems

<table>
<thead>
<tr>
<th>Ratio of concentration</th>
<th>Antimicrobial</th>
<th>Micro-organism</th>
<th>Matrix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twofold</td>
<td>Carvacrol</td>
<td><em>L. monocytogenes</em></td>
<td>Skimmed-milk</td>
<td>Karatzas et al, 2001, JAM, 90,463-469</td>
</tr>
<tr>
<td>Ten-fold</td>
<td>Rosemary extract</td>
<td><em>L. monocytogenes</em></td>
<td>Pork liver sausage</td>
<td>Pandit et Shelef, 1994, Food Microbiology, 11, 57-63</td>
</tr>
<tr>
<td>50-fold</td>
<td>Carvacrol</td>
<td><em>B. cereus</em></td>
<td>Soup</td>
<td>Ultee and Smid, 2001, IFFM, 64,373-378</td>
</tr>
<tr>
<td>25- to 100-fold</td>
<td>Plant essential oil from Spain</td>
<td><em>L. monocytogenes</em></td>
<td>Soft-cheese</td>
<td>Mendoza-Yepes et al, 1997, JFS, 17,47-55</td>
</tr>
</tbody>
</table>
II- Natural antimicrobials in surface decontamination

Log N

Time

Bacterial inhibition

Bacterial inactivation

Food surfaces

Meat carcasses

Fruits and vegetables

Food contact surfaces
Decontamination of meat products by lactic acid

Inoculation of chicken carcasses with 7-8 log/cm² *Listeria monocytogenes* or *Salmonella* Enteritidis.
Decontamination by immersion in lactic acid at different concentrations during 20 min

![Graph showing log reduction at 20 min for Listeria and Salmonella](chart)

- In USA, lactic acid is recognized as antilisterial agent and is allowed in food products (Food Safety and Inspection Service, 2000)
- In Europe it is approved by the European commission (directive 95/2/CE)

Anang *et al* (2007) Food Control, 18, 961-969
Decontamination of meat products by nisin

Inoculation of meat pieces with 4.5 log UFC/cm² of *Listeria innocua*. Decontamination by spraying nisin solution at 5000 UI/mL on meat pieces.

Cutter et Siragusa (1996) Food Microbiology, 13, 23-33

![Graph showing the comparison of Listeria counts in adipose and lean tissues over time for untreated, water-treated, and nisin-treated samples.](image)
Inactivation of *Salmonella* Typhimurium with carvacrol on stainless steel

Decontamination with 5 mM carvacrol

Salmonella counts (log CFU/mL) vs. time (min)

Detection threshold

Biofilm 3D-structure

Adherent cells 2D-structure

Planktonic cells
Activity on biofilms

Observation with Confocal Laser Scanning Microscopy (CLSM)

Bridier et al., 2009 – Eurobiofilms Roma

*Pseudomonas aeruginosa* biofilm

Green: Syto 9 (all the bacteria) and Red/ yellow: Propidium Iodide (Membrane altered cells)
III- Natural antimicrobials in active-packaging

Surface coating with natural antimicrobials

Active packaging

Pack
Environment
Food product
Release of natural antimicrobials from a sachet

Incorporation of natural antimicrobials in package

Coating of the packaging with natural antimicrobials
Inactivation of *Salmonella* on sliced tomatoes by carvacrol in vapor-phase

Decontaminated tomatoes were sliced, cut into 4 pieces and inoculated with *Salmonella*.

Carvacrol in vapor-phase is more active when temperature is low

Obaidat and Frank (2009) JFP, 72,315-324
Active packaging containing nisin

Slices of cheddar were inoculated with *Listeria innocua* or *Staphylococcus aureus* at 2-4 \(10^5\) CFU/g

Bioactive inserts were prepared allowing adsorption of nisin on cellulose packaging paper (7650 UI/cm\(^2\)) and placed over the slices of cheese.

Cheese were stored at 4°C in MAP

![Graph showing bacterial growth over time]

*Listeria*: 3 units log drop
*S. aureus*: 2 log units drop

Filled symbols: untreated inserts
Empty symbols: films prepared with nisin

Scannell et al. (2000) IJFM, 60,241-249
Active packaging containing essential oils

Bologna slices were inoculated with *Listeria monocytogenes* at 10^3 CFU/cm². Alginate-based edible films immerged in 20% calcium chloride and containing 1% essential oils of oregano, cinnamon or savory were applied to slices to prevent pathogen growth.

- Cinnamon-based films were the most effective on *Listeria* growth inactivation
- 2.5 log more reduction in 4 days with cinnamon and in 5 days for oregano and savory

Ouissalah *et al* (2007) JFP, 70, 4, 901-908
IV- Combinations of treatments

There is some eventual smelly and tasty effects of antimicrobials when used alone at efficient levels

« Hurdle Technology »

Gould (1985)

1st hurdle  2nd hurdle  3rd hurdle

Combination of antimicrobials with non-thermal processes

… or « Multifactorial Preservation »

Roller (2000)

3 simultaneous hurdles

Combination of antimicrobials
### Numerous potentialities of combinations

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Examples</th>
<th>Micro-organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriocin</td>
<td>Essential oil</td>
<td><em>Nisin-carvacrol</em></td>
<td><em>B. cereus</em></td>
<td>Periago et Moezelaar, 2001, IFFM, 68, 141-148</td>
</tr>
<tr>
<td>Bacteriocin</td>
<td>Essential oil</td>
<td><em>Nisin-thymol</em></td>
<td><em>L. monocytogenes, B. subtilis</em></td>
<td>Ettayebi et al, 2000, FEMS Microbiology Letters, 183, 191-195</td>
</tr>
<tr>
<td>Bacteriocin</td>
<td>Organic acid</td>
<td><em>Nisin-lactic, acetic acid or salts</em></td>
<td><em>L. monocytogenes</em></td>
<td>Samelis, 2005, Lebensm. –Wiss. U.-Technol. 38, 21-28</td>
</tr>
<tr>
<td>Bacteriocin</td>
<td>EDTA</td>
<td><em>Nisin-EDTA</em></td>
<td><em>Pseudomonas, Brochothrix</em></td>
<td>Economou et al, 2009, Food Chemistry, 114, 1470-1476</td>
</tr>
<tr>
<td>Bacteriocin</td>
<td>Irradiation</td>
<td><em>Pediocin-irradiation 2kGy</em></td>
<td><em>L. monocytogenes</em></td>
<td>Chen et al, 2004, JFP, 67, 1866-1875</td>
</tr>
<tr>
<td>Essential oil</td>
<td>Essential oil</td>
<td>20 plant aromatic extracts</td>
<td><em>S. Typhimurium</em></td>
<td>Nazer et al, 2005. Food Microbiology, 22, 391-398</td>
</tr>
<tr>
<td>Essential oil</td>
<td>Essential oil</td>
<td><em>Cymene-thymol</em></td>
<td><em>B. cereus</em></td>
<td>Delgado et al, 2004, Food Microbiology, 21, 327-334</td>
</tr>
<tr>
<td>Essential oil</td>
<td>Organic acid</td>
<td><em>Thymol, carvacrol-acetic, lactic citric acids</em></td>
<td><em>S. Typhimurium</em></td>
<td>Zhou et al, 2007, JFP, 70, 1704-1709</td>
</tr>
<tr>
<td>Essential oil</td>
<td>MAP</td>
<td><em>Eugenol or thymol - MAP</em></td>
<td><em>Spoilage flora</em></td>
<td>Valero et al, 2006, Postharvest Biology and Technology, 41, 317-327</td>
</tr>
<tr>
<td>Essential oil</td>
<td>refrigeration</td>
<td><em>Carvacrol, cinnamaldehyde or thymol - refrigeration</em></td>
<td><em>Bacillus cereus</em></td>
<td>Valero et al, 2006, Food Microbiology, 23, 68-73</td>
</tr>
<tr>
<td>Essential oil</td>
<td>HHP</td>
<td><em>Carvacrol-HHP</em></td>
<td><em>L. monocytogenes</em></td>
<td>Karatzas et al, 2001, JAM, 90, 463-469</td>
</tr>
<tr>
<td>Organic acids</td>
<td>HHP</td>
<td><em>Lactate-HHP</em></td>
<td><em>L. monocytogenes and Salmonella</em></td>
<td>Aymeric et al, 2005 – JFP, 68, 173-177</td>
</tr>
</tbody>
</table>

Most of these studies analyze cumulative effects of treatments but not really synergistic effects.
Combination with two antimicrobials

Isobolographic analysis

Isobole = curve connecting equivalent doses of both compounds that lead to total bacterial inhibition
Numerous potentialities of combinations

Whatever the application, nisin is often used in combination with other treatments: organic acids, EDTA, hexametaphosphate, lysosome, thermal treatment…

Nisin  Heat stable
hypoallergenic,
Degraded by proteolytic enzymes in the human intestinal tract

Example of hurdle technology:

Combinations of nisin and High Hydrostatic Pressure (HHP) on mechanically recovered poultry meat

Multifactorial preservation consequences

Sub-inhibitory doses of each antimicrobial can cause:

- bacterial STRESS?
- increasing of tolerance to bactericidal activity?
**Bacterial reactivity in presence of sub-inhibitory doses of organic acids**

### Saturation of membrane in presence of organic acids

- **Salmonella Typhimurium**

### Stress proteins synthesis

- Bi-dimensional electrophoresis

- At acidic pH in comparison with neutral pH
Bacterial reactivity in presence of sub-inhibitory doses of organic acids

Acid Tolerance Response (ATR)

Bacterial adaptation to organic acids

Increasing of tolerance
- to acidic environmental conditions
- to disinfectants

DDAB = didecyl dimethyl ammonium bromide
(quaternary ammonium compound)


Graph:
- pH 7 cultures
- pH 5.5 cultures

Listeria counts (log CFU/mL)

DDAB concentration (x 10^-2 mg/L)
Bacterial reactivity in presence of sub-inhibitory doses of aromatic compounds

Fatty acids membrane composition of *Salmonella* Typhimurium

When cells enter stationary phase

In presence of thymol

Similar results for carvacrol, citral and eugenol

Induction of antimicrobials tolerance?
Conclusion

The interest for natural antimicrobials has expanded in recent years in response to consumer demand for greener additives.

Their applications are likely to increase in the future:
- as food preservatives
- for surfaces decontamination
- in active-packaging

In all cases, we have underlined the interest of combined systems.

Further studies are still necessary to validate and optimize these systems.
Thank you for your attention!

Contact:
florence.dubois-brissonnet@jouy.inra.fr