USE OF POTENTIAL PROBIOTIC STRAINS TO REDUCE CAMPYLOBACTER JEJUNI IN BROILERS:

RECENT DEVELOPMENTS USING LACTOBACILLUS SALIVARIUS SMXD51

MANUEL J. SAINT-CYR¹, MURIEL GUYARD-NICODÈME², BERNARD TAMINIAU³, MICHEL AMELOT⁴, GEORGES DAUBE³, MARIANNE CHEMALY², XAVIER DOUSSET¹, NABILA HADDAD¹

¹SECALIM UNIT UMR1014, ONIRIS, INRA, UNIVERSITÉ BRETAGNE LOIRE, NANTES, FRANCE
²HYGIENE AND QUALITY OF POULTRY AND PORK PRODUCTS UNIT, ANSES, PLOUFRAGAN/PLOUZANÉ LABORATORY, UNIVERSITÉ BRETAGNE LOIRE, FRANCE
³FARAH, DEP. OF FOOD SCIENCES, LIÈGE UNIVERSITY, BELGIUM
⁴DEPARTMENT OF POULTRY EXPERIMENTATION, ANSES, PLOUFRAGAN/PLOUZANÉ LABORATORY, UNIVERSITÉ BRETAGNE LOIRE, FRANCE
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

PROBIOTICS: ATTRACTIVE AND NATURAL ANTIMICROBIAL AGENTS

• Health benefits
  • Basic nutritional advantages (Mountzouris 2007)
  • Protection against pathogens (Higgings 2008)

• Alternative to antibiotics

• Probiotic bacteria mainly used and studied
  • Lactobacilli (gasseri, plantarum, reuteri, rhamnosus, salivarius, …)
  • Bifidobacteria (longum, adolescentis, …)
  • Others lactic acid bacteria (Enterococcus, Lactococcus, Pediococcus, …)
  • Bacillus spp.

• Origin
  • Intestinal gut (human, pig, chicken, …)
  • Food (milk, cheese, …)
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXDS1

PROBIOTICS: ADVANTAGES

- Poultry industry
  - Enhancing growth performance of broilers

- Comparison to others strategies in progress to control Campylobacter

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccination</td>
<td>-Easy to use</td>
<td>-Antigenic variability of Campylobacter strains</td>
</tr>
<tr>
<td>Bacteriophage therapy</td>
<td>-Rapid action</td>
<td>-Selection of resistant Campylobacter strains</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Diversity of Campylobacter strains</td>
</tr>
<tr>
<td>Probiotics</td>
<td>-Easy to produce and to use</td>
<td>-Variable sensitivity of Campylobacter strains</td>
</tr>
<tr>
<td></td>
<td>-Mix of multiple species</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Different ways of inhibiting Campylobacter</td>
<td></td>
</tr>
</tbody>
</table>

adapted (Saint-Cyr 2016 in press)
PROBIOTICS AGAINST *CAMPYLOBACTER* IN BROILERS: EXAMPLE OF *L. SALIVARIUS* SMXDS1

PROBIOTICS: POTENTIAL ACTIVITIES AGAINST *CAMPYLOBACTER*

1. Potential mechanisms involved (Mohan 2015)

1. Acidic compounds production and pH reduction (Neal-McKinney 2012)
2. Competition for nutrients (Aho 1992)
3. Bactericidal substances production (bacteriocins, $H_2O_2$) (Messaoudi 2012)
4. Strengthening tight junctions of intestinal epithelium (Messaoudi 2012)
5. Competition for adhesion site (Wine 2009)
6. Co-aggregation (Nishiyama 2014)
7. Modification of the avian gut microbiota (Sanders 2011)
8. Modulation of the immune system (Brisbin 2011)

(Saint-Cyr 2016 in press)
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXDS1

PROBIOTICS: SCREENING FOR ANTI-CAMPYLOBACTER ACTIVITY

- General strategy

**In vitro** screening of anti-Campylobacter activity
  - Inhibition of growth
  - Reduction of virulence

**In vitro** evaluation of probiotic abilities
  - Survival in simulated gastrointestinal conditions
  - Safety assays
  - Adhesion to intestinal cells
  - Antimicrobial assays
  - Immunomodulation

**In vivo** testing of anti-Campylobacter activity
  - Therapeutic activity
  - Preventive activity
  - Host colonization
  - Safety for animals

adapted (Saint-Cyr 2016 in press)
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

PROBIOTICS: SCREENING FOR ANTI-CAMPYLOBACTER ACTIVITY

- In vivo testing of anti-Campylobacter activity

Campylobacter load reduction (log\(_{10}\) CFU/g)

- 0.5 log Low reduction
  - *Lb. crispatus* (Neal-McKinney 2012)
  - *B. subtilis* sp. mixture (Aguiar 2013)
  - *Lb. gasseri* (Nishiyama 2014)
  - *B. subtilis* (Guyard-Nicodème 2015)
  - *Bacillus* spp. (Arsi 2015)

- 2 log Moderate reduction
  - *E. faecium* + *P. acidilactici* + *Bf. animalis* + *Lb. salivarius* + *Lb. reuteri* (Ghareeb 2012)
  - *Lb. paracasei* + *Lb. rhamnosus* + *Lb. lactis* (Cean 2015)

- 0.2 log Very low reduction
  - *B. subtilis* (Fritts 2000)
  - *Bf. longum* (Baffoni 2012)
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51

INTRODUCTION: CAMPYLOW PROJECT

- French interregional project

- Institutional partners

- Laboratories
  - Secalim unit (ONIRIS, INRA)
  - Hygiene and Quality of Poultry and Pork Products unit (ANSES)

- Industrial partners

- Aim
  - Reduce Campylobacter carriage in broiler chickens using a potential probiotic strain
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

CHAPTER 1: IN VITRO EVALUATION

- **Lb. salivarius SMXD51 isolation**

- **In vitro** screening of anti-Campylobacter activity

- Evaluation of probiotic abilities

- Good candidate to control *Campylobacter* colonization in chickens

(Messaoudi 2011, 2012)
STORY OF THE POTENTIAL PROBIOTIC *LACTOBACILLUS SALIVARIUS* SMXD51
CHAPTER 2: *IN VIVO* EVALUATION

- **Trial 1 design**
  - Four groups of 30 day-of-hatch Ross PM3 chickens

<table>
<thead>
<tr>
<th>2</th>
<th>4</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>21</th>
<th>23</th>
<th>25</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>35 Days post-hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MRS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cell-free supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Laboratory freeze-dried (LFD) SMXD51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SMXD51 Culture</td>
</tr>
</tbody>
</table>

- **Ceca analyses**
  - *Campylobacter* loads by decimal dilution method
  - *Lb. salivarius* loads by quantitative real-time PCR

adapted (Saint-Cyr 2016 in revision)
STORY OF THE POTENTIAL PROBIOTIC *Lactobacillus salivarius* SMXD51

CHAPTER 2: *IN VIVO* EVALUATION

- Trial 1 results
  - *Lb. salivarius* presence

- Day 14
  - No detection of *Lb. salivarius* for control and supernatant (LOD=3 log bacteria/g)
  - 7.8±0.2 log bacteria/g

- Day 35
  - No detection of *Lb. salivarius* for control and supernatant
  - 7.9±0.5 log bacteria/g

- Increase of *Lb. salivarius* specie in ceca with SMXD51 treatment

Kruskal-Wallis test followed by a Dunn’s multiple comparisons test. ***P < 0.001

adapted (Saint-Cyr 2016 in revision)
STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51
CHAPTER 2: IN VIVO EVALUATION

- Trial 1 results
  - *Campylobacter* colonization

- Day 14
  - 0.8 log reduction for culture
  - Trend for others treatments

- Day 35
  - 1.5 log reduction for LFD SMXD51
  - 2.8 log reduction for SMXD51 culture

- No preventive effect but curative effect of SMXD51
- Need the bacteria
- Greater effect for culture than freeze-dried form

Kruskal-Wallis test followed by a Dunn’s multiple comparisons test.
* P < 0.05; *** P < 0.001

adapted (Saint-Cyr 2016 in revision)
STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51
CHAPTER 2: IN VIVO EVALUATION

• Trial 1 conclusions

  • *In vivo* anti-*Campylobacter* activity of SMXD51

  • Effective model for determining modes of action

  • BUT delivery route and administration form irrelevant for industrial application

• Trial 1 perspectives

  • Investigations on mechanism involved

  • Incorporation in diet
STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51
CHAPTER 2: IN VIVO EVALUATION

- Trial 2 design
- Treatments

Industrial freeze-dried (IFD) SMXD51 treatments

Freeze-drying of SMXD51 at pilot scale

Incorporation in diet

Ad libitum

IFD SMXD51 (diet)

Freeze-drying of SMXD51 at laboratory scale

30 minutes rehydration in tryptone salt broth

Per os administration

IFD SMXD51 (per os)

Laboratory freeze-dried (LFD) SMXD51 treatment

30 minutes rehydration in tryptone salt broth

Per os administration

LFD SMXD51 (per os)
PROBIOTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

CHAPTER 2: IN VIVO EVALUATION

- **Trial 2 design**
  - Four groups of 30 day-of-hatch Ross PM3 chickens

**Control**

![Control Chart](image1)

**Treated groups**

![Treated Groups Chart](image2)

- **Ceca analyses**
  - *Campylobacter* loads by decimal dilution method
  - *Lb. salivarius* loads by quantitative real-time PCR

adapted (Saint-Cyr 2016 in preparation)
STORY OF THE POTENTIAL PROBIOTIC *LACTOBACILLUS SALIVARIUS SMXD51*

CHAPTER 2: *IN VIVO* EVALUATION

- Trial 2 results
  - Diet supplementation

- 10.5 log of culturable lactobacilli / kg diet
- 11 log *Lb. salivarius* bacteria / kg diet
- Increase (3 log) of culturable lactobacilli and *Lb. salivarius* specie in diet with SMXD51 supplementation

![Graph showing bacterial concentration in diet](image)

**Bacterial concentration in diet**

- Culturable lactobacilli (MRS plates)
- *Lb. salivarius* (QPCR)
STORY OF THE POTENTIAL PROBIOTIC *LACTOBACILLUS SALIVARIUS* SMXD51

CHAPTER 2: *IN VIVO* EVALUATION

- Trial 2 results
  - *Lb. salivarius* presence (day 35)

- 6.6±0.4 log CFU/g in control
- 7.2±0.4 log CFU/g in treated groups

- Presence of autochthonous *Lb. salivarius*
- But significant increase of *Lb. salivarius* specie in ceca with SMXD51 treatment

Kruskal-Wallis test followed by a Dunn’s multiple comparisons test. Different superscript letters are significantly different at $P < 0.05$

adapted (Saint-Cyr 2016 in preparation)
STORY OF THE POTENTIAL PROBIOTIC *LACTOBAILLUS SALIVARIUS* SMXD51
CHAPTER 2: IN VIVO EVALUATION

- Trial 2 results
  - *Campylobacter* colonization (day 35)

  - High variability in control
  - No reduction
  - No curative effect
  - No reproductible curative effect for LFD SMXD51
  - Interference of autochthonous *Lb. salivarius*?

Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

adapted (Saint-Cyr 2016 in preparation)
PROBI OTICS AGAINST CAMPYLOBACTER IN BROILERS: EXAMPLE OF L. SALIVARIUS SMXD51

STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51

CHAPTER 2: IN VIVO EVALUATION

- Trial 2 results
  - Broiler growth performance

### Trial 2 results

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LFD (<em>per os</em>)</th>
<th>IFD (<em>per os</em>)</th>
<th>IFD (diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>a</td>
<td>ab</td>
<td>ab</td>
<td>b</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Increase of weight (+ 300 g) for IFD in diet
- Stable feed intake
- Improvement of growth performance in broilers challenged with *C. jejuni*

Kruskal-Wallis test followed by a Dunn’s multiple comparisons test. Different superscript letters are significantly different at $P < 0.05$
TRIAL 2 CONCLUSIONS

- No curative effect of freeze-dried SMXD51 on Campylobacter colonization with the freeze-drying processes used
  - Competition with autochthonous Lb. salivarius?
  - Influence of freeze-drying processes?

- Improvement of poultry growth performance after a Campylobacter challenge

TRIAL 2 PERSPECTIVES

- Identification of parameters influencing the in vivo anti-Campylobacter activity

- Evaluation of the impact of freeze-drying processes

adapted (Cell Host & Microbe cover 2013)
STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51
CHAPTER 3: CULTURE VS. FREEZE-DRIED FORMS

- Trial 1: Cecal Campylobacter loads

- Trial 1: Dose of SMXD51 administered

➢ Negative effect of freeze-drying processes or dose-effect relationship?

➢ No dose-effect relationship
➢ Possible negative effect of freeze-drying processes?

Native Lb. salivarius SMXD51

Freeze-dried Lb. salivarius SMXD51 forms

Laboratory

Industrial
STORY OF THE POTENTIAL PROBIOTIC LACTOBACILLUS SALIVARIUS SMXD51
CHAPTER 3: CULTURE VS. FREEZE-DRIED FORMS

Native *Lb. salivarius* SMXD51

Freeze-dried *Lb. salivarius* SMXD51 forms

Kruskal-Wallis test followed by a Dunn’s multiple comparisons test. **P < 0.01
***P < 0.001

Survival to conditions of digestive tract

Adhesion to avian epithelial cells

Production of bacteriocins anti-*Campylobacter*

Immune response modulation

**Freeze-dried forms less adherent**

**Freeze-dried forms produce less bacteriocins**

**Freeze-dried forms more sensitive**

**Adapted (Saint-Cyr 2016 in preparation)**
STORY OF THE POTENTIAL PROBIOTIC \textit{LACTOBACILLUS SALIVARIUS SMXD51}

CHAPTER 3: CULTURE VS. FREEZE-DRIED FORMS

**Conclusions**

- Significant differences observed* (native vs. freeze dried)
- Different trends observed (laboratory freeze-dried form vs. laboratory freeze-dried form)
- Impact on probiotic abilities of SMXD51

\[\text{Native Lb. salivarius SMXD51} = \text{Freeze-dried Lb. salivarius SMXD51 forms}\]

- **Adhesion**
- **Bacteriocin production**
- **Bile tolerance**
- **pH tolerance**

*adapted (Saint-Cyr 2016 in preparation)
In vivo anti-Campylobacter activity of SMXD 51
- Interesting (reduction >1 log)
- No reproducible
- Negative effect of freeze-drying processes (2.8 vs. 1.5 log)

Improvement of growth performance (+300g) of broilers challenged with C. jejuni and treated with SMXD51

Improvement of freeze-drying processes
- Maintain probiotic abilities of SMXD51

Investigation on mechanisms of in vivo anti-Campylobacter activity
- Understand the mechanisms
- Identify parameters influencing the activity
- Screen conditions enhancing the activity
ACKNOWLEDGMENT

ONIRIS-INRA
- SECALIM Unit
  - Xavier DOUSSET
  - Nabila HADDAD

ANSES
- HQPAP Unit
  - Marianne CHEMALY
  - Muriel GUYARD
  - Typhaine POEZEVARA
  - Ségalène QUESNE
  - Fabrizio TATONE

AGROCAMPUS OUEST-INRA
- SELEAC Unit
  - Michel AMELOT
  - David COURTOIS
  - Alassane KEITA
  - Bernard LE BERRE
  - Thierry LE COQ
  - Jean-Michel MANGART
  - Anthony VENDEMBEUCH

- STANDA
  - Riwanon LEMEE
  - Emma REDON

PAO
- Stéphan ROUVERAND
- PAYS DE LA LOIRE and BRITTANY regions

JEFO
- Jean-Christophe BODIN
- Nicolas DESTOMBES

TERRENA
- Cécile GUILLON

NUTREA
- Florence QUERE

Thank you for your attention