What can we realistically expect from probiotics* in poultry health and nutrition?

Christophe Bostvironnois
Global Poultry Commercial Development

*Probiotic: a micro-organism that when consumed maintains or restores beneficial bacteria to the digestive tract (WHO definition)
Acknowledgment
What a Crazyisis World!

CRISIS

危 机

Danger  Opportunity
What can we realistically expect from probiotics in poultry health and nutrition?

1. Important reminders in Microbiology

2. Microbiome Agility Why is it Important?

3. What does an ideal Microbiome look like?

4. An example of Good Bacteria: Bacilli’s Mode of Action
1. Important reminders in Microbiology
Naming of microorganisms

- Life
- Domain
- Kingdom
- Phylum
- Class
- Order
- Family
- Genus
- Species

+ sometimes subspecies and serotypes!
Classification of Bacteria based on:

- Oxygen demand

- **Bacillus**
- **Lactic Acid Bacteria**
Classification of Bacteria based on:

- **Temperature**
  - Bacillus
  - Lactic Acid Bacteria

- **pH**
  - Bacillus
  - Lactic Acid Bacteria
    - acidophile
    - neutrophile
    - alkaliophile
Cell morphology

**COCCI**
- Diplococci (Streptococcus pneumoniae)
- Tetrad
- Staphylococci (Staphylococcus aureus)
- Sarcina (Sarcina ventriculi)

**BACILLI**
- Streptococci (Enterococcus faecalis (M74))
- Chain of bacilli (Lactobacillus rhamnosus (LGG))
- Flagellate rods (Escherichia coli)
- Spore-former (Bacillus subtilis (CH201))

**SPIRAL**
- Vibrios (Vibrio cholerae)
- Spirilla (Helicobacter pylori)
- Spirochaetes (Brachyspira hyodysenteriae)
Bacteria growth in the laboratory

- Lag phase: Few live cells
- Exponential growth phase: Many live cells
- Stationary phase: Some cells remain viable
- Death phase: Dead cells

Total cells in population, live and dead, at each phase.

Binary Fission

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How to study bacteria

1. Microscopy
   - Shape of bacteria
   - Bacteria staining

Flagella staining

Gram staining

Endospore staining

green endospores inside bacterial cells
How to study bacteria

2. Culture in Medium

- Type of media:
  - Complex
  - Defined
  - Selective
  - Differential

- Blood agar

- Escherichia coli
- Staphylococcus aureus
- Salmonella enterica

(a) Nutrient agar
(b) MacConkey agar
(c) MacConkey agar

Beta Hemolysis
Alpha Hemolysis
Gamma Hemolysis
How to study bacteria

3. Molecular techniques

**PCR based**
- Presence/absence
- Limited resolution

**Quantitative PCR (qPCR)**
- Absolute abundance
- Resolution: only culturable bacteria

**Sequencing**
- The 16S-based approach
  - Amplify and sequence 16S rRNA
  - Group similar sequences into OTUs
  - Use database to identify OTUs

- Quantitative PCR (qPCR)
  - Absolute abundance
  - Resolution: only culturable bacteria

- 16S RNA sequencing
  - Shotgun sequencing (metagenome)

- DNA sequencing

- Studying bacteria

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Why Strains Matter: Why is it important to select the right *Bacillus*?

*Bacillus subtilis* species have more than 1,000 strains recorded in international strain banks. Selecting and differentiating strains matters greatly for the health and food industries as different strains have unique properties. There is huge difference between strains in e.g enzyme production, inhibition of pathogenic bacteria abilities, germination etc.
Sporeformers as Bacilli are excellent survivors
Bacillus life cycle from Feed to Litter!

11. Life cycle of an endospore-forming bacterium. Sporulation produces resting endospores in preparation for environmental changes or extremes. In the presence of water and nutrients, endospores may germinate and re-engage in the vegetative cycle. DPA is released during stage I germination. (Adapted from Figure 6, page 78 in Principles of Microbiology 6th edition (2001)).
# Probiotics? Which One? Bacillus vs. Lactic Acid Bacteria

<table>
<thead>
<tr>
<th></th>
<th>Lactic Acid Bacteria</th>
<th>Bacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram</strong></td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Growth</strong></td>
<td>Facultative-anaerobic</td>
<td>Aerobic/facultative-anaerobic</td>
</tr>
<tr>
<td><strong>Upstream</strong></td>
<td>Easy (generally)</td>
<td>Challenging</td>
</tr>
<tr>
<td><strong>Downstream</strong></td>
<td>Easy (generally)</td>
<td>Challenging</td>
</tr>
<tr>
<td><strong>Stability/Shelf life</strong></td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Bacteriocin production</strong></td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Enzymes production</strong></td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Immunomodulatory effect</strong></td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Lactic acid production</strong></td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Natural environment</strong></td>
<td>Gut, dairy products</td>
<td>Water, air, soil</td>
</tr>
<tr>
<td><strong>Safety</strong></td>
<td>All GRAS and QPS</td>
<td>Case by base</td>
</tr>
<tr>
<td><strong>Resistance to : pH</strong></td>
<td>++</td>
<td>++/+++</td>
</tr>
<tr>
<td><strong>Bile acid</strong></td>
<td>++</td>
<td>+/++++</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>+</td>
<td>+/++++</td>
</tr>
<tr>
<td><strong>High Aw</strong></td>
<td>+++</td>
<td>+ (Germination)/+++</td>
</tr>
</tbody>
</table>
2. Microbiome Agility
Why is it Important?
Microbiome Agility
Maintaining Function AND Diversity, a paradigm shift in modern broilers

Diversity Potential

Unstable
Stable

Functional Potential

Traditional Way, the Base of Nutritional Strategy

Is There another Nutritional Way?

Stable
Unstable

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Microbiome Agility, a Science-Based Platform

- Microbiome Agility

**Definition:** Microbiome Agility is the ability of the intestinal microorganisms to adapt to changing conditions in order to maintain **microbial balance** and **productivity**.

Agility noun [ˈædʒɪlɪtɪ] /əˈdʒɪl·ɪ·tɪ/
The **ability** to **move** about **quickly** and **easily**
3. What does an ideal Microbiome look like?
Microbiome Agility*: The scientific Fundation of Stability, Variability and Reproducibility

Key Learnings:
1. For microbiome data, multiple samples are needed (at least 15),
2. Consider the functionality of the bacteria (not only the family),
3. Sample from the intestinal segments, not only feces.
4. Microbiota of the mucus is different from the one in the lumen
5. Microbiota is age dependent (7 weeks in mice, 7 years in Human),
6. Co-housing limit the microbiome variability between individuals

*Moore R.J., Stanley D., Experimental design considerations in microbiota/inflammation studies, Clinical and Translational Immunology (2016)
Chicken Microbiome of high FCR (low performing-left) vs low FCR (High performing-right) broilers is different at family (first line) or genus (second line) level.

High Performing Birds have both DIFFERENT bacteria family and genus in their microbiome, but also a more DIVERSE microbiota, NUMERICALLY and QUALITATIVELY.
4. An example of Good Bacteria: Bacilli’s Mode of Action
Example of the Mode of Action of *B. subtilis*: Coating Effect of Chr Hansen *Bacillus* and protection of the epithelium by a biofilm

6 CH Innovation Laboratory
Quorum Sensing: the Language of Bacteria*

1 Bacillus subtilis has two kind of demonstrated Auto-Inducers
2 Lux-I-Like catalyze the formation of a specific autoinducer (green pentagons)
3 The autoinducer diffuses and accumulates at high cell density
4 The Lux-Receptor bind their auto-inducer
5 The Receptors activate transcription

*Schauder et Al., The languages of Bacteria

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**Quorum Sensing: the Language of Bacteria**

1. **Bacillus subtilis** has two kinds of demonstrated Auto-Inducers.
2. **Lux**-**I**-Like catalyze the formation of a specific autoinducer (green pentagons).
3. The autoinducer diffuses and accumulates at high cell density.
4. The **Lux**-**Receptor** binds their auto-inducer.
5. The Receptors activate transcription.

*Schauder et Al., the language of Bacteria*
Beta Diversity can be improved with Bacillus-Based Probiotics Turkey trial

Table 9. Spearman correlations of genera with probiotic dose in jejunum, ileum, and cecum samples. All genera with a significant correlation before correction for multiple hypothesis testing are shown.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>genus</th>
<th>rho</th>
<th>p-value</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>Bacillus</td>
<td>0.54</td>
<td>1.43 × 10^-6</td>
<td>0.00061</td>
</tr>
<tr>
<td>Ileum</td>
<td>Bacillus</td>
<td>0.54</td>
<td>1.21 × 10^-5</td>
<td>0.00045</td>
</tr>
<tr>
<td>Ileum</td>
<td>Clostridium sensu stricto</td>
<td>0.29</td>
<td>0.030</td>
<td>0.41</td>
</tr>
<tr>
<td>Ileum</td>
<td>Clostridium XIVa</td>
<td>0.28</td>
<td>0.035</td>
<td>0.41</td>
</tr>
<tr>
<td>Cecum</td>
<td>Collinsella</td>
<td>-0.27</td>
<td>0.035</td>
<td>0.14</td>
</tr>
<tr>
<td>Cecum</td>
<td>Barnesiella</td>
<td>-0.27</td>
<td>0.035</td>
<td>0.14</td>
</tr>
<tr>
<td>Cecum</td>
<td>Odoribacter</td>
<td>-0.41</td>
<td>0.0010</td>
<td>0.028</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Bacteroidetes</td>
<td>-0.30</td>
<td>0.018</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Lactobacillus</td>
<td>0.38</td>
<td>0.0032</td>
<td>0.043</td>
</tr>
<tr>
<td>Cecum</td>
<td>Clostridium XIVa</td>
<td>-0.29</td>
<td>0.025</td>
<td>0.12</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Lachnospiraceae</td>
<td>-0.41</td>
<td>0.0011</td>
<td>0.028</td>
</tr>
<tr>
<td>Cecum</td>
<td>Anaerobium</td>
<td>-0.33</td>
<td>0.011</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Clostridium IV</td>
<td>-0.30</td>
<td>0.020</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Ruminococcaceae</td>
<td>-0.31</td>
<td>0.017</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Clostridiales</td>
<td>-0.26</td>
<td>0.041</td>
<td>0.15</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Rhodospirillaceae</td>
<td>-0.31</td>
<td>0.018</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Bilophila</td>
<td>-0.30</td>
<td>0.020</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Unknown Bacteria</td>
<td>-0.33</td>
<td>0.010</td>
<td>0.10</td>
</tr>
<tr>
<td>Cecum</td>
<td>Akkermansia</td>
<td>0.40</td>
<td>0.0015</td>
<td>0.028</td>
</tr>
</tbody>
</table>

False discovery rate was used to correct for multiple hypothesis testing.

Odoribacter can produce short-chain fatty acids such as acetic acid, succinic acid, and butyric acid which are important for both microbial and host epithelial cell growth (Meehan and Belko 2014) and the genus has been associated with improved performance in chickens (Bortoluzzi et al. 2017). A previous experiments where poultry were given the probiotic B. subtilis found an increased abundance of Odoribacter in the cecum (Li et al. 2018).

Akkermansia is a mucin-degrading bacterium of the phylum Verrucomicrobia recently detected in the human colon. (Geerlings et al. 2018) In humans, Akkermansia has shown to have several beneficial physiological effects, including protection from IBD, obesity, and diabetes (Zhang et al. 2009).


In summary, treatment with the probiotic is associated with an enrichment and depletion of Akkermansia and Odoribacter, respectively, which may both have potential beneficial physiological effects on their hosts.
Probiotics and Health
Direct Inhibition by Bacillus-Based Probiotics
Materials & Methods

Agar well diffusion assay

Agar with pathogen poured into the plate
Wells created

DSM32325/32324/25840 added to selected wells

Incubation
Inspection of inhibition zones

Salmonella enterica serovars tested:
Albany, Braenderup, Enteritidis, Gallinarum, Heidelberg, Infantis, Kentucky, Mbandaka, Minnesota, Muenchen, Schwarzengrund, Stanley, Tennessee and Typhimurium
## FOOD SAFETY in VITRO data

*Salmonella – in VITRO data*

<table>
<thead>
<tr>
<th>Entero-pathogens</th>
<th>Serogroup</th>
<th>DSM32325/32324/25840 inhibition Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Albany</em></td>
<td>C2</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Braenderup</em></td>
<td>C1</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Gallinarum</em></td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Infantis</em></td>
<td>C1</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Kentucky</em></td>
<td>C2</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Mbandaka</em></td>
<td>C1</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Muenchen</em></td>
<td>C2</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Stanley</em></td>
<td>B</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Schwarzengrund</em></td>
<td>B</td>
<td>Y</td>
</tr>
<tr>
<td><em>Salmonella Tennessee</em></td>
<td>C</td>
<td>Y</td>
</tr>
</tbody>
</table>

Ref: Chr. Hansen Innovation Laboratories, M 1076
Materials & Methods

Inhibition in feed matrix (Time Kill)

Feed autoclaved (kills all bacteria). DSM32325/32324/25840 ($10^5$ CFU/g of feed) added. Incubation.

Pathogen added ($10^3$ CFU/g of feed). Incubation.

Samples taken at different time points. Added to plates, incubated and counting of pathogens (e.g., *Salmonella* & *E. coli*).

*Salmonella enterica* serovars tested:

Heidelberg and Typhimurium
Inhibition - Time Kill Assay

Salmonella Stanley

Counts for control and test samples (Preliminary results)

Results
FOOD SAFETY in VITRO data

E. Coli

A triple strain bacillus-based probiotic can reduce E.coli risk by inhibiting directly this pathogen

Multiple in vitro trials have been done to determine the activity of the Bacillus strains against pathogenic microorganisms.

The three strains all show their effectiveness to inhibit pathogenic bacteria.

<table>
<thead>
<tr>
<th>King</th>
<th>Queen</th>
<th>Knight</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Entero-pathogens

- \( E. coli O149:K91:F4 \)
- \( E. coli O101:K::F5 \)

Ref: Chr. Hansen Innovation Laboratories
FOOD SAFETY in VITRO data

E. Coli

A triple strain bacillus-based probiotic (DSM32325/32324/25840) can reduce E.coli risk by inhibiting directly this pathogens in the feed matrix

![Graph showing Escherichia coli counts for control and test samples in the feed](image)
Bacillus licheniformis DSM17236 inhibits the proliferation of Clostridium perfringens type A and C

Protocol: Patented Chr. Hansen

Bacillus licheniformis produces lichenysin (antimicrobial peptides) disrupting of bacterial cytoplasmic membranes of C. perfringens (hypothesis in the literature)

Reference: Internal data: B. licheniformis - Poultry Science Association Annual Meeting 2015
Bacillus-based Probiotics are Enzyme factory in the gut of chickens: They reduce the availability of undigested substrate for pathogens (ie Protein for *C. perfringens*)

<table>
<thead>
<tr>
<th>Enzymes produced by <em>B. subtilis</em> determined by ApiZYM and API20E</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Phosphate (LPS of Gram neg. bact)</td>
</tr>
<tr>
<td>Estearase (C4)</td>
<td>Fat</td>
</tr>
<tr>
<td>Estearase lipase (C8)</td>
<td>Fat</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>Protein</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>Protein</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Phosphate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes produced by <em>B. subtilis</em> determined by ApiZYM and API20E</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-galactosidase</td>
<td>NSP*</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>NSP*</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td>Carbo**</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>NSP*</td>
</tr>
</tbody>
</table>

*NSP: non-starch polysacharides  
**Carbo: other carbohydrates  
Source: Chr. Hansen, Innovation CD News M1006
An example of Immune system modulation with a novel three-strain Bacillus-based probiotic on a mild-necrotic enteritis challenge

**DSM32325/32324/25840 combination performed as an immune modulator**

IL-1b, IL-6, and IL-18 are upregulated in the T4 group, but not in the other groups, indicating immune modulation properties by the probiotic.

**DSM32325/32324/25840 combination modulates the immune response.**

Once the challenge was presented, the T4 group had an increase in cytokine gene expression vs T1, T2 and T3.

There is no up-regulation observed of the pro-inflammatory cytokine gene expression by the three-strain probiotic **without** challenge (T2 vs T1 group).

Ref: TRIAL nr 80423
Probiotics, what about the future?
Microbiome, the forgotten Organ

‘Transplantation’ of behaviour via feces....

BALB/c
Shy and anxious

NIH Swiss
Agressive

Microbiota transplant
Recip

Germ free

Shy and anxious

Agressive

Germ free

Bercik et al Gastroenterology 2016
The 24 hours Life of a chicken during heat stress*

*Wang W.C. et Al, Supplementation of Bacillus subtilis-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens
The 24 hours Life of a layer under a double strain Bacillus-Based Probiotic (CH201/CH200)*: Reduction of Aggresivness

Utilização de **probiótico** para galinhas poedeiras vermelhas, pós-pico de produção: produtividade, qualidade de ovos e bem-estar

Caio César dos Ouros¹, Ibiara Correia de Lima Almeida Paz¹, Alberto Inoue¹, Gustavo Henrique Coelho Chaves¹, Marconi Italo Lourenço da Silva¹, Andressa Silva Jacinto¹

¹ UNESP, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Produção Animal e Medicina Veterinária

<table>
<thead>
<tr>
<th>Tratamento</th>
<th>Arranque de penas (%)</th>
<th>Brigas (%)</th>
<th>Ave escandon (%)</th>
<th>Ovos sujos (%)</th>
<th>Ovos trincados no útero (%)</th>
<th>Falhas de pigmentação (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controle</td>
<td>24,81 A</td>
<td>7,43 A</td>
<td>21,38 A</td>
<td>5,87 A</td>
<td>14,03 A</td>
<td>10,76 A</td>
</tr>
<tr>
<td>200</td>
<td>8,09 B</td>
<td>3,52 B</td>
<td>15,97 B</td>
<td>2,25 B</td>
<td>4,30 B</td>
<td>4,28 B</td>
</tr>
<tr>
<td>400</td>
<td>8,70 B</td>
<td>3,21 B</td>
<td>12,44 B</td>
<td>1,70 B</td>
<td>5,27 B</td>
<td>5,58 B</td>
</tr>
<tr>
<td>Valor de P</td>
<td>0,01</td>
<td>0,01</td>
<td>0,02</td>
<td>0,01</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>CV (%)</td>
<td>12,30</td>
<td>8,87</td>
<td>10,43</td>
<td>7,09</td>
<td>6,77</td>
<td>9,34</td>
</tr>
</tbody>
</table>

* Under review
What can we realistically expect from probiotics in poultry health and nutrition? Microbiome Agility.

In Summary,

- Bacillus-Based probiotics can be considered as the probiotics of choice in Poultry,

- They have 3 main benefits:
  - **Application Benefits**: Bacillus are perfectly adapted to in-feed application (stability, compatibility with Ab, acids, Acox, resistance to heat, ...)
  - **Intestinal Functionality Benefits**: Biofilm production, protection of the top of the villi, better surface of absorption, production of local enzymes,
  - **Health Benefits**: competitive exclusion (space, nutrients, quorum sensing, remember the forest), direct inhibition (production of bacteriocins), Immune-Modulation

- Still to explore:
  - **Welfare Benefits**: calmer behavior, less aggressiveness, heat stress management (intestinal neural link?? Production of beneficial compounds for the brain???)
26th World's Poultry Congress

The French WPSA branch is waiting for you in Paris for WPC2020.

16-20 August 2020
PARIS PALAIS DES CONGRÈS

www.wpcparis2020.com
Acknowledgment