In Ovo translational model for functional assessment of probiotics through metabolome analysis

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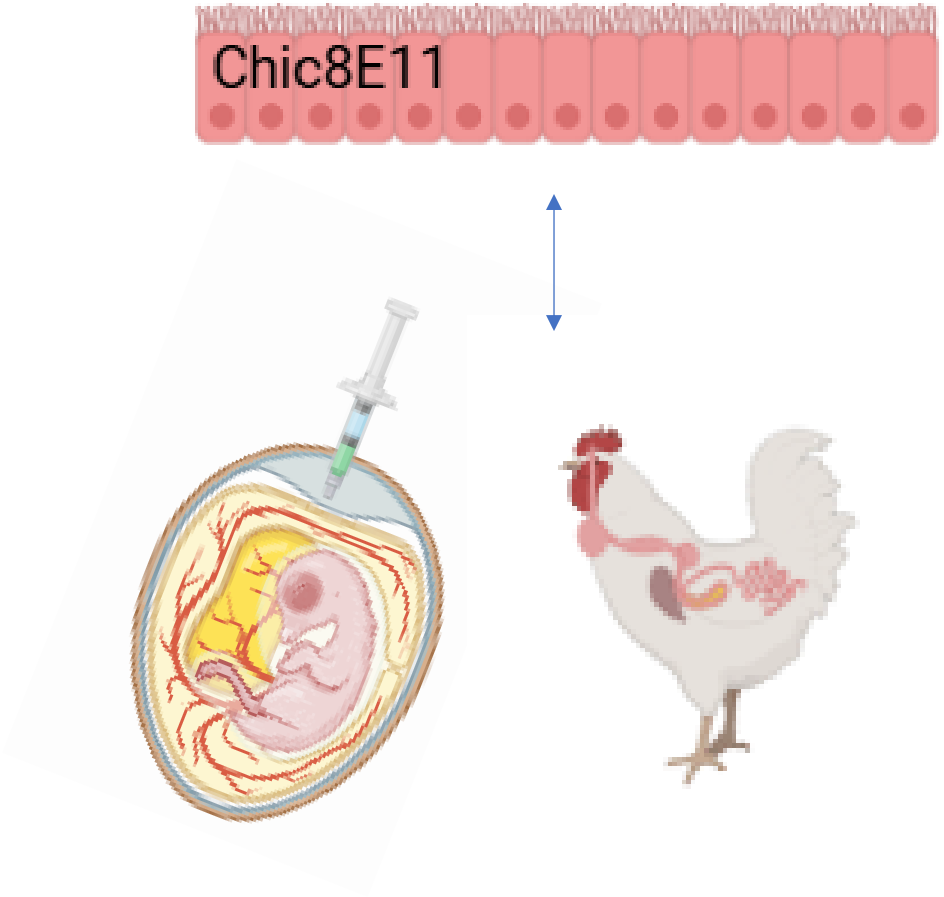
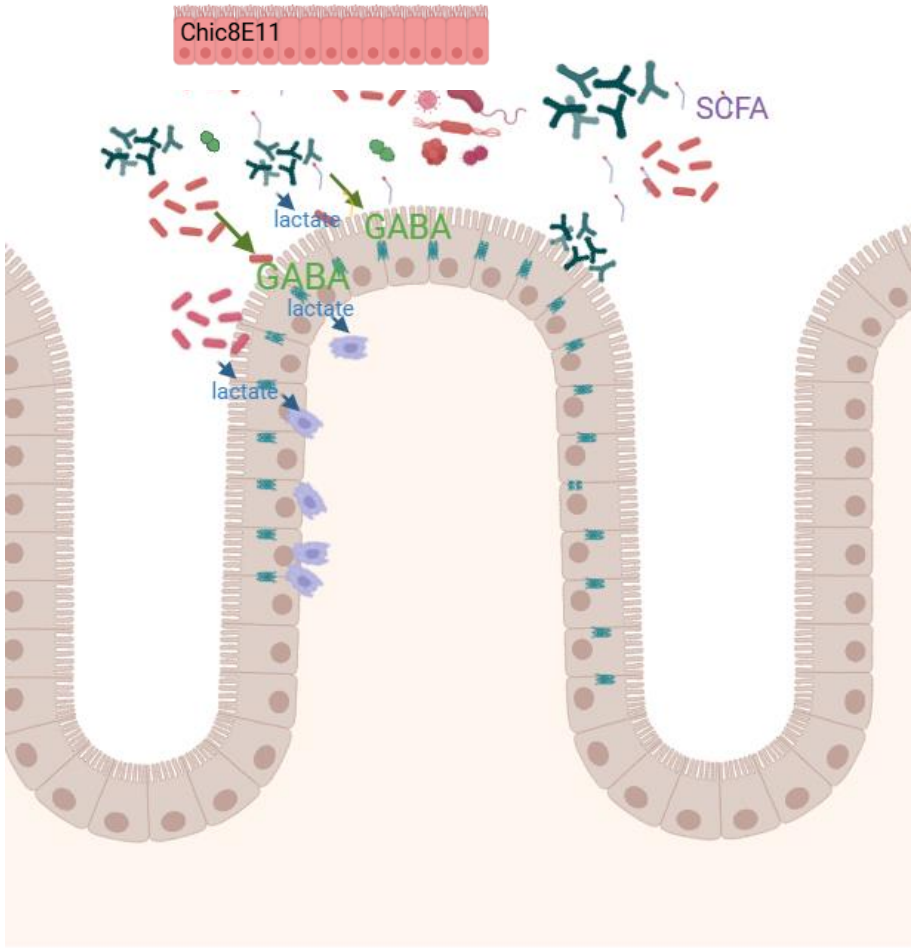


Background



Candidate/ probiotics, beneficial microbial products, phytobiotics, have known benefits but mechanism unclear

Gap in knowledge and techniques→ need for translational models linking *in vitro* and *in vivo* effects

→ evidence-based development of strategies to manage health by nature based solutions: bioactive compounds, immunomodulators

In vitro tests → early life/prenatal treatment → life long track of effects

Study Objectives

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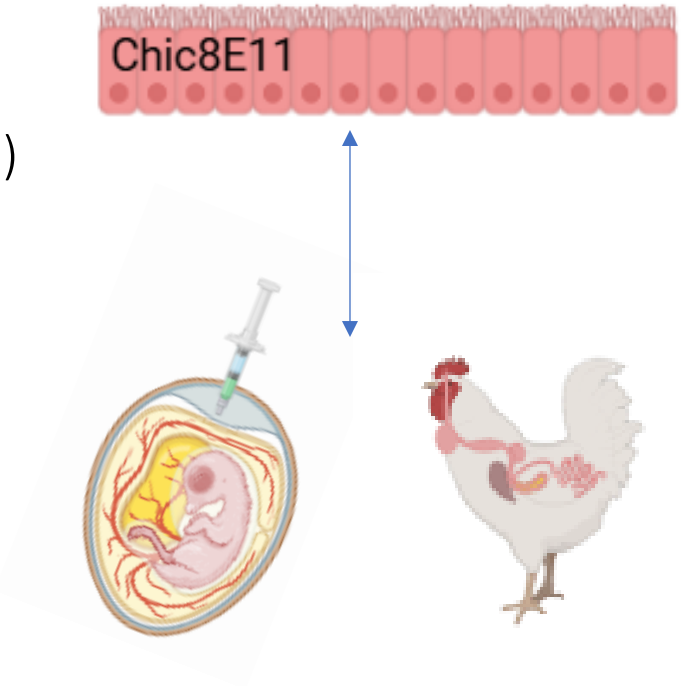
Study Objectives

•Characterize metabolite changes in **chicken intestinal cell line** (Chick8E11) treated with *Bifidobacterium lactis* NCC2818 [Akhavan et al., 2023, Frontiers in Microbiology]

•Analyze metabolome changes in **gut content** of chickens injected *in ovo* with *B. lactis* alone or in a synbiotic combination of *B. lactis* with Astragalus polysaccharides synbiotic (Xi'an Weizhen Biotechnology Co., Ltd.)

•Establish correlations between *in vitro* and *in ovo/in vivo* models

•Identify key metabolic pathways affected by probiotic treatment



Material and methods

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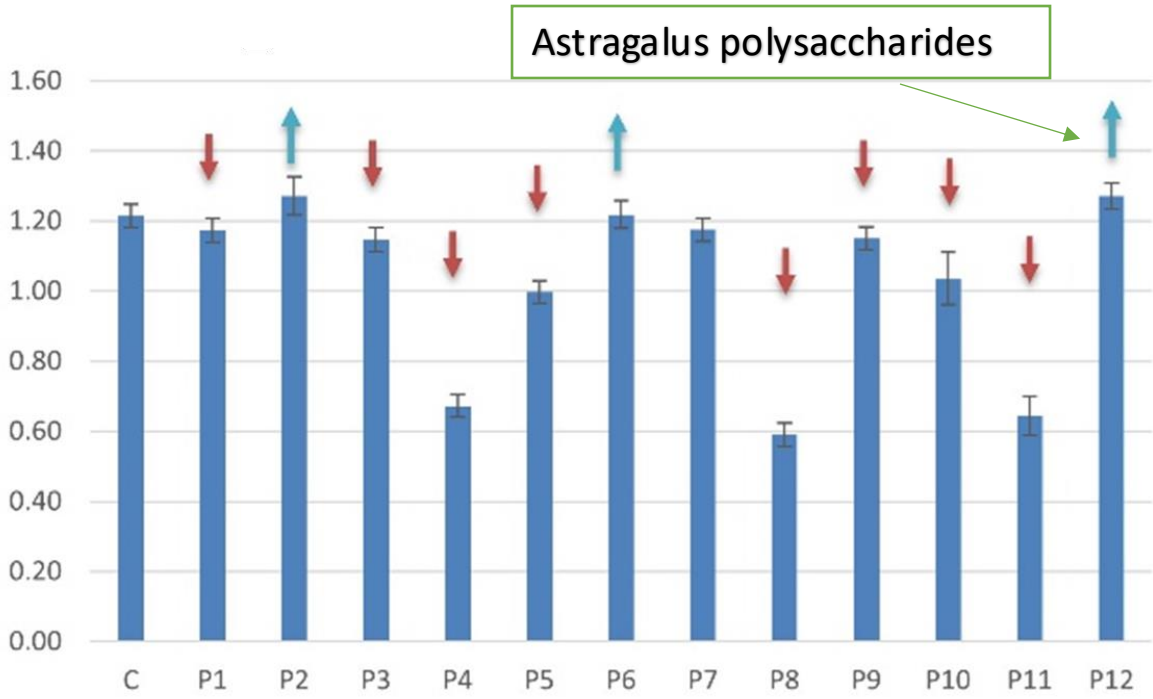
The pairs of candidate probiotic (n=2) and prebiotic (n=2) compounds were selected *in vitro* by testing 12 prebiotics and 12 probiotics:

Candidate Prebiotics	Candidate Probiotics
P1 Beta glucan	*Lactobacillus plantarum*
P2 Vegetable protein hydrolysate	*Bifidobacterium lactis*
P3 Liquid seaweed extract	*Lactobacillus rhamnosus*
P4 Short chain inulin	*Lactobacillus plantarum*
P5 Long chain inulin	*Carnobacterium divergens*
P6 Raffinose	*Propionibacterium thoenii*
P7 Galactooligosaccharides	*Clostridium butyricum*
P8 Snow crab chitooligosaccharides	*Bacillus strain* (5 were tested)
P9 Saccharicterpenin	
P10 Lentinus	
P11 Mannan oligosaccharides	*P1: F981M156B, P2: B001P335, P3: A114P252 (BioAtlantis Ltd.)*
P12 Astragalus polysaccharides	

***Bifidobacterium lactis* growth**



The one-way ANOVA with Tukey's test as post hoc (Newman-Keuls) comparisons ($p \leq 0.05$). Blue arrows show stimulation of the probiotic growth and red arrows show the inhibition of the probiotic growth compared to the control variant (C: Control with glucose)

Selections are based on in vitro trials in Akhavan et al., Front Microbiol. 2023 & Zuo et al., 75.EAAP Abstract #2216306

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Co-cultures: **The intestinal cells (Chic-8E11,** Tentamus Pharma & Med Deutschland GmbH) were inoculated with the *Bifidobacterium lactis (10:2 [CFU: cells] ratio)* and **cell free supernatants (CFS, at 20% v/v)** derived from probiotic cultures that were adjusted to 10^6 CFU

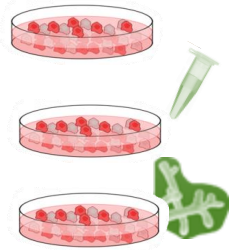
The ***Bifdobacterium*** **CFS** were produced by collecting the probiotic culture supernatants followed by centrifugation at 10,000 x g for 10 min and filtration through a 0.45 µm pore size filters (Millipore, Merck Group, Poland)

Co-cultures were maintaned for 24h at 37°C, 5% CO_2 in in a complete DMEM/F12 medium, 3 independent experiments (biological repetitions) per each combination

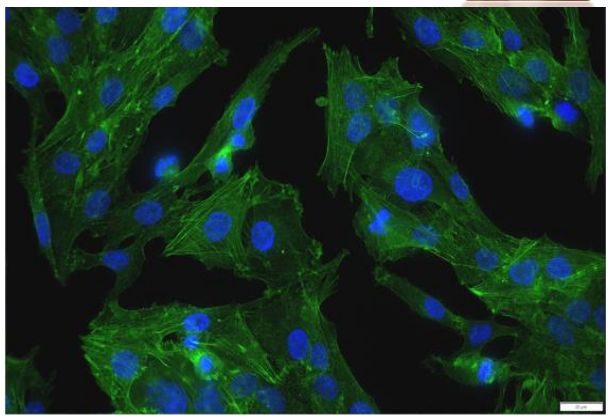
Control Chic-8E11

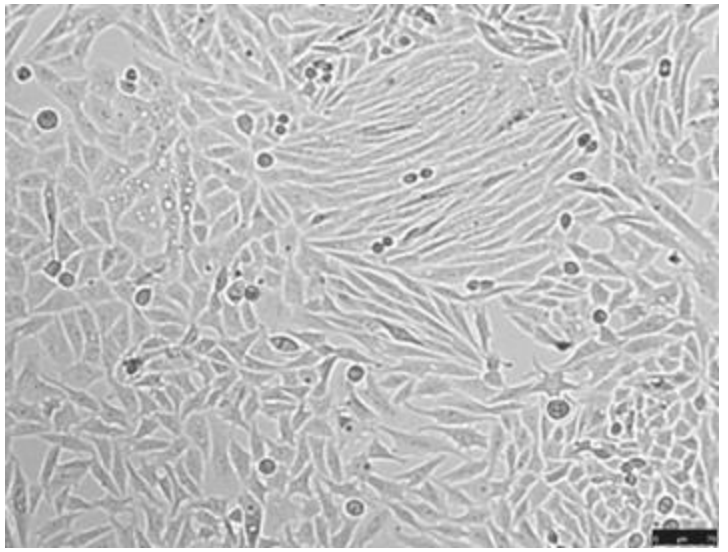
Chic-8E11 + Bifido CFS

Chic-8E11 + Bifido



targeted metabolomic analysis protocol of the MxP® Quant 500 kit (Biocrates, Austria)





Material and methods

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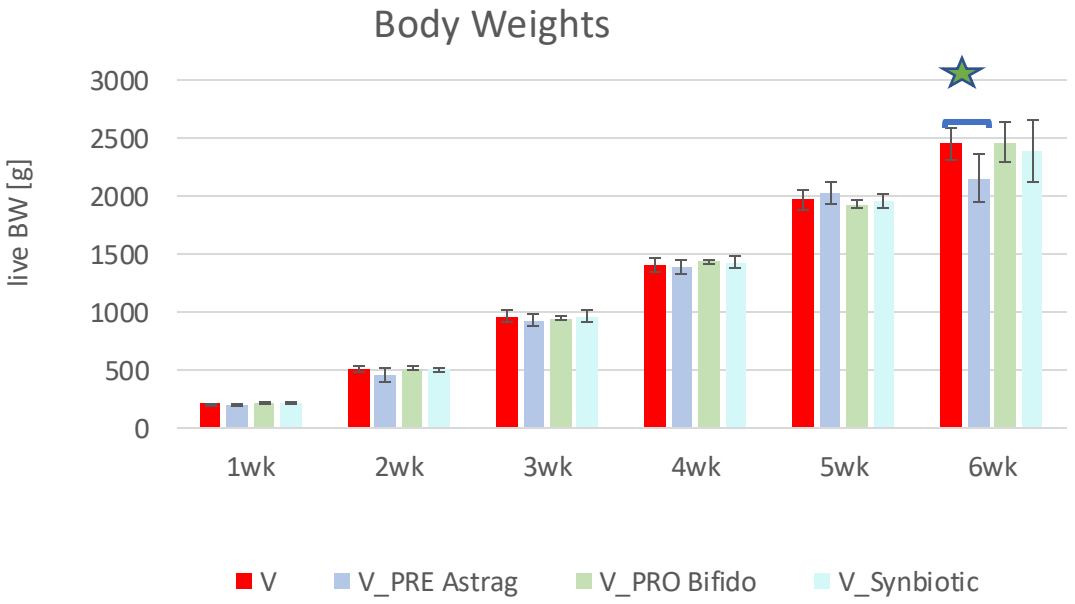
In ovo injections were performed automatically to the amnion, using the Vinovo vaccination technology (Contributor: Viscon Group, Gravendeel The Netherlands). All the embryos were vaccinated (IBD, Phivax)

50 µl/egg,
140 eggs/group
18.5 day of embryonic incubation



Control
Prebiotic 1mg AP
Probiotic B. Lactis 10^6 CFU
Synbiotic 10^3 CFU B. lactis + 1 mg AP

(4 replicates per group, 15 birds per pen) with females only

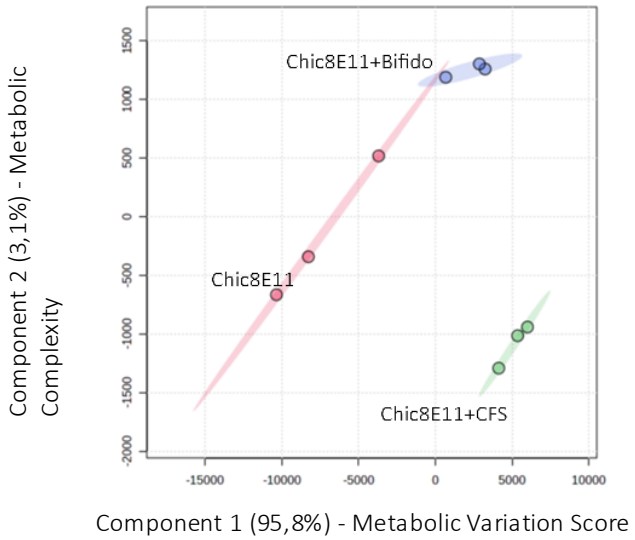
Metabolomic fingerprint profiles & key metabolites

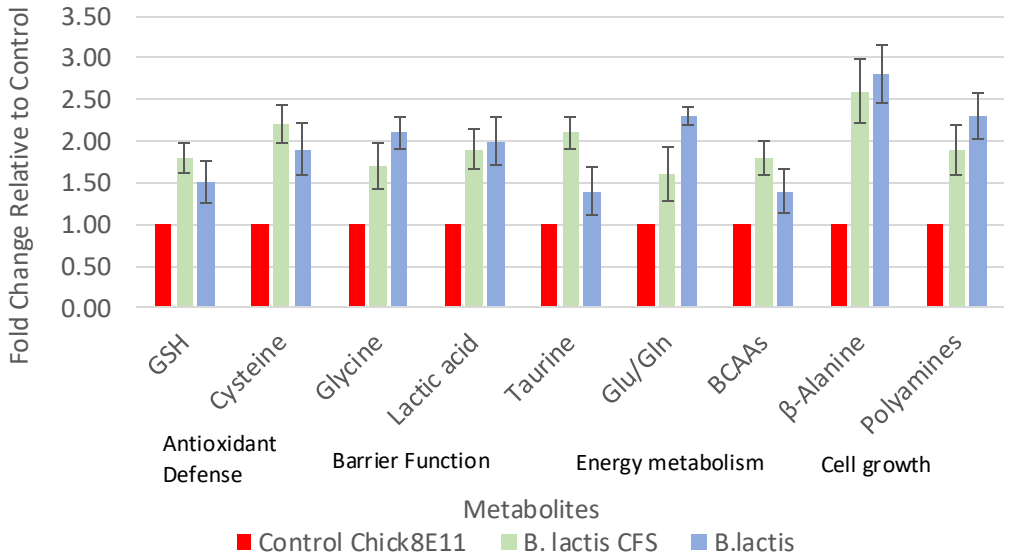
Control Chic-8E11

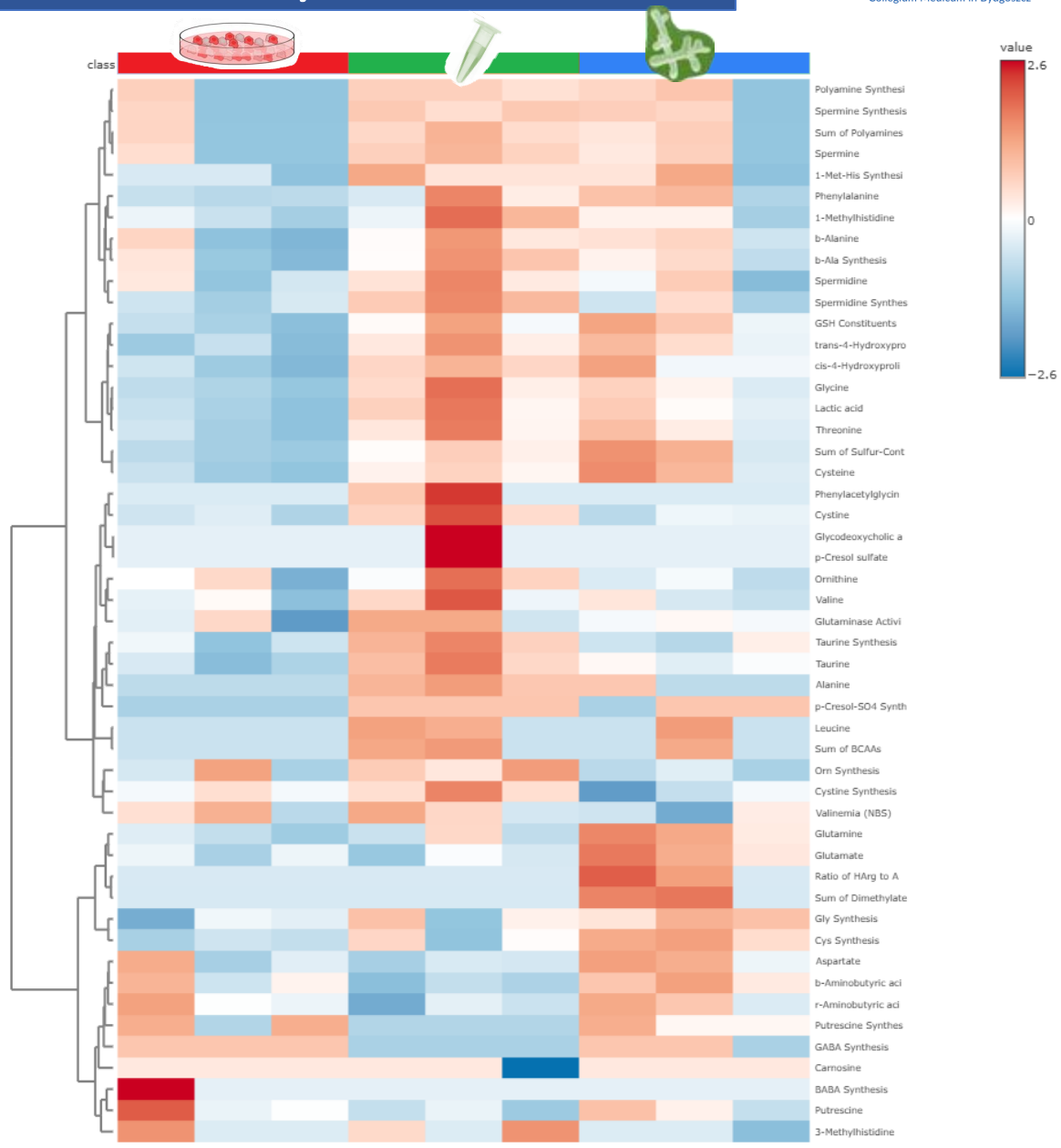
Chic-8E11 + Bifido CFS

Chic-8E11 + Bifido

Partial Least Squares Regression-DA plot

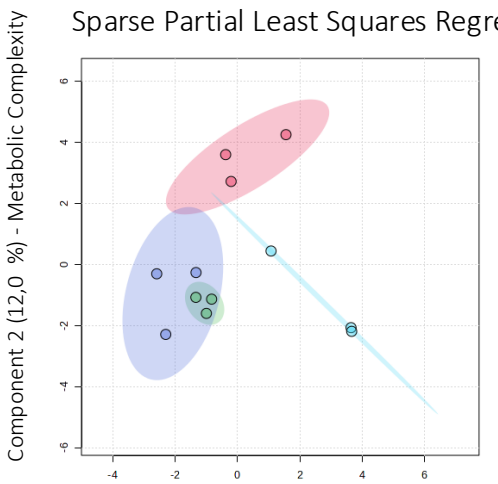






Individual samples are shown in columns and metabolites in rows

In ovo metabolomic profiles confirm treatment effects



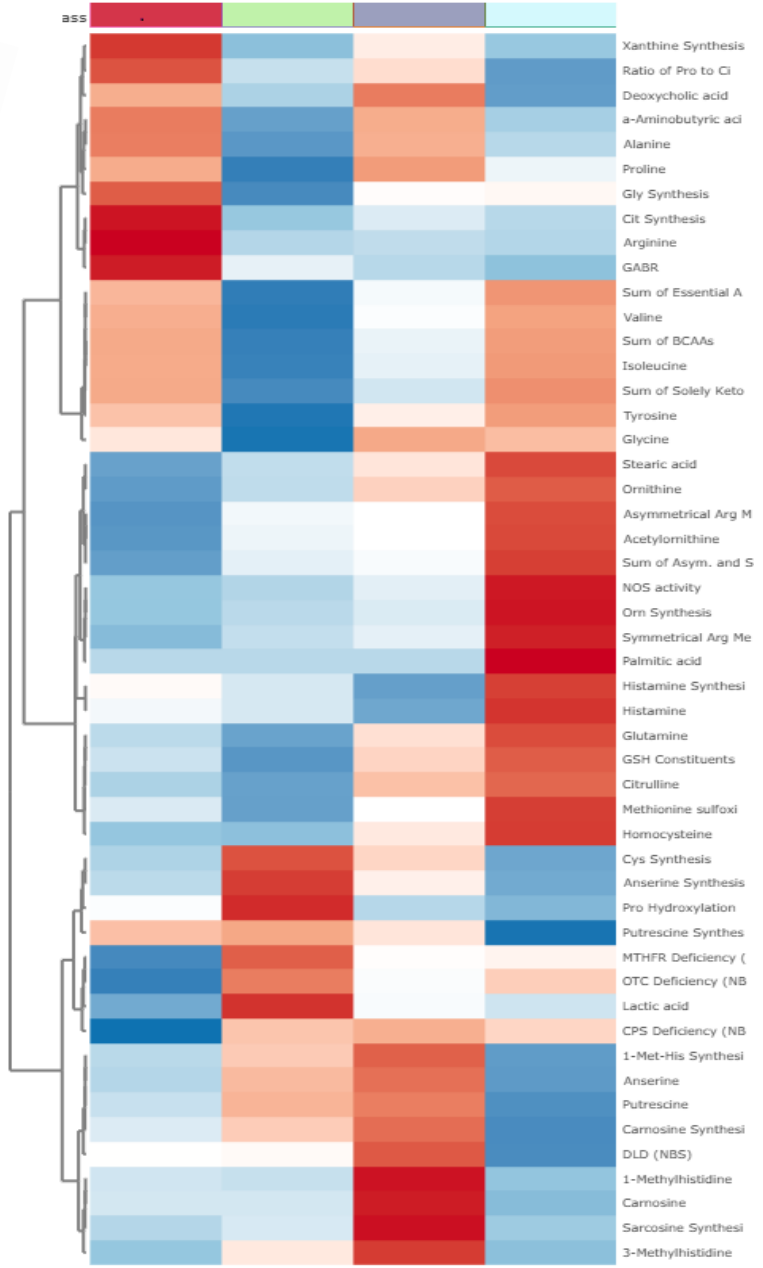
Control

Probiotic B. Lactis 10^6 CFU

Prebiotic 1mg AP

Synbiotic 10^3 CFU B. lactis + 1 mg AP

→distinct metabolic signatures for each treatment group
→ treatment-specific metabolite clustering patterns
→ synbiotic treatment provides the most balanced metabolic profile
→ changes represent metabolic adaptations rather than clear "beneficial" or "harmful" effects



Metabolite Class	Metabolite	Probiotic	Prebiotic	Synbiotic	Functional significance
Antioxidants	GSH constituents	++	++	+++	Cellular protection, detoxification
	Cysteine synthesis	+++	+	++	Supports antioxidant production
Amino Acids	Citrulline	+	++	+++	Nitric oxide (NO) cycle, mucosal barrier regulation
	Ornithine	+	++	+++	Ammonia detoxification
Energy metabolism	Lactic acid	+++	+	++	Antimicrobial, energy substrate
	Glutamine	+	++	+++	Primary enterocyte fuel
Barrier function	Anserine synthesis	+++	+++	+	Antioxidant, pH buffering
	Pro hydroxylation	+++	++	++	Collagen stability
Lipid metabolism	Fatty acids	+	++	+++	Membrane integrity, signaling
Bile Acids	Deoxyholic acid	+	++	+	Reduced inflammation

+Low levels ++ Moderate levels +++ High levels

Key metabolic pathways affected across models

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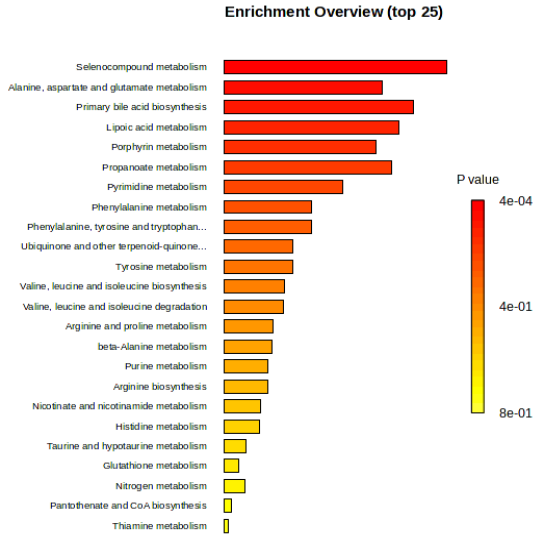
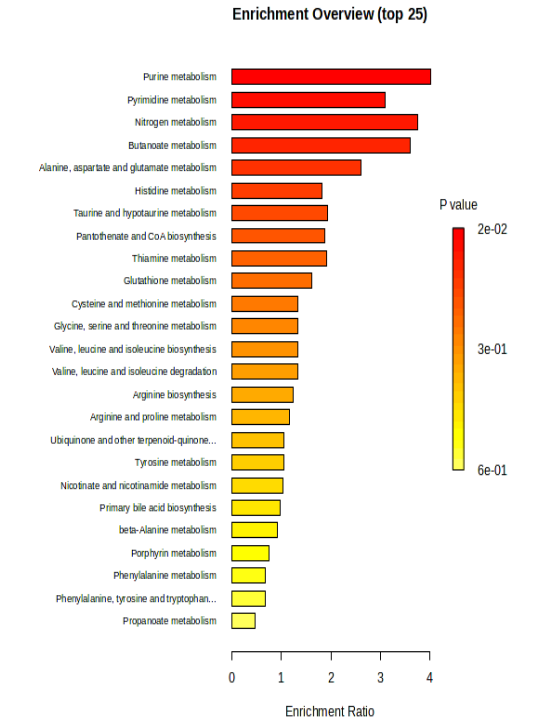
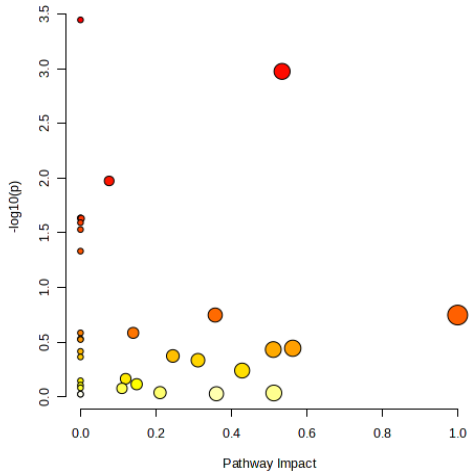


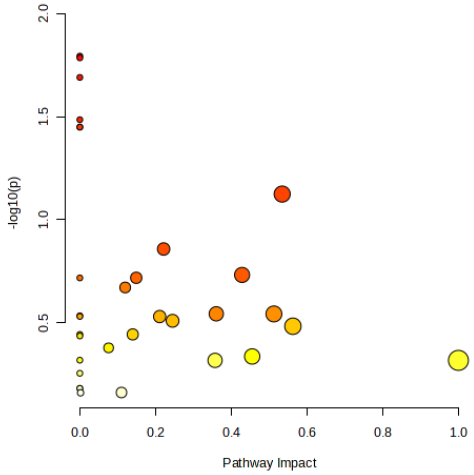


Pathways affected between control cells and cells treated with *Bifidobacterium lactis* CFS



→ most significant pathways: amino acid metabolism, TCA cycle

Pathways affected between control cells Chic-8E11 and cells co-cultured with *Bifidobacterium lactis*



→ differential activation patterns between direct contact vs. supernatant

Key metabolic pathways affected across models

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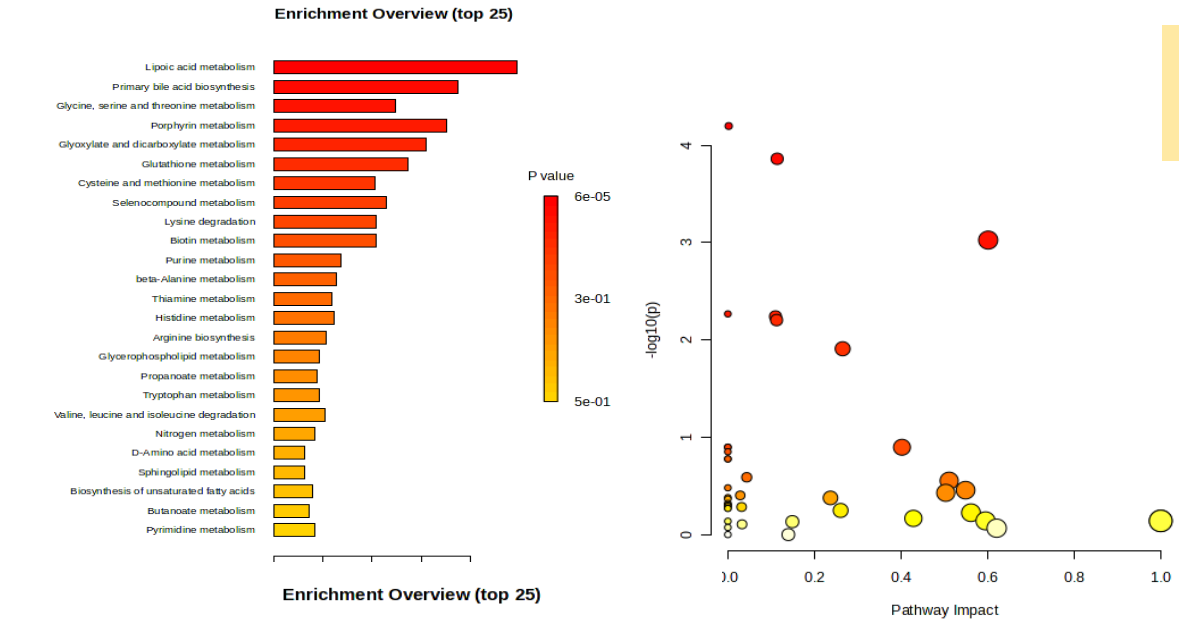
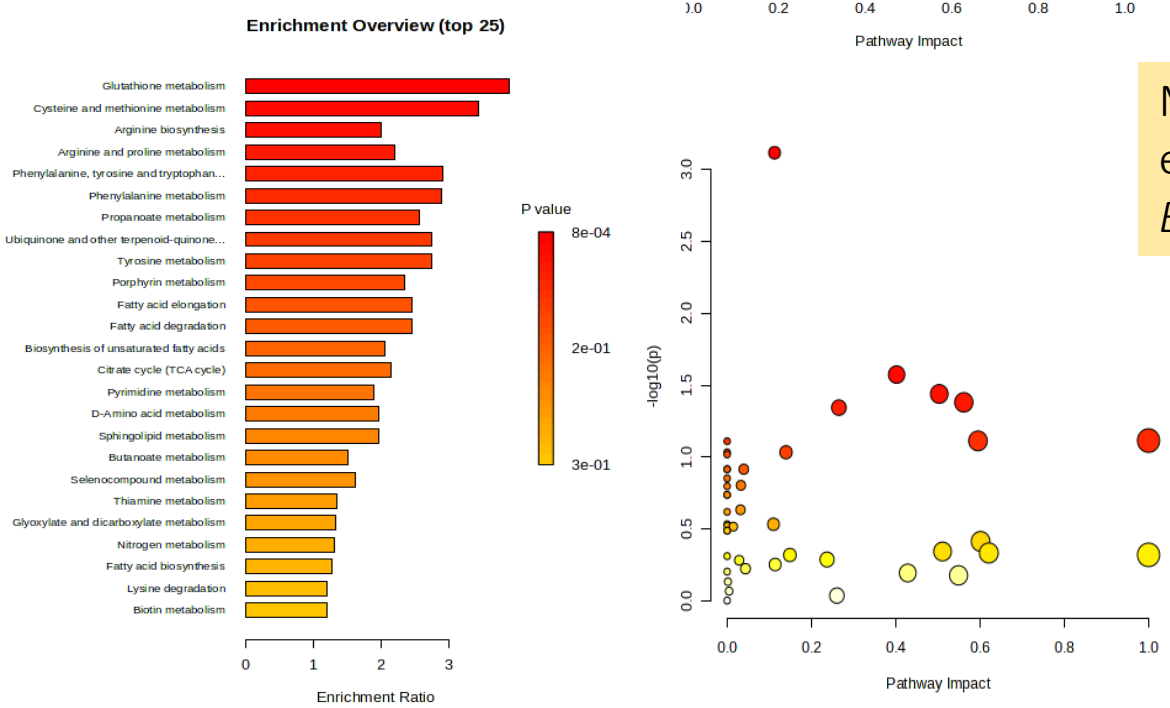


Metabolic pathways affected by differential metabolite enrichment between *in ovo* control and *Bifidobacterium* probiotic

← probiotic-specific pathway activation



Metabolic pathways affected by differential metabolite enrichment between control and synbiotic group (*10^3 CFU Bifidobacterium* + 1 mg Astragalus polysaccharides),

← synergistic effects not seen in single treatments
← consistent metabolic pathway activation patterns across experimental models with enhanced effects in synbiotic treatment

Translational Model Validation

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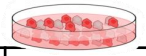
Validating the translational model: In Vitro to In Ovo

Key metabolite comparison table

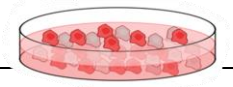


Metabolite Class	Key Metabolites	In Vitro Model	In Ovo Model	Functional Significance
Carboxylic Acids	Lactic acid	↑ in co-culture	↑ in probiotic group	Antimicrobial, energy source
	Succinate	↑ in co-culture	↑ in probiotic & synbiotic	Energy metabolism, homeostasis
Antioxidants	GSH constituents	↑ in both treatments	↑↑ in synbiotic group	Cellular protection, detoxification
	Cysteine	↑ in both treatments	↑ in probiotic group	Host's antioxidant defense capacity
Amino Acids	Glutamine/Glutamate	↑ in both treatments	↑↑ in synbiotic group	Enterocyte energy, barrier function
	Ornithine	↑ with supernatant	↑↑ in synbiotic group	Nitrogen metabolism
Growth Factors	Polyamines	↑ in both treatments	↑ in probiotic & synbiotic	Cell growth, differentiation
	β-Alanine	↑ in both treatments	↑ in probiotic group	Carnosine synthesis

→Metabolite consistency
→Pathway conservation
→Model complementarity

Translational model validation

Functional significance of metabolic alterations



In vitro specific

Direct cellular responses
Immediate metabolic changes
Tight junction regulation

Shared metabolic pathways

AA metabolism
TCA cycle
Antioxidant pathways
Polyamine synthesis
Lactic acid production
Barrier function
Metabolites

In ovo specific

Bile acid metabolism
Microbial community interactions
Host immune modulation

Functional significance

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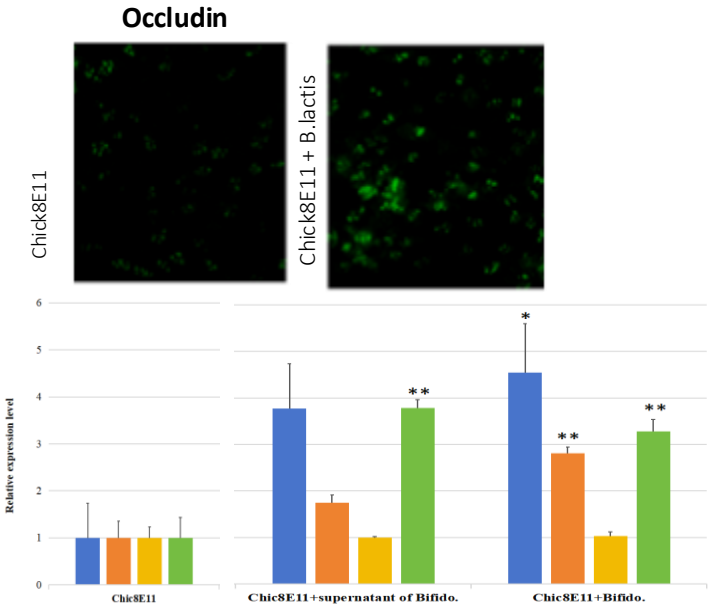
Integrating Metabolomics & Metagenomics

Enhanced intestinal barier integrity

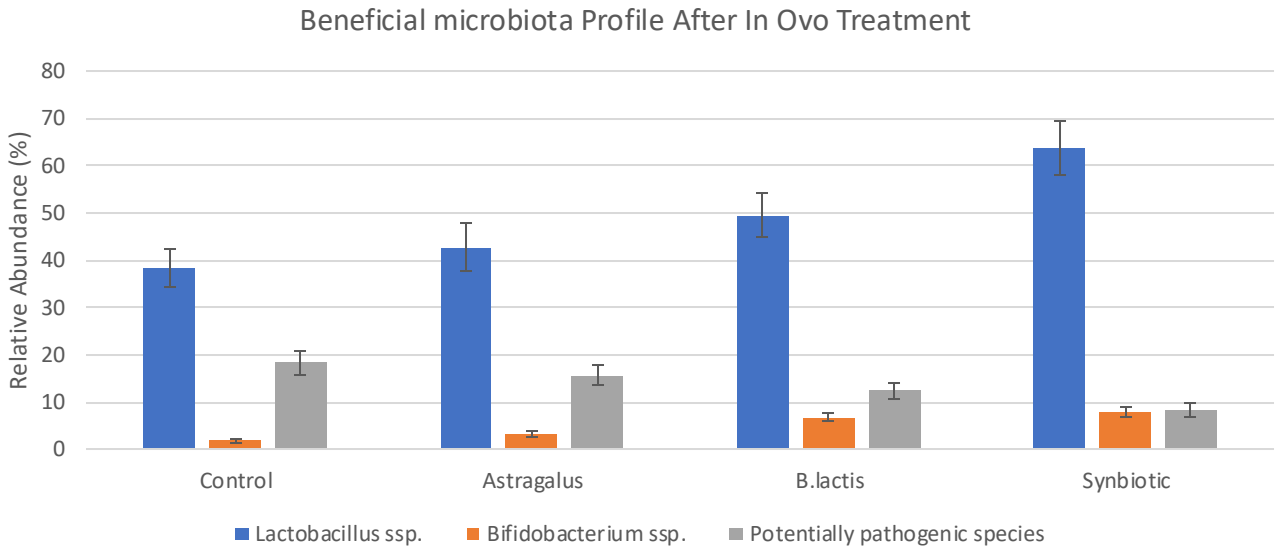
In vitro study:



Barrier Function Enhancement

Metagenomic data, in ovo:

Lactobacillus → GABA



- *Villin*
- *CK18*
- *Zona occludens*
- *Occludin*

MECHANISM:

→ Occludin gene significantly up-regulated ($p<0.01$) in Chick-8E11 cells with *B. lactis* treatment
→ GABA production (↑ 5.2-fold, $p<0.05$)
→ Lactobacillus strengthens tight junctions through occludin expression
→ Synbiotic treatment increases beneficial Lactobacillus abundance that directly supports barrier function

Functional significance

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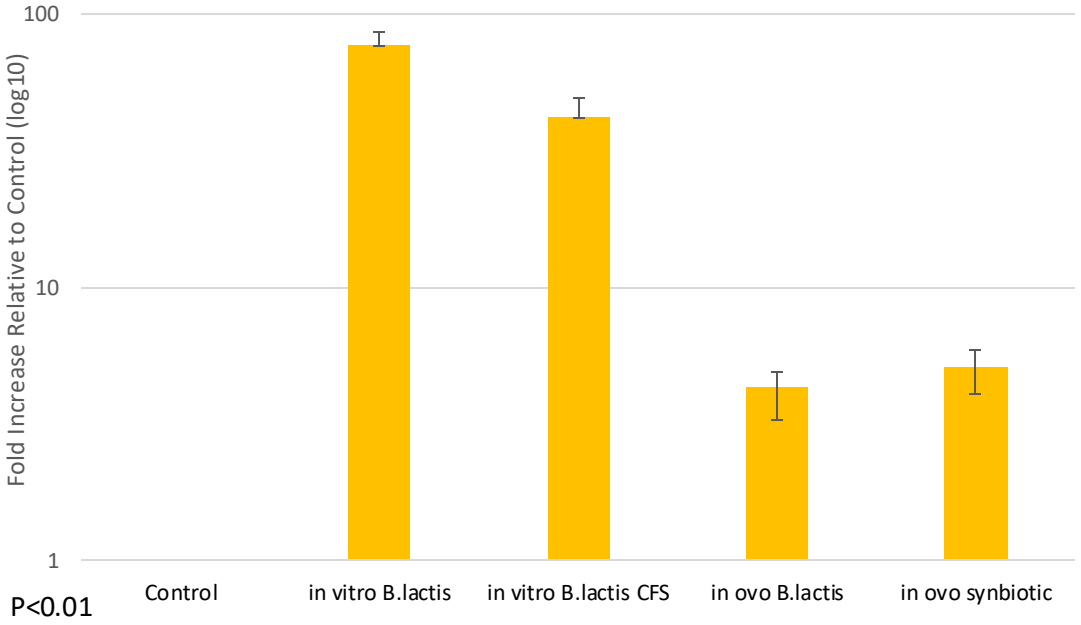
Anti-inflammatory effects

Metabolomic evidence:

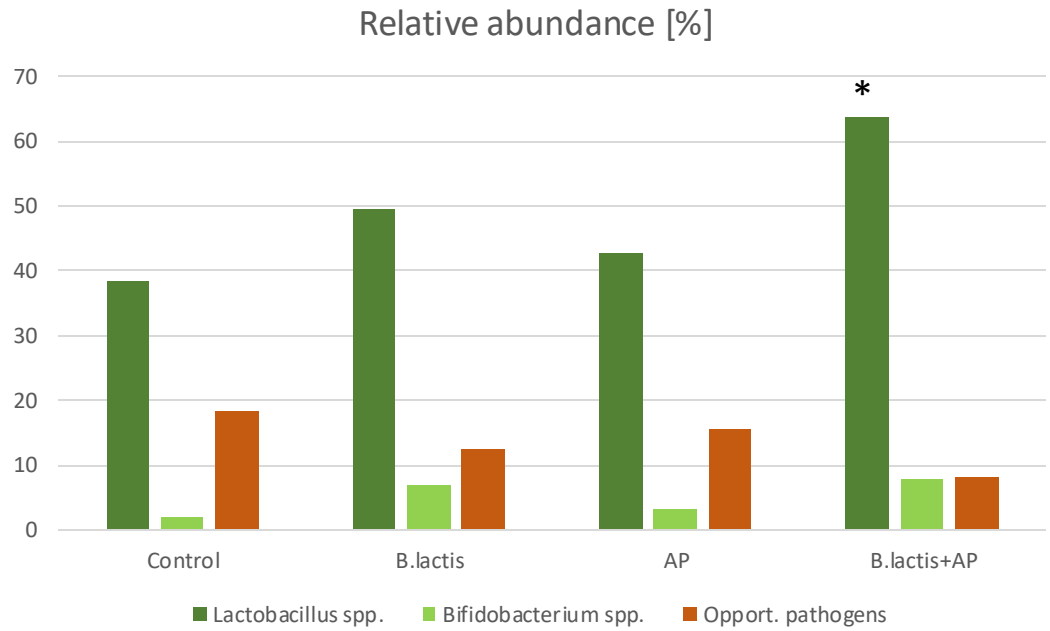
Significant lactate elevation with *B. lactis* treatment



P<0.01

Metagenomic suport:

Reduced abundance of inflammatory-associated bacteria (*Staphylococci, Streptococci, Corynebacteria*)



Mechanism: Lactate production by beneficial bacteria inhibits pathogens and modulates immune response

Anti-inflammatory effects are achieved through both direct metabolite action and suppression of pathogenic bacteria

Functional significance

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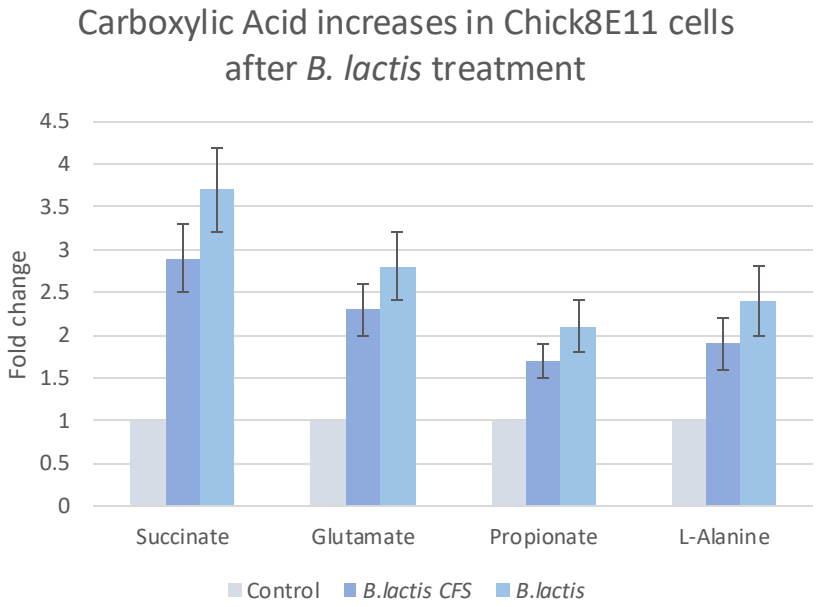
Host- mediated effect



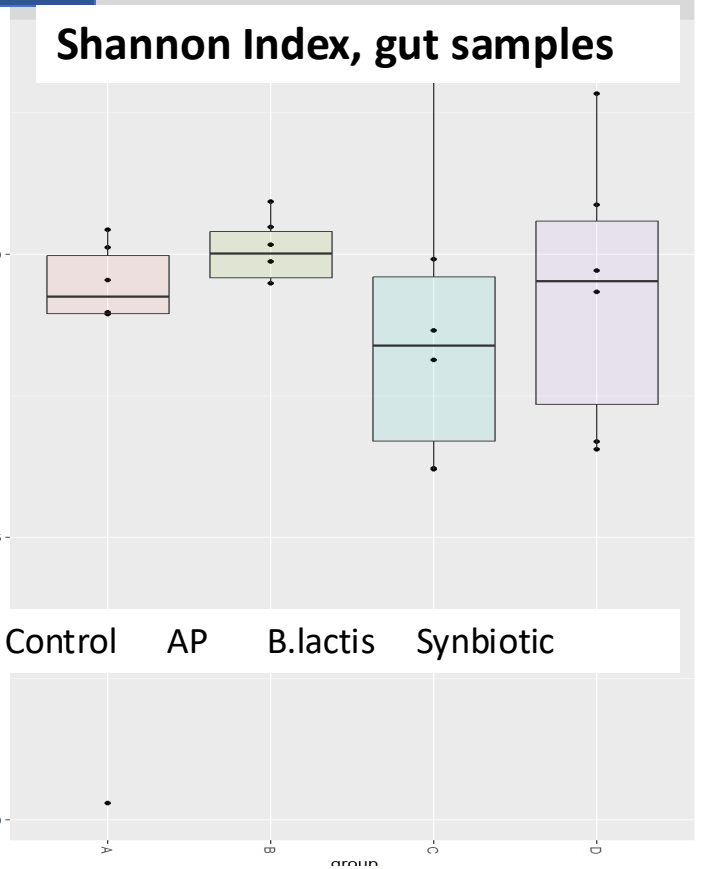
All treatment conditions show statistically significant increases ($p < 0.01$) compared to control

←Carboxylic acids, important for cellular energy metabolism show increases after *B. lactis* treatment.

← Direct co-culture shows stronger effects

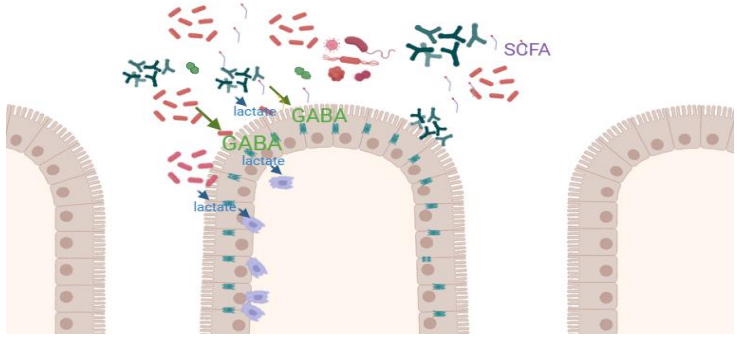
B.Lactis showed lower diversity of microbial community→

Synbiotic group showed a more balanced microbial interaction→



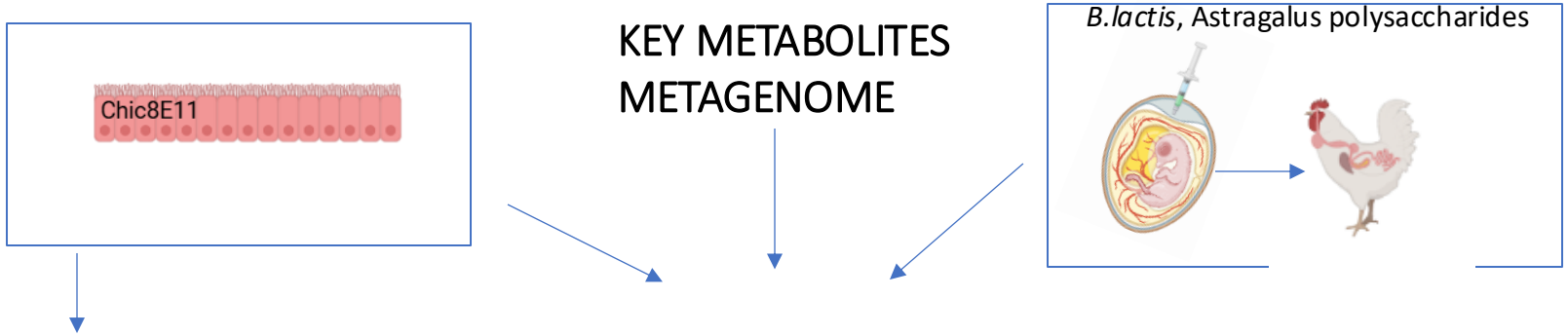
Integrative Model

Combining metabolomics and metagenomics explained the mechanisms of probiotic action



Conclusions

Translational, predictive model linking in vitro and in ovo effects



PROBIOTIC METABOLIC SIGNATURES
Carboxylic acids ↑
GSH constituents ↑
polyamines ↑

Mapped key affected pathways:
Amino acid metabolism,
Energy production
Antioxidant defense

FUNCTIONAL INTERPRETATION
Barrier function↑
Energy metabolism↑ and redox balance
Microbiota balance↑
Demonstrated synergistic effects of prebiotic-probiotic combinations

- Applications for probiotic screening
- Further validation in post-hatch chickens
- Potential for personalized probiotic formulations

Acknowledgements

Krzysztof Skowron

Katarzyna Hrynkiewicz

Waldemar Studziński

Cinzia Randazzo

Giuseppe Maiorano

Niloofar Akhavan

Sanling Zuo

Mengjun Wu

Mariam Ibrahim

Anna Bajek

Research is funded by the Polish National Science Centre 2019/35/B/NZ9/03186-Ovobiom.