Synbiotics for in ovo application - in vitro design

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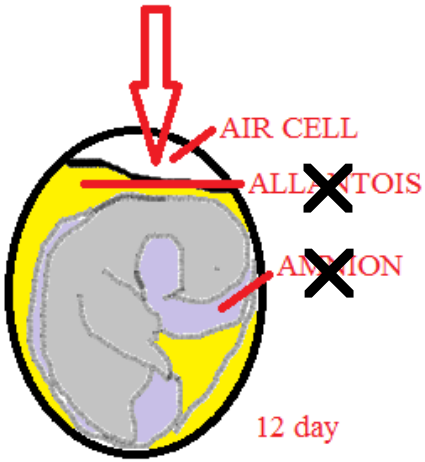
How may *in ovo* technologies work in industry

3 modes of *in ovo* treatments:

- *In ovo* vaccination (19-19.5 day of egg incubation)- amnion/muscle (no egg chamber)
- *In ovo* feeding (17-19.5 of egg incubation)- amnion
- *In ovo* modulation of a beneficial microbiome: using **prebiotic or a synbiotic (12. day** of egg incubation**)** or probiotic (17-19.5 of egg incubation)- air chamber/ amnion, yolk sac



Synbiotics are delivered early to improve life-long performance

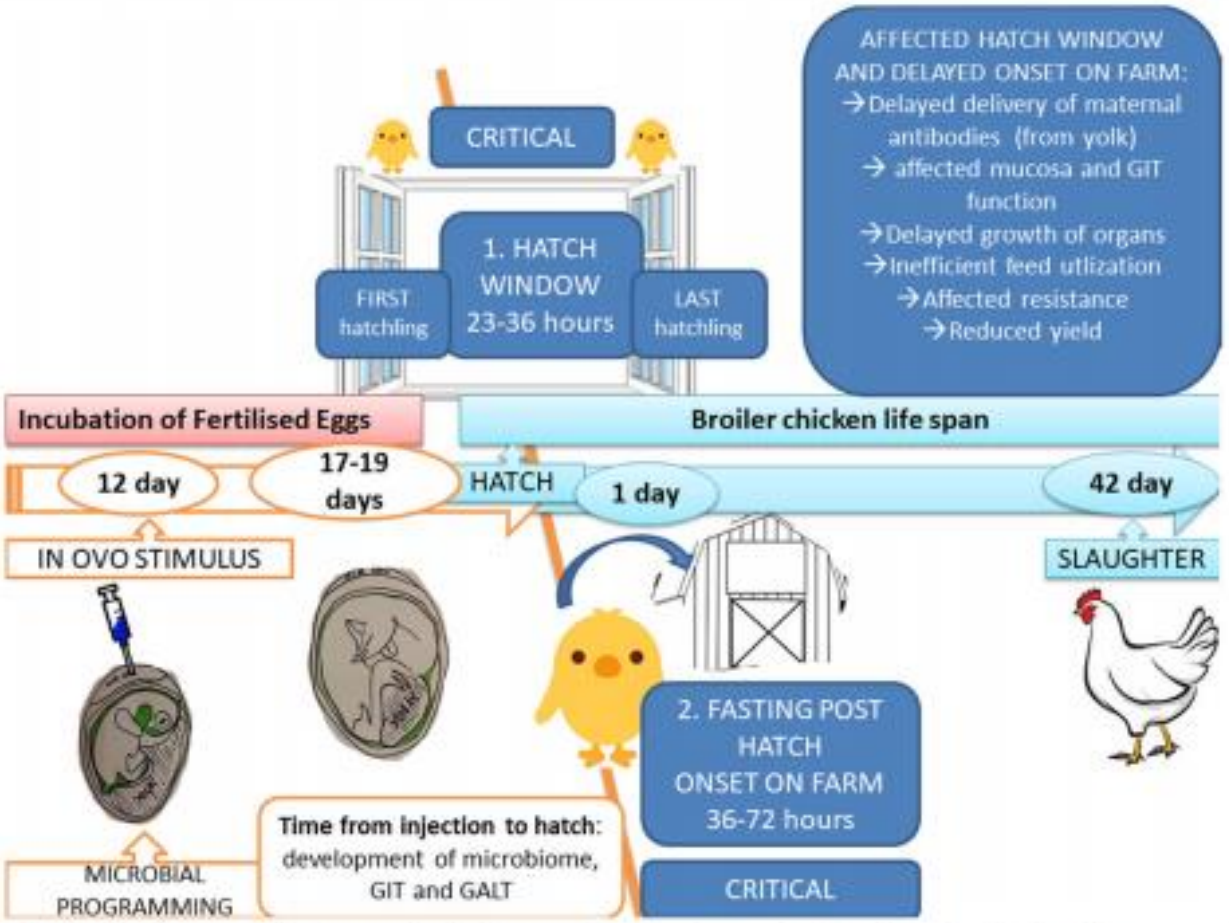
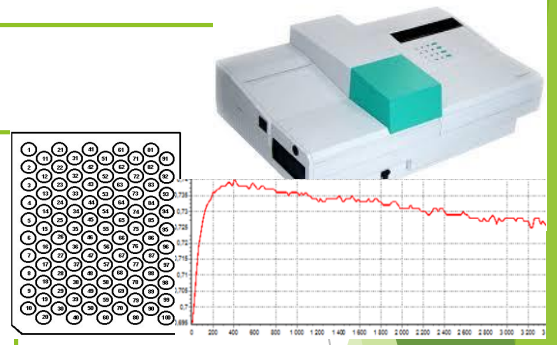


Fig. 2 Concept of early microbial programming *in ovo*. Prebiotic or probiotic given on day 12 of egg incubation influences embryonic factors (microbiome, GALT development and function, gene expression, nutrient absorption) which are critical for future phenotype of the broiler chicken. Two critical perinatal moments are shown (hatch window and fasting post-hatching), when the newly hatched chicken is the most receptive to environmental stressors

(BMC Vet Res., Siwek et al. 2018)

How the „Bioscreen C" works? Workflow for synbiotic optimization

Bioscreen C- system operation scale is economical, evidential and used for industry, eg. antibiotic testing



The proposed work flow of a synbiotic optimization protocol for poultry:

1. Bioscreen C microbiological growth test for *in vitro* pre-selection of bioactive components

2. *in ovo* PRE-SELECTION and dose optimization of synbiotic formulations; checking probiotic viability after hatch and hatch performance

3. *In vivo* selection of bioactives based on performance including gut health parameters and gene expression modulation. Validation.

Aim and scope

The interactions between bioactives were tested:

5 selected natural prebiotic carbohydrate substrates

- ALGAE 1
- ALGAE 2
- ALGAE 3

natural extracts containing carbohydrates

- Galactooligosaccharides (GOS)

from enzymatic transgalactosylation of the milk lactose by *Bifidobacterium*

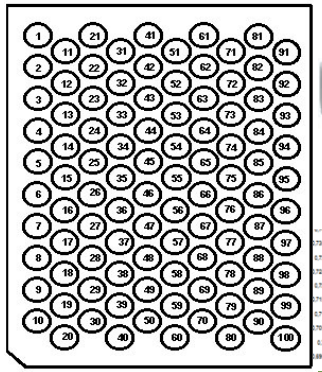
- Inulin (INU)

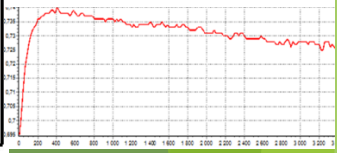
natural extract from chicory

10 probiotic bacteria:

- *Lactobacillus rhamnosus* GG (LGG)
- *Lactobacillus rhamnosus*FL2 (L FL2)
- *Lactobacillus rhamnosus* FL3 (L FL3)
- *Lactobacillus rhamnosus* FL4 (L FL4)
- *Lactobacillus rhamnosus* FLC5 (L FL5)
- *Lactobacillus rhamnosus* AT194 (L AT194)
- *Lactobacillus rhamnosus* AT195 (L AT195)
- *Lactobacillus rhamnosus* 39 (L 39)
- *Lactobacillus rhamnosus* H25 (L J25)
- and *Bifidobacterium* ATCC (Bifido)

Methods





- The probiotics were grown on:

1/ a standard De Man, Rogosa and Sharpe agar (M-MRS) with no saccharides (**negative control**)

2/ on modified MRS media with glucose (**positive control**)

3/ on modified MRS media where glucose was replaced with selected prebiotics (2% w/v) as a carbohydrate source (**experimental groups**)

(360 µl medium per well + prebiotic or glucose*
+40 µl probiotic inoculate onto honeycomb well plates)

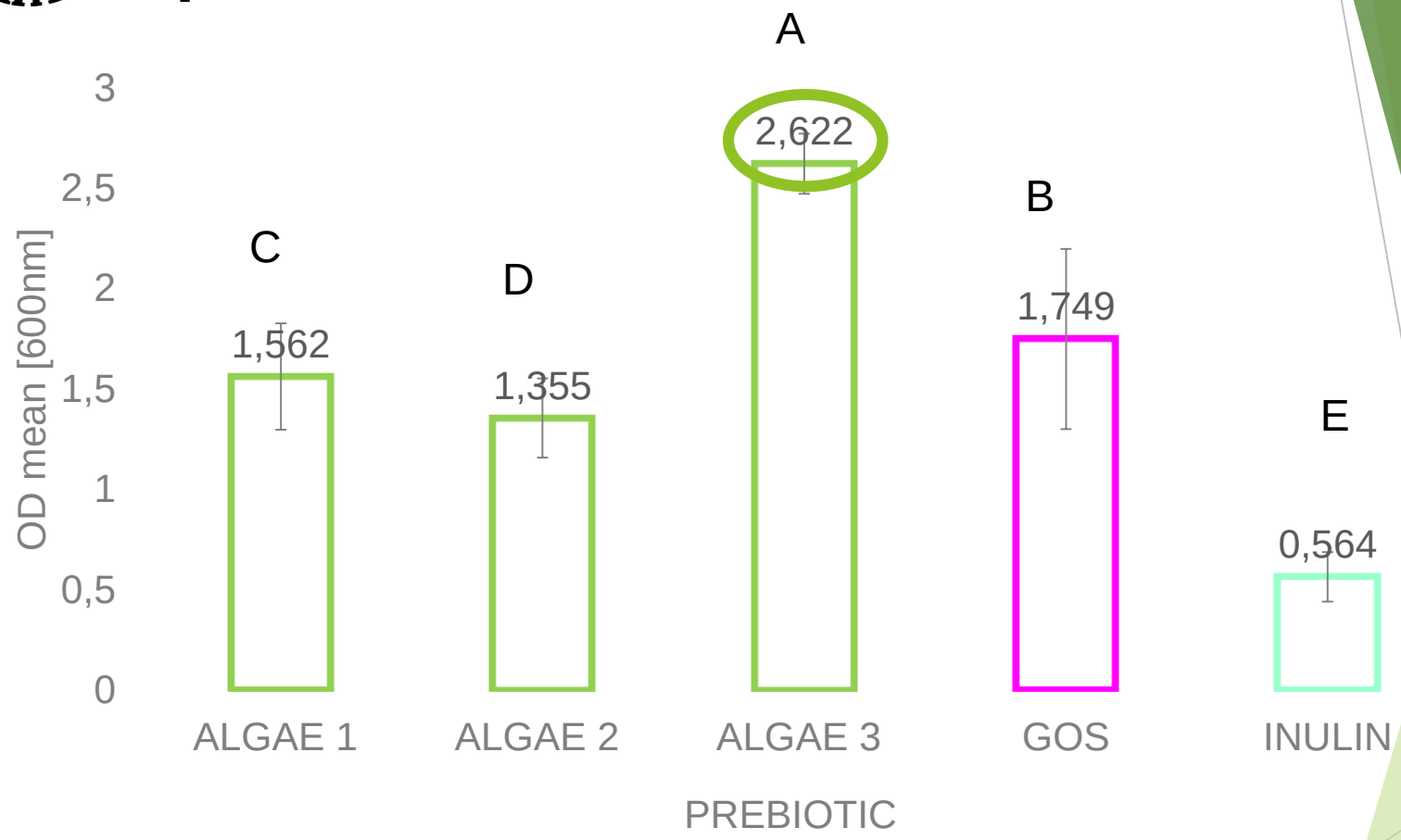
- The changes in optical density (OD) were plotted in real time (hourly) using Automated Microbiology Growth Curve Analysis System, Bioscreen C (Oy Growth Curves Ab Ltd)- in triplicates
- The growth kinetic parameters were estimated according to Baranyi and Roberts, 1994 method
- To verify significant differences in relation to the treatments, the data were evaluated by using 1-way ANOVA and means were separated by Scheffe's battery of pairwise tests

Results- growth kinetics parameters

Bacteria	Prebiotic/ saccharide	Growth rate [1/h]	Lag [h]	se(lag)	y_end [OD] (=Load increase Δ_y)
					the upper asymptote of the sigmoid curve (final load value)
Lb.rhamnosus FLC5	glucose	0,24	3,39	0,14	1,85
Lb.rhamnosus GG	glucose	0,2	2,88	0,21	1,82
Lb.rhamnosus FL4	GOS	0,2	3,58	0,27	1,48
Lb. rhamnosus H25	GOS	0,16	4,65	0,24	1,48
Lb.rhamnosus 39	GOS	0,16	3,48	0,21	1,44
Lb.rhamnosus GG	ALGAE 1	0,11	1,04	0,21	1,18
Lb.rhamnosus FL4	ALGAE 1	0,1	2,5	0,23	1,16
Lb.rhamnosus FL3	ALGAE 1	0,1	1,99	0,12	1,12
Lb.rhamnosus GG	ALGAE2	0,12	2,74	0,16	0,83
Lb.rhamnosus 39	ALGAE2	0,09	1,97	0,2	0,75
Lb.rhamnosus FL2	ALGAE 2	0,09	1,99	0,22	0,56
Lb.rhamnosus 39	no saccharides	0,05	3,91	0,39	0,52
Lb. rhamnosus H25	no saccharides	0,05	4,02	0,3	0,51
Lb.rhamnosus FL2	no saccharides	0,07	3,5	0,46	0,5
Lb.rhamnosus FL2	inulin	0,04	1,5	0,56	0,45
Lb.rhamnosus FL3	inulin	0,04	0,76	0,5	0,45
Lb.rhamnosus GG	no saccharides	0,03	1,04	0,63	0,41
Bifidobacteria ATCC	ALGAE 3	0,01	3,88	0,35	0,25
Lb.rhamnosus FLC5	ALGAE 3	0,02	3,39	0,39	0,18
Lb.rhamnosus GG	ALGAE 3	0,01	3,36	6,7	0,11

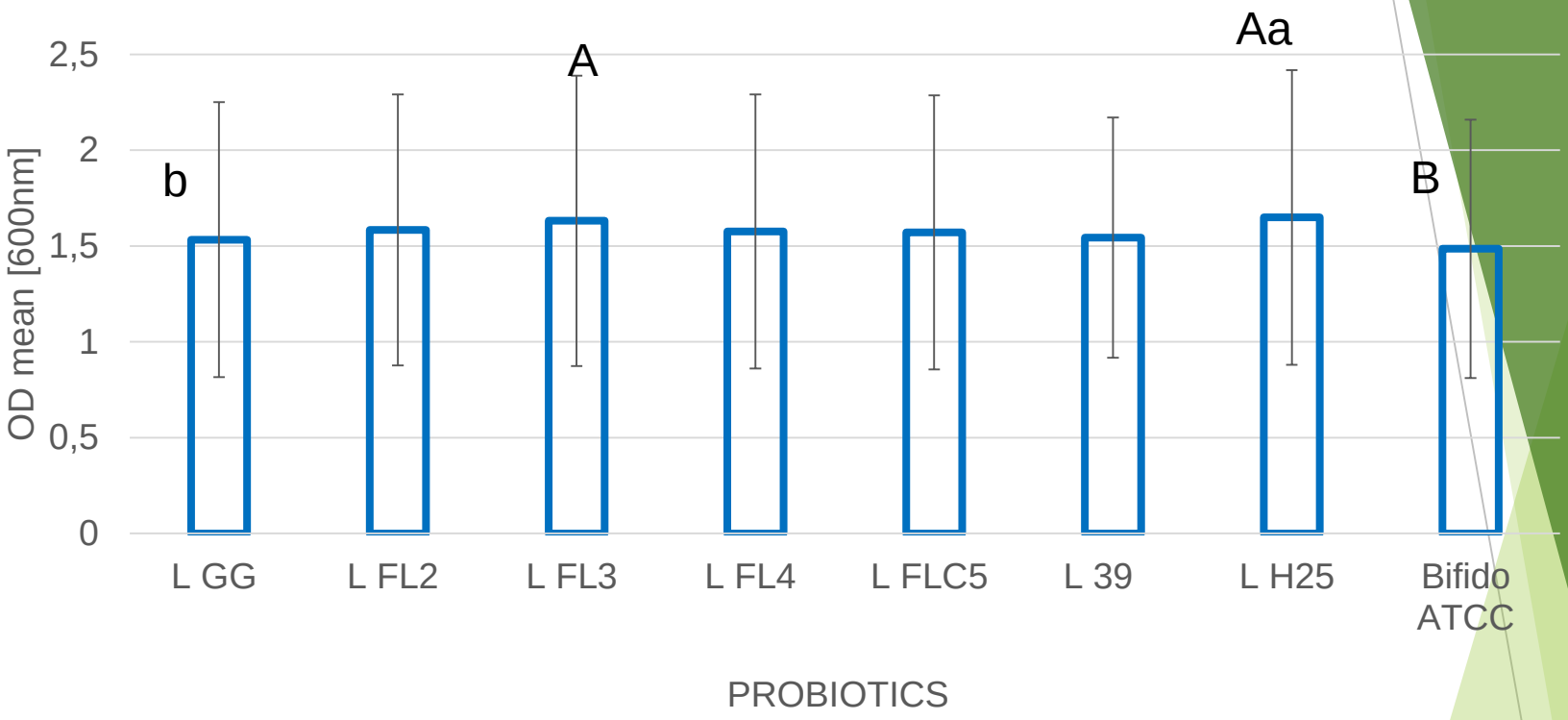
The growth kinetic parameters were estimated according to Baranyi and Roberts, 1994 method. Maximum specific growth rate (μmax), lag phase, initial load values (y_0= 0), final load values (y_end= Δ_y) and load increase (Δ_y).

Differences in relations to treatments- prebiotic effect



ALGAE 3 was the most universal substrate for probiotics tested (P≤ 00.1); SEM= 0.008

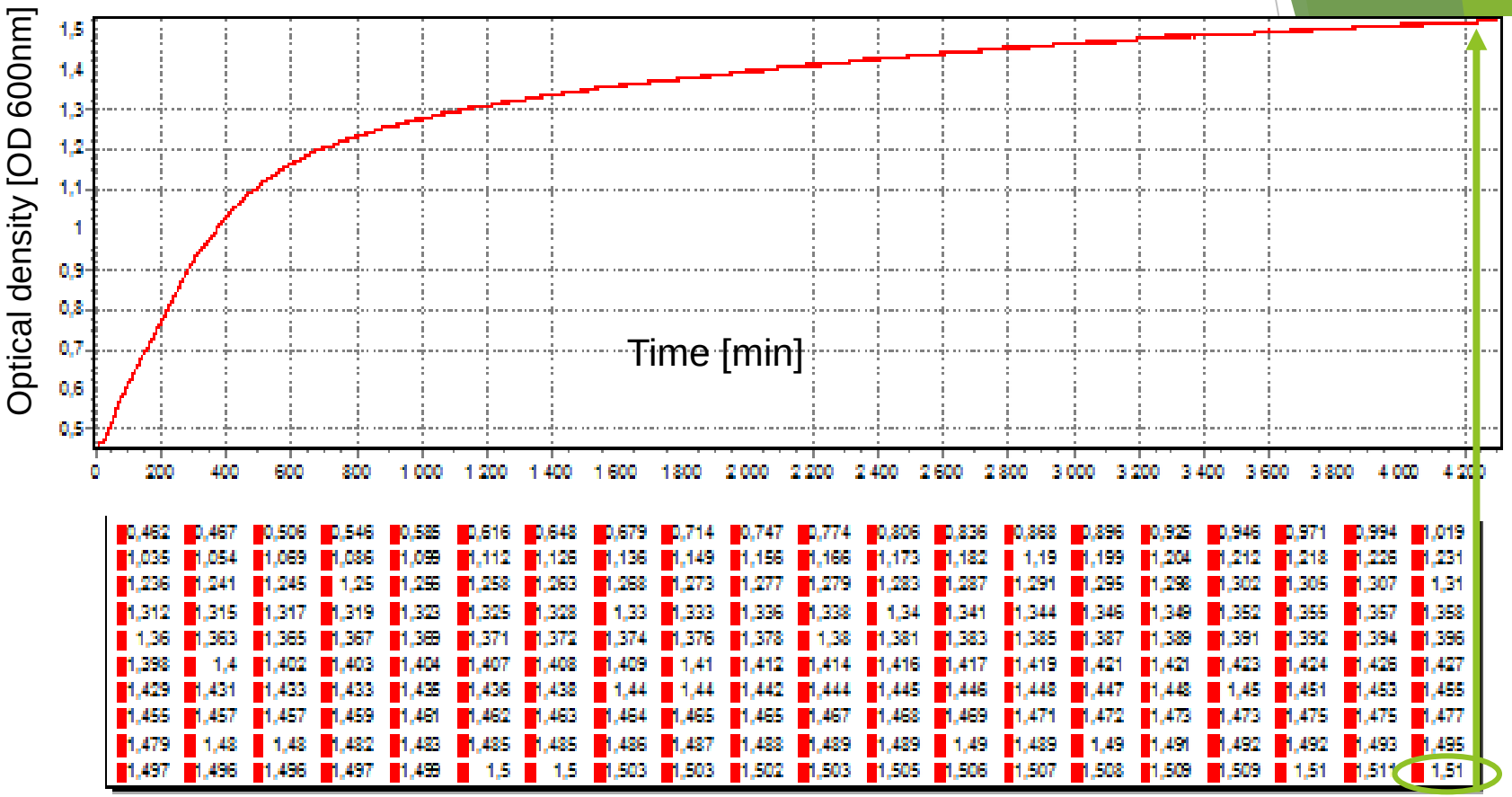
Differences in relations to treatments- efficiency of probiotic to metabolize prebiotics



The LFL3 and LH25 probiotics (mean OD = **1.63** ± 0.76; and **1.65** ± 0.77, respectively), used the prebiotics as an energy source significantly more effectively, compared to Bifido and LGG (mean OD = 1.49 ± 0.67; and 1.53 ± 0.72)

Results- growth curves

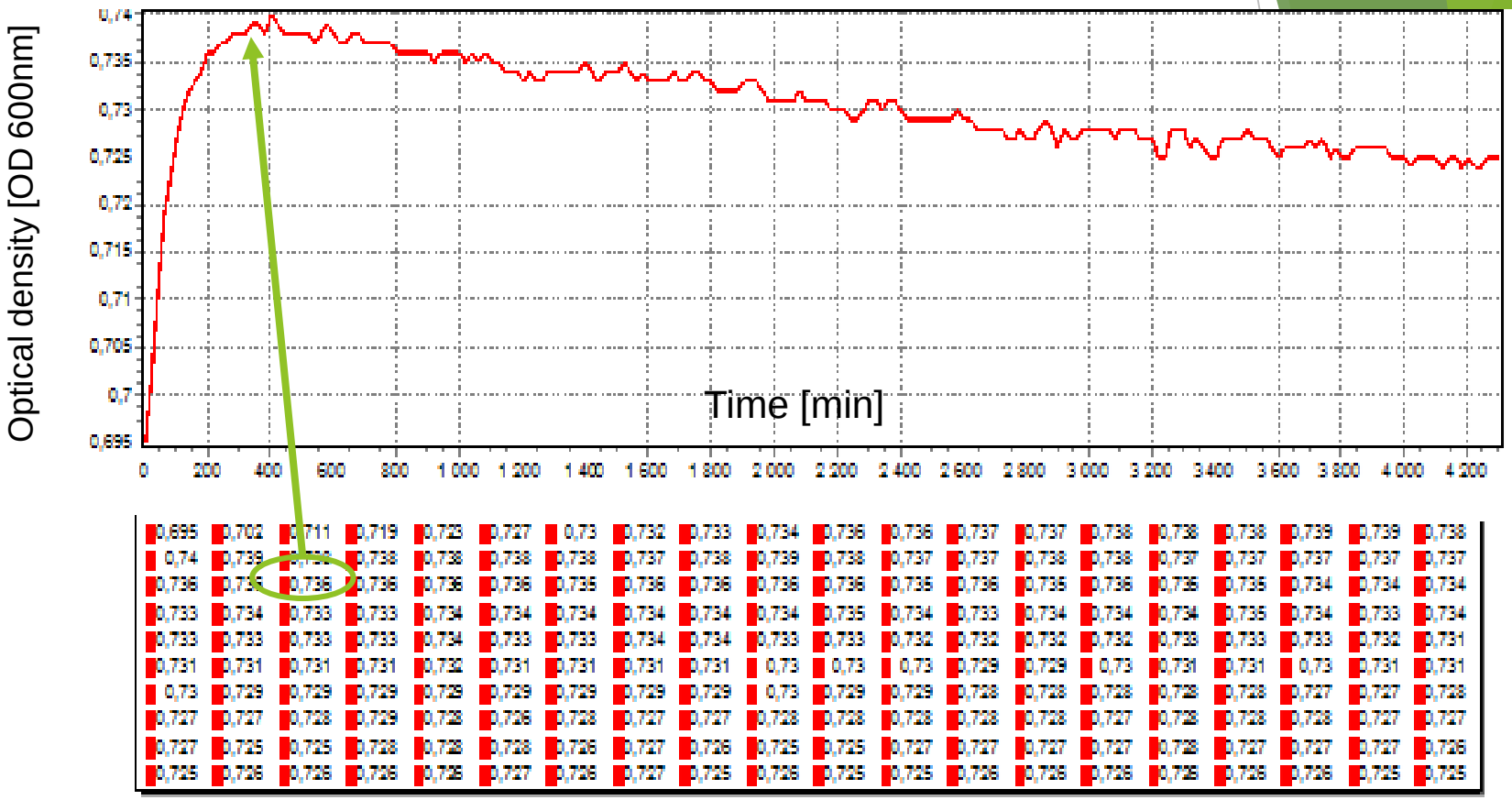
Mean for ALGAE 1 prebiotic with all probiotics



ALGAE 1 would guarantee stable performance as a substrate

Results- growth curves

Mean for ALGAE 2 prebiotic with all probiotics

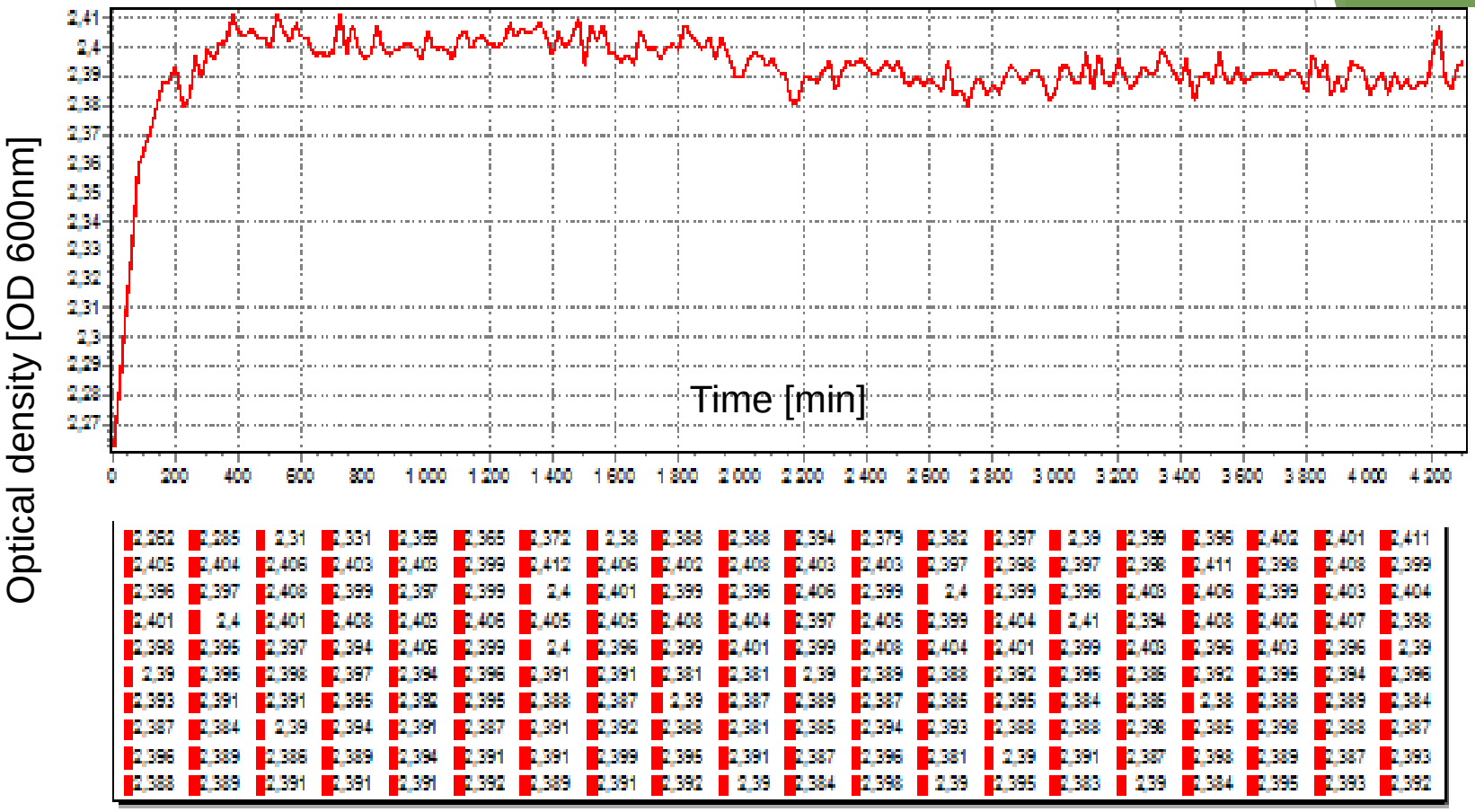


0,695	0,702	0,711	0,719	0,723	0,727	0,73	0,732	0,733	0,734	0,736	0,736	0,737	0,737	0,738	0,738	0,738	0,739	0,739	0,738
0,74	0,739	0,738	0,738	0,738	0,738	0,738	0,737	0,738	0,739	0,738	0,737	0,737	0,738	0,738	0,737	0,737	0,737	0,737	0,737
0,736	0,735	0,736	0,736	0,736	0,736	0,735	0,736	0,736	0,736	0,736	0,735	0,736	0,735	0,736	0,736	0,735	0,734	0,734	0,734
0,733	0,734	0,733	0,733	0,734	0,734	0,734	0,734	0,734	0,734	0,735	0,734	0,733	0,734	0,734	0,734	0,735	0,734	0,733	0,734
0,733	0,733	0,733	0,733	0,734	0,733	0,733	0,734	0,734	0,733	0,733	0,732	0,732	0,732	0,732	0,733	0,733	0,733	0,732	0,731
0,731	0,731	0,731	0,731	0,732	0,731	0,731	0,731	0,731	0,73	0,73	0,73	0,729	0,729	0,73	0,731	0,731	0,73	0,731	0,731
0,73	0,729	0,729	0,729	0,729	0,729	0,729	0,729	0,729	0,73	0,729	0,729	0,728	0,728	0,728	0,728	0,728	0,728	0,727	0,728
0,727	0,727	0,728	0,729	0,728	0,726	0,728	0,727	0,727	0,728	0,728	0,728	0,728	0,728	0,727	0,728	0,728	0,728	0,727	0,727
0,727	0,725	0,725	0,728	0,728	0,728	0,726	0,727	0,726	0,725	0,725	0,727	0,727	0,727	0,727	0,728	0,727	0,727	0,727	0,726
0,725	0,726	0,728	0,728	0,728	0,727	0,726	0,727	0,725	0,728	0,725	0,725	0,728	0,728	0,728	0,728	0,728	0,728	0,725	0,725

ALGAE 2 would not be a preferred substrate (low OD after lag with sharp exponential phase and fast entry into death phase)

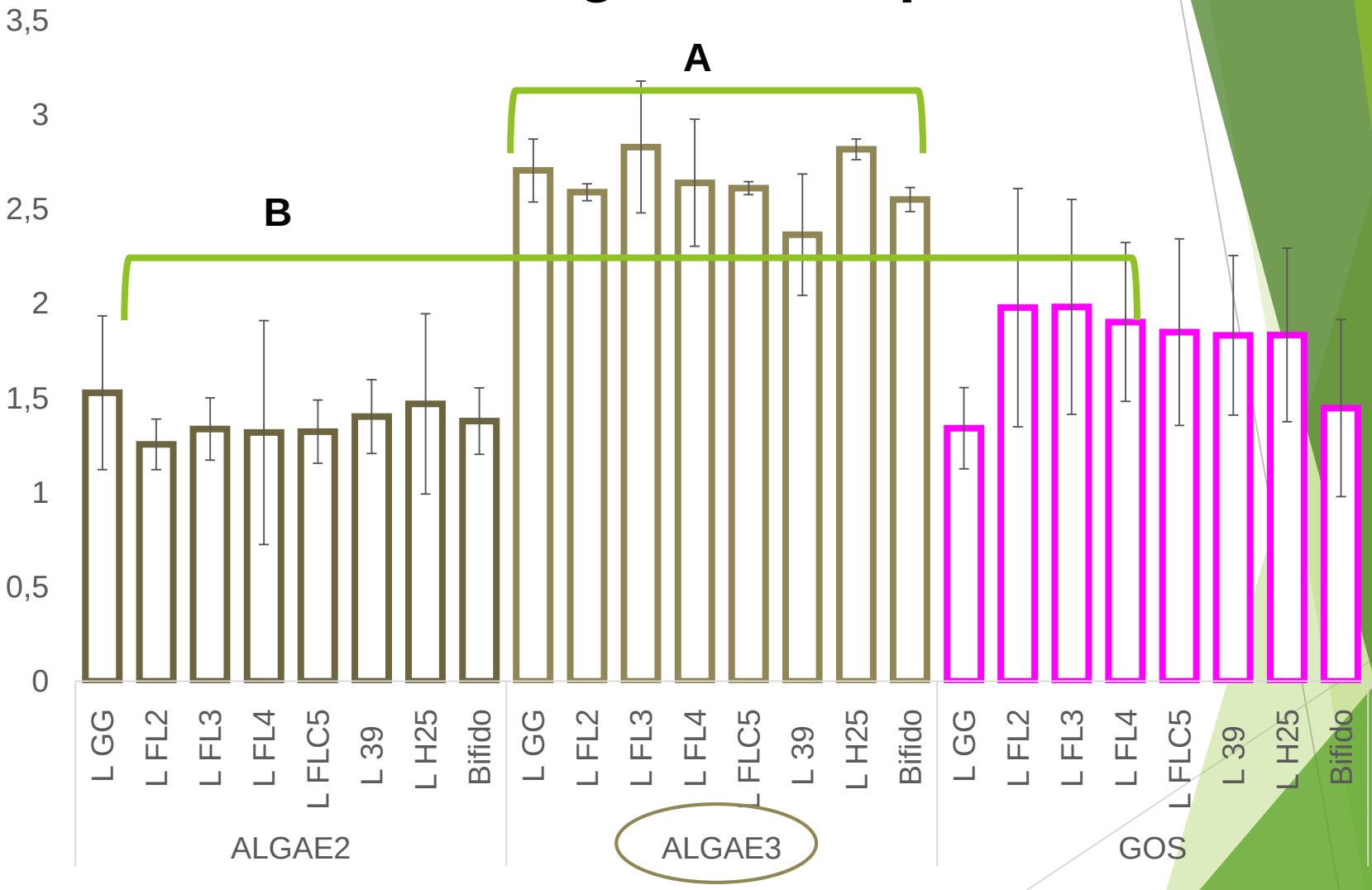
Results- growth curves

Mean for ALGAE 3 prebiotic with all probiotics



ALGAE 3 might represent a „universal substrate”

Results- OD means of probiotics metabolizing different prebiotics

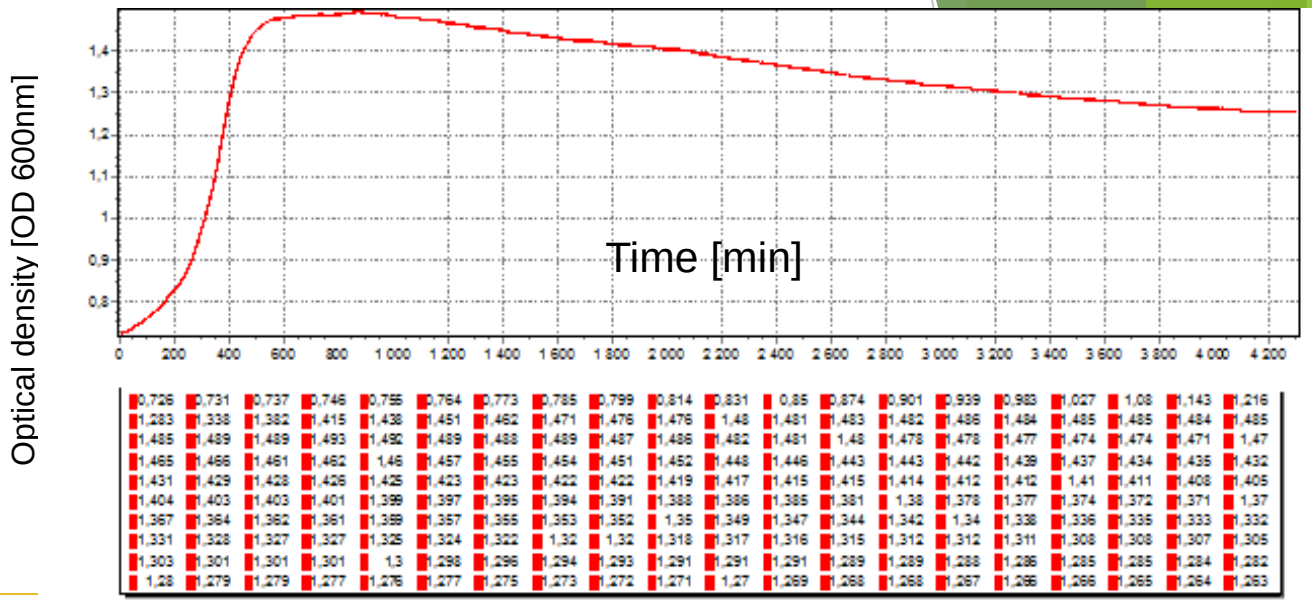


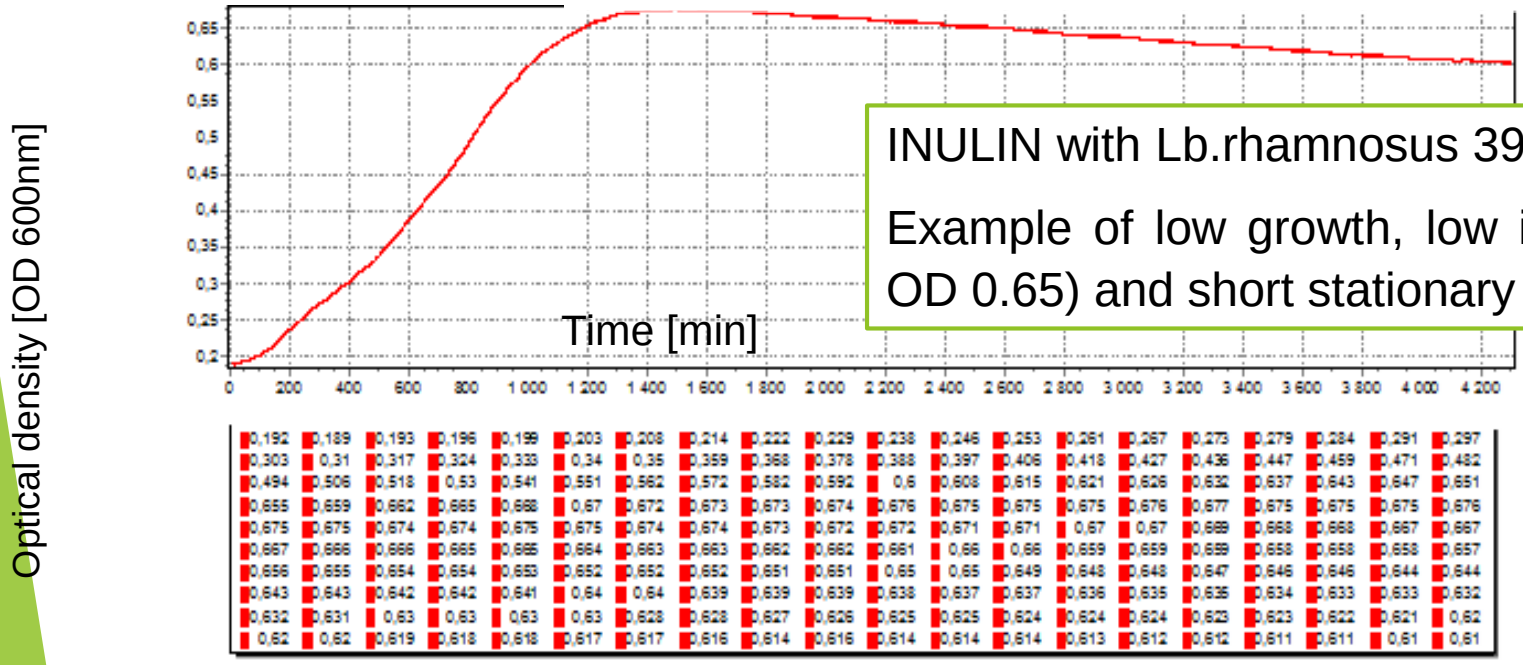
A,B; P<0.001

Results- growth curves

ALGAE 2 with Lb.rhamnosus FLC5

Example of low OD and short stationary phase (3.3h)





INULIN with Lb.rhamnosus 39

Example of low growth, low increase (max OD 0.65) and short stationary phase (3.3 h)

Conclusions

- The microbiological tests were only partially elucidative to select for optimal bioactive substances
- We cannot definitiely conclude whether the lag phase/adaptation [h] or change in probiotic load over culture should be the determinant for candidate choice in vitro
- Nevertheles:

1/ the BioscreenC protocol is cost effective (test of 100 prebiotic-probiotic combinations in vitro costs ab. 5 500 EUR (the major expense is for monitoring and sampling) and time effective (22500 curves in 2 months)

2/ allows for preselection of potentially unlimited number of pro/prebiotic combinations **prior** to using them in the *in vivo* poultry trials

3/ we assume the *in vivo* results will give conclusions as to **which growth kinetics parameters of a probiotic** should be considered **in vitro selection criteria**

What are the next steps?

- Validate the candidate probiotics and combinations *in vivo* using *in ovo* technique
- Understand the optimal growth curves based on *in vivo* results and metabolome (probiotic footprint)
- Once understood, to use the BioscreenC routinely as *in vitro* pre-selection method
- In parallel- testing protective role of prebiotic on probiotic to survive gut stress
- Is fecal microbial transplant (*in ovo*) an option to isolate species- specific probiotic candidates and set new synbiotic formulations?

Thank You

The Collaborators:

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Poznań Science and Technology Park

BioAtlantis

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