Simulation of chicken probiotic biology: in vitro versus in vivo

The gastrointestinal tract of poultry can be compartmentalised into three distinct microbiome based on oxygen and carbon-dioxide tension.

by Dr Susim Mukul Ray, Group Product Manager, Zydus AH, Cadila Healthcare Ltd, India. susim.ray@zydusahl.com

It is leveraged by several different factors including:

- Differential anatomy and physiology of the chicken gut leading to spatial and temporal differences in digestion and absorption of various nutrients.

- Fast growing aerobes and facultative anaerobes in the early part of the chick's life (day 1-12) consuming oxygen and ending up in higher carbon dioxide tension in the hind gut.

- Overgrowing facultative anaerobes in the mid and hind gut replacing aerobes.

- Dominance of obligatory anaerobes in the hind gut by the second week of the chick's life.

Finally, by day 12 the gastrointestinal tract of the chicken shelters a complex plethora of micro-organisms – aerobes, facultative anaerobes and obligatory anaerobes.

In vitro probiotic biology

When probiotics are grown in a batch culture, the resulting growth curve usually has four phases: lag, exponential (log), stationary, and death (Fig. 1).

- Lag phase:

When micro-organisms are introduced into a fresh culture medium, no immediate increase in cell number usually occurs. This period is called the lag phase.

It is not a time of inactivity; rather cells identify a new environment (for example new media) and synthesise new components essential for proliferation (doubling i.e. from one to two).

- Exponential phase:

During the exponential (log) phase, probiotics start growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions.

The growth rate is constant during the exponential phase i.e., they complete cell cycle and double in numbers at regular intervals. Thus, this phase of growth essentially requires more energy input which is leveraged by aerobic/anaerobic respiration (36-38 moles of ATP produced per cycle) only.

- Stationary phase:

In a closed system, such as a batch culture, population growth

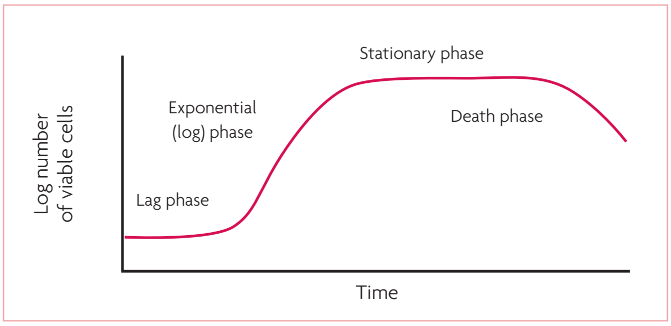


Fig. 1. Probiotic growth curve in a closed system/in vitro conditions.

eventually ceases and the growth curve becomes horizontal owing to several factors, such as nutrient depletion, limited O_2/CO_2 tension (for example aerobes and anaerobes), accumulation of toxic waste products, population reaching critical level in numbers etc. This is called the stationary phase. The transition of log phase to stationary phase requires lower energy inputs. Thus, during this transition the maintenance energy required is leveraged by fermentation (2 moles of ATP per cycle).

- Death phase:

During the death phase, the number of viable cells decline exponentially, with cells dying at a constant rate. There are two alternative events:

– Viable but non-culturable (VBNC) cells result when they are only temporarily unable to grow, at least under laboratory conditions used. Once the appropriate conditions are available (for example a change in temperature or passage through the host), VBNC probiotics resume growth.

– Programmed cell death occurs in a fraction of the microbial population and leaks nutrients enabling eventual growth of those cells in the population that did not initiate cell death.

In vivo probiotic biology

Within the chicken gut, the environment differs significantly from laboratory (in vitro) conditions.

Based on various research data published on the life cycle of probiotics in the chicken gut, it turns out that the phases of the growth curve have considerable resemblances and may be classified

Continued on page 13

Fig. 2a. Hypothesised growth curve (in a challenging environment) of Bacillus subtilis in the chicken gut based on published data and one time feeding of spores.

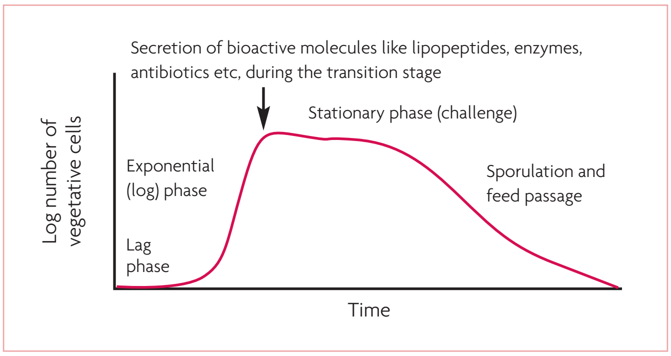
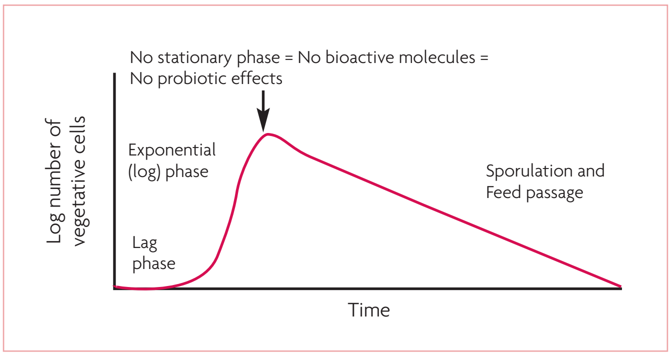


Fig. 2b. Hypothesised growth curve (without challenge) of Bacillus subtilis in the chicken gut based on published data and one time feeding of spores.



Continued from page 11
into four phases – lag, exponential (log), stationary/challenge and sporulation and feed passage (Fig. 2a and 2b). For better elucidation of the in vivo growth curve, let us consider the example of Bacillus subtilis, the widely used probiotic in poultry feed.

● Bacillus subtilis enters the chicken gut predominantly in endospore form. Specialised receptors in spores identify a favourable environment (for example nutrient, oxygen tension etc) and germinate into vegetative cells.

It has been reported that up to 90% of spores fed to chickens can germinate within an hour in the crop to gizzard section of the gut (upper GIT) possibly owing to favourable conditions for aerobes (higher O_2 tension). It is followed by colonisation on gut epithelial cells and preparation for proliferation.

This phase of the probiotic life cycle within the chicken gut can be defined as the lag phase. The number of spores per gram of feed and the rate of germination within the same period of time significantly influences the population density in the next phase.

● The exponential (log) phase of Bacillus subtilis in the chicken gut is shorter than in vitro conditions. This is owing to continuous feed passage, cellular sloughing, competition with other micro-organisms and a hostile gut environment. However, the rate of germination and feed retention in the crop triggers fast doubling of cell numbers, enough to create its own ecological niche. In fact, few research reports suggest Bacillus subtilis undergoes a full cycle of germination and sporulation during its journey from crop to cloaca.

● The transition of the log phase to the stationary phase for Bacillus subtilis is critical to envisage the benefits of using probiotics. Unlike in vitro conditions where this phase is precipitated primarily by nutrient depletion, in the chicken gut, it is owing to decreasing O_2 tension in the lower GIT and competition evoked by other bacteria/fungi.

A report published on Bacillus subtilis suggests that during the transition state it excretes several enzymes (to degrade complex substrates), immunomodulators (systemic resistance), antibiotics, bioactive compounds (for example lipopeptides) etc, to kill competitors and preventing them from invading its ecological niche.

These substances are produced by fermentation which simulates their biology in in vitro conditions (for example penicillin and enzyme production).

Interestingly, in commercial broiler trials, insignificant differences in growth performance parameters (for example BWG and FCR) between the probiotic control (Bacillus subtilis) and the negative control may be suggestive of a lack of stationary phase in the chicken gut.

Moreover, a lack of stationary phase often leads to overgrowth of probiotics resulting in competition with host (poultry) nutrition.

Such incidences are recorded in several field trials (Fig. 3 and Table 1) where the treatment group performs significantly better at a lower inclusion level.

● Sporulation is usually considered the last resort to be embarked upon when all other attempts to grow, compete and survive have been exhausted. Various research workers have reported that the sporulation

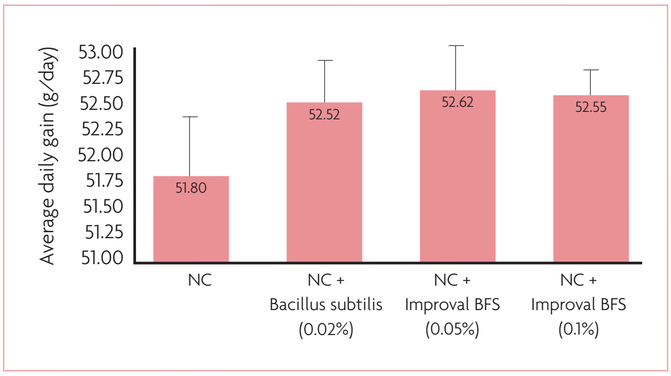


Fig. 3. Average daily gain in broilers (day 0-42) fed single strain of Bacillus subtilis (0.02%) and Improval BFS (0.05% and 0.1%) (Zydus AH R&D trial data). Bacillus subtilis = Total potency NLT 20 x 10^{11} spores/kg, Improval BFS = Microencapsulated spores of Bacillus subtilis, Bacillus coagulans, Bacillus licheniformis, Clostridium butyricum HJCB998 (Total potency NLT 5 X 10^{11}/kg), NC = Negative control.

tendency of Bacillus subtilis increases in the chicken hind gut (ileum to cloaca) relative to the upper-and-mid gut (crop to jejunum).

Such response has been attributed to factors like strict anoxic (CO_2) conditions, elevated NH_3 concentration and efficient nitrate/amino acid/nutrient utilisation by obligatory anaerobes (for example Cl. perfringens) outrunning Bacillus subtilis.

Conclusion

● The vegetative form of probiotics is biologically important for poultry.

● Considering the short feed passage time in poultry, spore forming probiotics must germinate fast in concurrence to vegetative form.

● A well defined log phase in the chicken gut should be followed by the stationary phase to yield beneficial bioactive molecules.

● Single strain probiotics like Bacillus subtilis tend to sporulate in the hind gut and thereby undergo feed passage. Hence, competitive exclusion of Clostridium perfringens is not possible leading to an unaddressed threat of necrotic enteritis from day one.

● In field conditions, multistrain probiotics (a strategic combination of aerobes and anaerobes) are more effective at lower inclusion level relative to single strain. It is probably because of the enhanced stationary phase yielding beneficial bioactive molecules, amplifying the protective spectrum against microbial infections and better utilisation of the gut microbiome. ■

Table 1. Villus height and crypt depth in the experimental birds at day 42 (Zydus AH R&D trial data).

Parameters	NC	NC + Bacillus subtilis (0.02%)	NC + Improval BFS (0.05%)	NC + Improval BFS (0.1%)
Villus height (µm)	634.1	657.6	659.1	648.4
Crypt depth (µm)	187.4	198.8	189.7	189.8
Villus height : crypt depth	3.39	3.32	3.49	3.47

Male birds, jejunum only. Mean of 10 male birds from each dietary treatment (one bird from each pen)

References are available from the author on request