Animal Science Papers and Reports vol. 36 (2018), no. 1, 87-98
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Long-term study on single and multiple species probiotic preparations for Florida softshell turtle (*Apalone ferox*) nutrition*

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(Accepted January 17, 2018)

The aim of the study was to assess the effects of two dietary probiotic preparations on the growth performance and gastrointestinal tract microecology of the Florida softshell turtle (*Apalone ferox*). In this 52-week experiment, 36 young animals were randomly distributed into three experimental groups: the control (CON) group, which was treated with no additives; the single species probiotic (SSP) group, which was treated with *Bacillus subtilis* PB6; and the multiple species probiotic (MSP) group, which was treated with a probiotic containing multiple strains. The study showed that the SSP preparation resulted in increases in body weight and in both the length and width of the carapace and plastron, and it had positive effects on shell mineralisation parameters, including the concentration of crude ash and Ca as well as the Ca:P ratio. Fluorescent in situ hybridization (FISH) showed that the SSP treatment had bacteria-suppressing effects on the total number of bacteria as well as *Clostridium perfringens* and *Salmonella* ssp., while the MSP treatment led to an increased number of bacteria in the digesta from the small intestine. These results showed that dietary probiotics are a highly specific, but efficient tool for turtle nutrition.

* The current study was supported by the following grant: NCN PRELUDIUM: UMO-2013/11/N/NZ9/04624, financed by the National Science Center (Poland).
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KEY WORDS: *Apalone ferox* / gastrointestinal tract microbiota / probiotic / Softshell turtle / shell mineralization

The Florida softshell turtle (*Apalone ferox*) is the largest softshell turtle species native to North America. The species is frequently farmed in the USA and Asia, and it has the potential to become one of the most commercially important turtle species after the Chinese softshell turtle (*Pelodiscus sinensis*). To our knowledge, the present study is the first to perform growth performance and microbiological trials on *A. ferox* focusing on the use of dietary probiotics. Additionally, the long-term (52 weeks) nature of the study allows for a complex analysis of the probiotic effects in turtles, which are one of the most long-lived animals. The gastrointestinal tract (GIT) microbiota is currently considered to be one of the most important factors affecting animal health, feed digestibility, growth performance and immune response [Montalban-Arques *et al*. 2015]. In modern animal management, gut health is a key goal achieved through proper nutrition and hygiene, it has been frequently studied in mammals and birds. A wide spectrum of feed additives is used for microbiota stabilisation and positive modification of GIT development. Prebiotics, probiotics, synbiotics, organic acids, antibiotics and bacteriocins are considered effective and as such have been widely used both in experimental studies and large-scale animal farming [Alloui *et al.* 2013, Rawski *et al*. 2016, Józefiak *et al.* 2013, Świątkiewicz *et al.* 2010]. However, few studies have focused on the microbiota of the GIT and the nutrition of reptiles [Keenan *et al.* 2013], while most of the available publications on turtle nutrition address the animal's requirements for basic nutrients and vitamins. For reptilian microbiota, most studies have focused on a narrow spectrum of the Enterobacteriaceae family, particularly Salmonella spp., in the turtle GIT and environment, and it has been widely discussed in terms of reptile-associated salmonellosis. Additionally, most previous studies have been based on traditional culture techniques or faecal sampling, which restricts their usefulness for assessing the GIT microbiota functions [Keenan *et al*. 2013]. The study of microbial communities in chelonians should be emphasised because of the global decline of turtle populations, frequent outbreaks of diseases in wild turtle populations and the low survival and breeding rates of turtles in captivity [Perry 2015, Hausmann *et al.* 2015, He *et al*. 2010]. Moreover, the increasing popularity of turtle farming requires new methods of enhancing growth performance among captive turtles and the development of suitable feed additives to increase meat yield from turtle farms [Zou *et al.* 2012]. Probiotics are considered one of the most effective feed additives due to their beneficial effect on the animal microbiota, health and growth performance [Fuller 1989, Montalban-Arques *et al.* 2015]. They have been utilised since ancient times and are consumed in a variety of fermented products [Fuller 1989, Dicks and Botes 2010]. Their properties as feed additives have been widely studied in both animals and humans [Dicks and Botes 2010, Świątkiewicz and Koreleski 2007]. Therefore, this study was designed to provide essential data to provide insight into the mode of action of probiotics in the turtle GIT. The potential positive effects of probiotics on the microbiota of the turtle GIT may represent an important factor allowing their keepers

to improve biosecurity, while they could also help secure suitable financial results for turtle farms and promote turtle rearing conducted for reintroduction programmes. In view of the above, *A. ferox* was chosen as a model species. The objective of this study was to evaluate how dietary supplementation with different probiotic preparations containing single or multiple species affects growth performance, shell composition and intestinal microbiota in *A. ferox*.

Material and methods

Ethics statement

This study was conducted in strict accordance with the recommendations of the National Ethics Commission (Warsaw, Poland). All procedures and experiments complied with the guidelines and were approved by the Local Ethics Commission of the Poznań University of Life Sciences (Poznań, Poland) with respect to animal experimentation and the care of the animals used for the study. All efforts were made to minimise suffering (Permit number: 22/2012). The animals were euthanised by decapitation according to the AVMA Guidelines for the Euthanasia of Animals [Leary et al., 2013]. Euthanasia was performed as part of a 3-step protocol (injectable anaesthetic, decapitation and pithing). The first step was an injection of ketamine (50 mg/kg IM) and decapitation was subsequently performed using a guillotine. To ensure death and avoid unnecessary suffering, the brain structure was destroyed by pithing.

Animals and diets

A 52-week growth experiment was conducted on 36 young *A. ferox* (mean body weight of 13.69 g and mean straight carapace length of 47.17 mm). The turtles were randomly allocated to plastic tanks (20x15x15 cm) filled with 2 l of water. One turtle was allotted per tank. They were acclimatised for one month to the experimental tanks and diets (with no feed additives). In the first week after settlement, the animals were fed live bloodworms (*Chironomidae*), while subsequently they were fed the experimental phase gelatine-based diets (Tab. 1) [Rawski *et al*. 2016, Rawski *et al*. 2017]. Water and air temperatures were controlled by a thermostat and maintained at a constant level of 28°C throughout the experiment. The water in the turtle tanks was changed every 48 h. The animals were assigned to 3 dietary treatments: the control (CON) with no additives; a single species probiotic (SSP) treatment with *Bacillus subtilis* PB6 at $2 \cdot 10^9$ colony forming units/g (CFU/g) of preparation (Kemin Industries, USA); and a multiple species probiotic (MSP) treatment containing (*Lactobacillus plantarum* at $1.26 \cdot 10^7$ CFU/g, *L. delbrueckii* subsp. *bulgaricus* at $2.06 \cdot 10^7$ CFU/g, *L. acidophilus* at $2.06 \cdot 10^7$ CFU/g, L. rhamnosus at $2.06 \cdot 10^7$ CFU/g, Bifidobacterium bifidum at $2.00 \cdot 10_7$ CFU/g, *Streptococci salivarius* subsp. *thermophilus* at $4.10 \cdot 10^7$ CFU/g, *Enterococcus faecium* at $5.90 \cdot 10^7$ CFU/g, *Aspergillus oryzae* at $5.32 \cdot 10^6$ CFU/g, *Candida pintolepessi* at $5.32 \cdot 10^6$; in the treatments the total number of live microorganisms was $2.0 \cdot 10^8$ CFU/g of preparation (Probiotics International Ltd.,

Table 1. Nutritional value of the diet in dry matter

Nutrient	Share
Crude Protein	34.55%
Crude Fat	6.91%
Crude Fibre	1.10%
Crude Ash	9.65%
Ca	4.47%
P	1.63%
Vitamin A	40000 IU/kg
Vitamin D_3	8000 IU/kg
Vitamin E	80 mg/kg
Vitamin K	70 mg/kg
Biotin	200 mg/kg
Choline chloride	800 mg/kg
Fe	180 mg/kg
Mn	340 mg/kg
Zn	240 mg/kg
Cu	32 mg/kg
Fe	180 mg/kg

IU – international units

Lopen Head, South Petherton, Somerset, UK). The probiotic preparations were added to the diets '*on top*' according to the manufacturer's recommendations for poultry (SSP at 500 ppm and MSP at 500 ppm).

Measurements and sampling

The growth and development of the experimental turtles were evaluated for body weight (BW) measurements using a laboratory scale (Radwag PS 600/C/2 Radom, Poland; accurate to ±0.01 g), straight carapace length (SCL), carapace width (CW), plastron length (PL) and plastron width (PW) measurements, which were performed using electronic callipers (accurate to ±0.01 mm). Shell measurements were conducted in a maximal straight line for the SCL, CW, and PL. For the PW the frontal edges of the shell bridge were chosen as representative for the survey. The weight of the turtles and their shell dimensions were measured after 1 day of fasting. These measurements were used for the straight carapace length gain (SCLG), carapace width gain (CWG), plastron length gain (PLG), plastron width gain (PWG), body weight gain (BWG) and condition index (CI) calculations [Jackson 1980, Willemsen and Hailey 2002, Rawski and Józefiak 2014].

Shell composition analysis

The dry matter (DM), crude ash, Ca and P concentrations were measured for the shell (carapace and plastron). The shells were cleaned from adherent tissue, dried (112°C for 12 h) and ashed (550°C for 14 h). The DM was calculated relative to the shell weight, while ash weight was calculated relative to the shell DM. The resulting crude ash was solubilised on a sand heater (300VC 15 min) in 10 ml of 6 N HCl and

30 ml of demineralised water. The solution was transferred after filtration (ashless filters) into a 100 ml volumetric flask. The Ca and P concentrations were measured by Atomic Absorption Spectrophotometry (VARIAN Techtron AA 475, Pty. Ltd., Springvale, Australia) as described in detail by Revy *et al.* [2004]. The Ca and P contents were calculated relative to crude ash.

Microbial community analysis by fluorescent in situ hybridization (FISH)

The gastrointestinal content samples collected during turtle dissection were immediately frozen and stored at -80°C for the FISH analysis. The procedure was performed in strict accordance with our previous study [Rawski *et al.* 2016]. To distinguish the total count of bacteria from other particles in the samples (performed via DAPI), oligonucleotide probes were labelled with DsRed and Alexa Fluor fluorochromes. The filters were visualised using a Carl Zeiss Microscope Axio Imager M2 [Józefiak *et al.* 2013, Rawski *et al*. 2016]. The counts of detected bacteria were expressed in CFU/g of digesta.

Table 2. Oligonucleotide probes used for fluorescent in situ hybridization (FISH) and intestinal microbiota analysis

Target	Probe	Sequence (5' to 3')	Reference
Enterobacteriaceae	Enter1432	CTTTTGCAACCCACT	Sghir *et al.* [2000]
Bifidobacterium sp.	Bif228	GATAGGGACGCGACCCCAT	Marteau *et al.* [2001]
Lactobacillus sp./*Enterococcus* sp.	Lab158	GGTATTAGCAYCTGTTTCCA	Harmsen *et al.* [1999]
Clostridium perfringens	Cpref191	GTAGTAAGTTGGTTTCCTCG	Fallani *et al.* [2006]
Bacteroides-Prevotella cluster	Bacto303	CCAATGTGGGGGACCTT	Manz *et al.* [1996]
Akkermansia muciniphila	Akk	ATCTGAAGCCAACCGCAAGG	Derrien *et al.* [2008]

Statistical analysis

All the recorded traits were tested for a normal distribution using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was conducted. The significance of differences between the groups was determined with Duncan's multiple range test at the significance level of $P \leq 0.05$. The calculations were performed using SAS 9.3 software [SAS Institute, 2011].

Results and discussion

The data on growth performance for the turtles are summarised in Table 3. The final BWG, SCLG, PLG, CWG and PWG (at the 52nd week) were significantly increased in the SSP group compared with the CON and MSP groups. The shell analysis results are presented in Table 4. The share of the shell in the turtle BW was not affected by any of the treatments. The DM of the shell was significantly reduced by the MSP preparation compared with the SSP preparation; however, the DM was not different compared with that of the CON treatment. The SSP treatment significantly increased the share of crude ash in the DM and Ca in the crude ash and increased the

Table 3. Growth performance of *Apalone ferox* at the 52nd week of the experiment

Item	CON	SSP	MSP	*P*-value
Body weight (g)	57.43[b]	83.31[a]	51.89[b]	0.0193
Body weight gain (g)	43.67[b]	69.39[a]	38.66[b]	0.0139
Straight carapace length (mm)	74.90[b]	85.11[a]	71.39[b]	0.0177
Straight carapace length gain (mm)	27.13[b]	38.25[a]	24.81[b]	0.0056
Plastron length (mm)	60.50[b]	69.17[a]	58.00[b]	0.0047
Plastron length gain (mm)	17.58[b]	27.13[a]	16.34[b]	0.0011
Condition index	0.74[b]	0.95[a]	0.72[b]	0.0328
Condition gain	0.46[b]	0.65[a]	0.44[b]	0.0194

CON – control treatment, SSP – single strain probiotic *Bacillus subtilis* PB6, MSP – multiple strain probiotic.
[ab]Means between treatments bearing different superscripts differ significantly at P≤0.05.

Table 4. Shell development and composition

Item	CON	SSP	MSP	*P*-value
Shell in body weight	28.03	27.78	27.55	0.8626
Dry matter in shell	35.13[ab]	37.78[a]	35.13[b]	0.0117
Crude ash in dry matter	8.05[b]	10.04[a]	8.10[b]	0.1470
Ca in crude ash	32.44[b]	34.52[a]	31.41[b]	0.0010
P in crude ash	16.22	16.27	16.63	0.5363
Ca:P ratio	2.00[b]	2.13[a]	1.90[b]	0.0210

CON – control treatment, SSP – single strain probiotic *Bacillus subtilis* PB6, MSP – multiple strain probiotic.
[ab]Means between treatments bearing different superscripts differ significantly at P≤0.05.

Table 5. Selected microbial populations in the small intestine

Item	CON	SSP	MSP	P-value
	LOG CFU/G			
Total number of bacteria	8.25[a]	8.01[b]	8.28[a]	0.0010
Bifidobacterium sp.	7.50[b]	7.45[b]	7.76[a]	<0.0001
Enterobacteriaceae	7.51[b]	7.41[b]	7.66[a]	0.0009
Clostridium perfringens	7.48[a]	7.32[b]	7.58[a]	0.0010
Lactobacillus sp./*Enterococcus* sp.	7.45[b]	7.46[b]	7.68[a]	0.0015
Akkermansia muciniphila	7.30[b]	7.29[b]	7.37[a]	0.0117
Bacteroides-Prevotella cluster	7.45[b]	7.42[b]	7.68[a]	<0.0001
Salmonella sp.	7.22[a]	7.08[b]	7.29[a]	0.0115

CON – control treatment, SSP – single strain probiotic *Bacillus subtilis* PB6, MSP – multiple strain probiotic.
[ab]Means between treatments bearing different superscripts differ significantly at P≤0.05.

Ca:P ratio. The microbial community analysis was conducted on the small intestine digesta applying FISH. The results (Tab. 5) showed that the SSP treatment lowered the total number of bacteria as well as the *Clostridium perfringens* and *Salmonella* sp. concentrations compared with those following the CON and MSP treatments.

For all the studied bacterial groups the highest levels were observed after the MSP treatment, which increased the counts of *Bifidobacterium* spp.*, Enterobacteriaceae, Lactobacillus* spp./*Enterococcus* spp., *Akkermansia muciniphila* and *Bacteroides-Prevotella* cluster compared with those following both the CON and SSP treatments. All tested specimens were confirmed as *Salmonella* spp. carriers. The concentration of this pathogen was lowered by the SSP treatment.

The mode of action of probiotics occurs via a complex mechanism that includes the reduction of pathogen colonisation by competitive exclusion, promotion of probiotic adhesion to the intestinal mucosa, reduced adhesion of potentially pathogenic strains and secretion of antimicrobial substances (bacteriocins, organic acids, etc.). Additionally, probiotics produce health-promoting substances (e.g. conjugated linoleic acid), enhance the epithelial barrier and support the immune system [Bermudez-Brito *et al.* 2012, Fuller 1989, Montalban-Arques *et al*. 2015]. These mechanisms cause significant changes in the GIT morphology and histological structure, improve the health status and lead to a better growth performance [Alloui *et al.* 2013]. Limited information is available and few studies have been performed on the microbiota and possible modes of action of probiotics in chelonians. Thus, in this study of the Florida softshell turtle (*A. ferox*) two different types of probiotic preparations were used: a single species preparation (SSP) and a multiple species preparation (MSP). The results of the experiment show that the SSP preparation had positive effects on BWG, SCLG and PLG, CL and WG as well as the condition index. No effects on growth performance were recorded for the MSP treatment. These results are consistent with the outcomes of previous studies, thus providing further evidence that dietary probiotics may represent effective growth promoters in animal nutrition [Fuller 1989, Montalban-Arques *et al.* 2015, Rawski *et al*. 2016]. However, as it was demonstrated in our previous study on *T. scripta* and *S. odoratus*, the effects of probiotics were dependent on the strain and species [Rawski *et al*. 2016]. The positive effects of *B. subtilis* were described in earlier experiments on poultry, piglets and Chinese softshell turtles (*P. sinensis*) [Jeong and Kim 2014, Teo and Tan 2006, Selvam *et al*. 2009, Lei and Yaohong 2010, Guan and Wang 2011]. *Bacillus subtilis* isolated from the grass carp (*Ctenopharyngodon idella*) improved the feed conversion ratio (FCR) and daily BWG and enhanced the sucrase, maltase, amylase, lipase and ATPase levels in P. *sinensis.* The studied probiotic decreased the bacteria richness in *P. sinensis*, but increased the total *Firmicutes* and Clostridia counts, although other groups of *Firmicutes* were decreased [Zhang *et al.* 2014]. The positive alteration of growth performance parameters by *B. subtilis* PB6 observed in this study was consistent with the results of a study on *Escherichia coli*-infected broiler chickens, which showed improved and comparable growth performance in the case of an antibiotic (zinc bacitracin and colistin sulphate) and *Bacillus subtilis* PB6 administration [Teo and Tan 2006]. The MSP treatment had no effect on growth performance, which was unexpected based on previous results [Rawski *et al*. 2016] and the general assumption that multiple-strain probiotics are more likely to inhabit

the GIT of the host. These probiotics should be more effective because of the synergistic effects of different probiotic bacterial strains, each having their own traits and properties for the inhibition of pathogens as well as a higher probability of promoting positive alterations of GIT function [Timmerman *et al*. 2004, Sanders and Huis in't Veld 1999, Kim *et al.* 2012]. In the present study the increased number of intestinal bacteria in the MSP treatment may have been an effect of overcolonisation of the turtle GIT by probiotic microorganisms. Additionally, the high levels of these microorganisms may have caused the underlying growth depression because of their ability to deconjugate bile salts and reduce the bile acid emulsification of fat properties, which has been widely investigated in chickens. Additionally, the increased bacterial numbers may decrease growth performance because of direct competition for nutrients, such as competition with the host for essential amino acids [Knarreborg *et al.* 2002, Harrow *et al*. 2007, Torok *et al*. 2011]. For captive chelonians, growth improvements are frequently considered a negative factor because growth may negatively affect shell mineralisation [Ritz *et al.* 2010]. In the present study we observed the best growth performance and highest mineral concentrations in the SSP group. Similarly to our previous study on *S. odoratus* as well as other works conducted on poultry, the results suggest that probiotic bacteria may increase mineral absorption from digesta [Rawski *et al.* 2016]. The Ca:P ratio in the shell of *A. ferox* varies in a range of 1.9:1 to 2.13:1, which confirms the assumption that for proper turtle nutrition the ratio should be approximately 2:1 [Mader 2005, Rawski *et al*. 2017]. From the veterinary perspective the improved shell mineralisation and Ca:P ratio may represent an important result for the study and could lead to better development of captive chelonians. Inefficient shell mineralisation is a key health issue in turtles and tortoises, having numerous health consequences, including pathologic fractures and secondary hyperparathyroidism metabolic bone disease (MBD) caused by an insufficient Ca:P ratio in the diet and malabsorption of Ca [Mans and Braun 2014]. Thus, the increase in shell mineralisation by probiotics may have a strictly practical role. Several mechanisms have been proposed to explain the role of probiotics in bone mineralisation. Bacterial metabolites, including vitamins D, C, K or folate, may play a key role, which may explain increased Ca levels in the shell. For the crude ash content, probiotic degradation of mineral-complexing phytic acid may occur, which was indicated by bacterial phytase production by the probiotic strain or affected microbial populations [Scholz-Ahrens *et al.* 2007]. In an earlier study on P. sinensis the experiments showed that an exogenous phytic acid in the diet reduced Mg absorption. The phytate increased the requirements to approximately 10% above the phytate-free diet level; therefore, phytic acid should be considered an important factor in the turtle shell mineralisation process [Chen *et al.* 2014]. Additionally, the positive effect of the MSP preparation on shell mineralisation in our previous study confirmed that MSP potentially contains phytase-producing strains [Rawski *et al.* 2016]. The improved absorption of minerals may also be related to the positive alteration of the GIT microstructure, such as increased villus height, which was observed in *T. scripta* and *S. odoratus* treated with

dietary probiotics [Rawski *et al.* 2016]. Furthermore, a study on Danio rerio showed that probiotics may accelerate bone deposition by stimulating the expression of key genes involved in ossification and suppressing bone formation inhibitors [Maradonna *et al*. 2013]. For microbial populations that inhabit the small intestine the number of total bacteria, *Clostridium perfringens* and *Salmonella spp*. were lowered by *B. subtilis* PB6, which may have been caused by the wide and direct activity of *B. subtilis* PB6 against pathogenic bacteria, including *C. perfringens, C. difficile, Streptococcus pneumonia, Campylobacter jejuni*, and *Campylobacter coli,* which was described earlier [Teo and Tan 2005]. The applied probiotic presents a wide spectrum of positive actions that exhibit antimicrobial as well as antidiarrhoeal and anti-inflammatory effects [Selvam *et al.* 2009, Foligné *et al.* 2012]. These effects may be caused by pathogen competitive exclusion and the secretion of surfactins - amphipathic cyclic lipoheptapeptides [Peys *et al.* 2007]. The decrease of these populations in the small intestine may be related to a direct effect at the bacterial level and changes in the intestinal microecology and GIT function in turtles (e.g. competitive exclusion of pathogens during histological structure remodelling). Multiple beneficial effects of microbiota modification on the GIT microecology were observed in earlier studies that supplemented the diets of *P. sinensis* with prebiotics (xylooligosaccharide) and probiotics (*B. subtilis*). Prebiotics supplementation resulted in an increase in amylase activity, decrease counts of total aerobic bacteria and Enterobacteria, and an increase in Bifidobacteria numbers [Guan and Wang 2011]. In our opinion, the GIT microbiota in turtles is still 'Terra Incognita'. However, the evidence discussed above shows that the use of microbiota has a latent potential to enhance animal growth and development as well as modulate metabolism. In the future, probiotics may be used for growth promotion, microbiome stabilisation and health status improvements through feed additives used in turtle farming, captive zoo breeding, conservation programmes or even keeping turtles as pets. The key factor for the efficient action of probiotic preparations appears to be connected with our understanding of the factors that determine colonisation and the positive effects of the applied strains of dietary probiotics in different turtle species.

REFERENCES

1. ALLOUI M.N., SZCZUREK W., ŚWIĄTKIEWICZ S., 2013 – The usefulness of prebiotics and probiotics in modern poultry nutrition: a review. ***Annals of Animal Sciences*** 13, 17-32.
2. BERMUDEZ-BRITO M., PLAZA-DÍAZ J., MUÑOZ-QUEZADA S., GÓMEZ-LLORENTE C., GIL A., 2012 – Probiotic mechanisms of action. ***Annals of Nutrition and Metabolism*** 61, 160-174.
3. CHEN C.Y., CHEN S.M., HUANG C.H., 2014 – Dietary magnesium requirement of soft-shelled turtles, *Pelodiscus sinensis*, fed diets containing exogenous phytate. ***Aquaculture*** 43, 280-284.
4. DERRIEN M., COLLADO M.C., BEN-AMOR K., SALMINEN S., DE VOS W.M., 2008 – The Mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. ***Applied and Environmental Microbiology*** 74, 1646-1648.
5. DICKS L.M., BOTES M., 2010 – Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. ***Beneficial Microbes*** 1,11-29.

6. FALLANI M., RIGOTTIER-GOIS L., AGUILERA M., BRIDONNEAU C., COLLIGNON A., EDWARDS C.A., CORTHIER G., DORÉ J., 2006 – *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. ***Journal of Microbiological Methods*** 67,150-161.
7. FOLIGNÉ B., PEYS E., VANDENKERCKHOVE J., DEWULF J., BRETON J., POT B., 2012 – Spores from two distinct colony types of the strain *Bacillus subtilis* PB6 substantiate anti-inflammatory probiotic effects in mice. ***Clinical Nutrition*** 31, 987-994.
8. FULLER R., 1989 – Probiotics in man and animals. ***Journal of Applied Bacteriology*** 66, 365-378.
9. HARMSEN H.J., ELFFERICH P., SCHUT F., WELLING G.W., 1999 – A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization. ***Microbial Ecology in Health and Disease*** 11, 3-12.
10. HARROW S.A., RAVINDRAN V., BUTLER R.C., MARSHALL J.W., TANNOCK G.W., 2007 – Real-time quantitative PCR measurement of ileal *Lactobacillus salivarius* populations from broiler chickens to determine the influence of farming practices. ***Applied and Environmental Microbiology*** 73, 7123-7127.
11. HAUSMANN J.C,, WACK A.N., ALLENDER M.C., CRANFIELD M.R., MURPHY K.J., BARRETT K., ROMERO J.L., WELLEHAN J.F., BLUM S.A., ZINK M.C., 2015 – Experimental challenge study of fv3-like ranavirus infection in previously fv3-like ranavirus infected eastern box turtles (*Terrapene carolina carolina*) to assess infection and survival. ***Journal of Zoo Wildlife Medicine*** 46, 732-746.
12. HE B., LIU Y., SHI H., ZHANG J., HU M., MA Y., FU L., HONG M., WANG J., JONATHAN F.J., 2010 – Captive breeding of the Four-eyed Turtle (*Sacalia quadriocellata*). ***Asian Herpetological Research*** 1, 111-117.
13. JACKSON O.F., 1980 – Weight and measurement data on tortoises (*Testudo graeca* and *Testudo hermanni*) and their relationship to health. ***Jorunal of Small Animal Practice*** 21, 409-416.
14. JEONG J., KIM I., 2014 – Effect of *Bacillus subtilis* C-3102 spores as a probiotic feed supplement on growth performance, noxious gas emission, and intestinal microflora in broilers. ***Poultry Science*** 93, 3097-3103.
15. JÓZEFIAK D., KIEROŃCZYK B., JUŚKIEWICZ J., ZDUŃCZYK Z., RAWSKI M., DŁUGOSZ J., SIP A., HØJBERG O., 2013 – Dietary nisin modulates the gastrointestinal microbial ecology and enhances growth performance of the broiler chickens. PloS one 8,1-11.
16. KEENAN S.W., ENGEL A.S., ELSEY R.M., 2013 – The alligator gut microbiome and implications for archosaur symbioses. ***Scientific Reports*** 3, 1-7.
17. KIM J., INGALE S., KIM Y., KIM K., SEN S., RYU M., LOHAKARE J., KWON I., CHAE B., 2012 – Effect of supplementation of multi-microbe probiotic product on growth performance, apparent digestibility, cecal microbiota and small intestinal morphology of broilers. ***Journal of Animal Physiology and Animal Nutrition*** 96, 618-626.
18. KNARREBORG A., ENGBERG R.M., JENSEN S.K., JENSEN B.B., 2002 – Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. ***Applied and Environmental Microbiology*** 68, 6425-6428.
19. LEARY S., UNDERWOOD W., ANTHONY R., CARTNER S., COREY D., GRANDIN T., GREENACRE C.B., GWALTNEY-BRAN S., MCCRACKIN M.A., MEYER R., 2013 – AVMA guidelines for the euthanasia of animals: 2013 edition. American Veterinary Medical Association. Schaumburg, IL, USA.
20. LEI G., YAOHONG Z., 2010 – Effects of *Bacillus subtilis* on Growth Performance, Digestive Enzyme Activities and Blood Biochemical Indices of *Pelodiscus sinensis*. ***Chinese Journal of Animal Nutrition*** 1, 42.

21. MADER D.R., 2005 Reptile medicine and surgery, 2nd ed., Elsevier Health Sciences, St. Louis, Missouri, USA.
22. MANS C., BRAUN J., 2014 – Update on common nutritional disorders of captive reptiles. ***Veterinary Clinics: Exotic Animal Practice*** 17, 369-395.
23. MANZ W., AMANN R., LUDWIG W., VANCANNEYT M., SCHLEIFER K.H., 1996 – Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. ***Microbiology*** 142, 1097-1106.
24. MARADONNA F., GIOACCHINI G., FALCINELLI S., BERTOTTO D., RADAELLI G., OLIVOTTO I., CARNEVALI O., 2013 – Probiotic supplementation promotes calcification in *Danio rerio* larvae: a molecular study. ***PLoS ONE*** 8, 1-10
25. MARTEAU P., POCHART P., DORÉ J, BÉRA-MAILLET C., BERNALIER A., CORTHIER G., 2001 – Comparative study of bacterial groups within the human cecal and fecal microbiota. ***Applied and Environmental Microbiology*** 67, 4939-4942.
26. MONTALBAN-ARQUES A., DE SCHRYVER P., BOSSIER P., GORKIEWICZ G., MULERO V., GATLIN D.M., GALINDO-VILLEGAS J., 2015 – Selective manipulation of the gut microbiota improves immune status in vertebrates. ***Frontiers in Immunology*** 6, 1-14.
27. PERRY K., 2015 – Deaths in Bellinger River snapping turtles. ***Australian Veteterinary Journal*** 93, 19.
28. PEYS E., VARGHESE J., SURESH P., VANDENKERCKHOVE J., VAN HEMEL J., CHANIYILPARAMPU R., SAS B. 2007 – Effects of *Bacillus subtilis'* PB6'(ATCC-PTA 6737) on *Clostridium difficile* associated diarrhea (CDAD) and inflammatory bowel disease (IBD) in animal models. ***American Journal of Infectious Diseases*** 3, 254-265.
29. RAWSKI M., JÓZEFIAK D., 2014 – Body condition scoring and obesity in captive African side-neck turtles (*Pelomedusidae*). ***Annals of Animal Science*** 14, 573-584.
30. RAWSKI M., KIEROŃCZYK B., DŁUGOSZ J., ŚWIĄTKIEWICZ S., JÓZEFIAK D., 2016 – Dietary probiotics affect gastrointestinal microbiota, histological structure and shell mineralization in turtles. ***PLoS ONE*** 11, 1-16.
31. RAWSKI M., MANS C., KIEROŃCZYK B., ŚWIĄTKIEWICZ S., BARC A., JÓZEFIAK D., 2017 – Freshwater turtle nutrition – a review of scientific and practical knowledge. ***Annals of Animal Science*** Article in press: DOI: 10.1515/aoas-2017-0025
32. REVY P.S., JONDREVILLE C., DOURMAD J.Y., NYS Y., 2004 – Effect of zinc supplemented as either an organic or an inorganic source and of microbial phytase on zinc and other minerals utilisation by weanling pigs. ***Animal Feed Science and Technology*** 16, 93-112.
33. RITZ J., GRIEBELER E.M., HUBER R., CLAUSS M., 2010 – Body size development of captive and free-ranging African spurred tortoises (*Geochelone sulcata*): high plasticity in reptilian growth rates. ***Herpetology Journal*** 20, 213-216.
34. SANDERS M., HUIS IN'T VELD J., 1999 – Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. ***Antonie Van Leeuwenhoek*** 76, 293-315.
35. SAS INSTITUTE, 2011 – SAS/IML 9.3 user's guide.
36. SCHOLZ-AHRENS K.E., ADE P., MARTEN B., WEBER P., TIMM W., AΣIL Y., GLÜER C.C., SCHREZENMEIR J. 2007 – Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. ***Journal of Nutrition*** 137, 838-846.
37. SELVAM R., MAHESWARI P., KAVITHA P., RAVICHANDRAN M., SAS B., RAMCHAND C., 2009 – Effect of *Bacillus subtilis* PB6, a natural probiotic on colon mucosal inflammation and plasma cytokines levels in inflammatory bowel disease. ***Indian Journal of Biochemistry and Biophysics*** 46, 79-85.

38. SGHIR A., GRAMET G., SUAU A., ROCHET V., POCHART P., DORE J., 2000 – Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. ***Applied and Environmental Microbiology*** 66, 2263-2266.
39. ŚWIĄTKIEWICZ S., KORELESKI J., 2007 – Feed additives enhancing immune responses in poultry. ***Medycyna Weterynaryjna*** 63, 1291-1295.
40. ŚWIĄTKIEWICZ S., KORELESKI J., ARCZEWSKA-WŁOSEK A., 2010 – Effect of prebiotic fructans and organic acids on mineral retention in laying hens. ***Acta Agriculturae Scandinavica Section A*** 60, 125-128.
41. TEO A.L., TAN H.M., 2006 – Effect of *Bacillus subtilis* PB6 (CloSTAT) on broilers infected with a pathogenic strain of *Escherichia coli*. ***Journal of Applied Poultry Research*** 15, 229-235.
42. TEO A.L., TAN H.M., 2005 – Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. ***Applied and Environmental Microbiology*** 71, 4185-4190.
43. TIMMERMAN H., KONING C., MULDER L., ROMBOUTS F., BEYNEN A., 2004 – Monostrain, multistrain and multispecies probiotics—a comparison of functionality and efficacy. ***International Journal of Food Microbiology*** 96, 219-233.
44. TOROK V.A, HUGHES R.J, MIKKELSEN L.L., PEREZ-MALDONADO R., BALDING K., MACALPINE R., PERCY N.J., OPHEL-KELLER K., 2011 – Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. ***Applied and Environmental Microbiology*** 77, 5868-5878.
45. WILLEMSEN R.E., HAILEY A., 2002 – Body mass condition in greek tortoises: regional and interspecific variation. ***Herpetology Journal*** 12, 105-114.
46. GUAN Y., WANG Z., 2011 – Effects of xylooligosaccharide on growth performance, activities of digestive enzymes, and intestinal microflora of juvenile *Pelodiscus sinensis*. ***Frontiers of Agriculture in China*** 5, 612-617.
47. ZHANG X., PENG L., WANG Y., LIANG Q., DENG B., LI W., FU L., YU D., SHEN W., WANG Z., 2014 – Effect of dietary supplementation of probiotic on performance and intestinal microflora of Chinese soft-shelled turtle (*Trionyx sinensis*). ***Aquaculture Nutrition*** 20, 667-674.
48. ZOU Y., AI Q., MAI K., ZHANG W., ZHANG Y., XU W. 2012 – Effects of brown fish meal replacement with fermented soybean meal on growth performance, feed efficiency and enzyme activities of Chinese soft-shelled turtle, *Pelodiscus sinensis*. ***Journal of Ocean University of China*** 11, 227-235.