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Neural Network Analysis of Age-Related Changes in the Biosystem of the Intestinal Microbiota of Broiler Chickens

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The study aims to evaluate age-related changes and mass accumulation activity in the biosystem of the intestinal microbiota of broiler chickens using fractal frequency-taxonomic profiles and neural network analysis. The molecular genetic terminal restriction fragment length polymorphism method and polymerase chain reaction were used to analyze the frequency-taxonomic profiles of the intestinal microbiota. These profiles were processed using the NONN computational neural network and trained to correctly convert digital profile data into age-related activity index (CSIfract) and mass accumulation activity index (CSImass). The visualization of the age dependence of mass accumulation activity was carried out using a two-dimensional Scale matrix. With the increasing age of broilers, a decrease was observed in the biochemical activity of their intestinal microbiota, associated with the accumulation of functional disorders in the microbial biosystem. Mass accumulative activity reached a maximum at 2-3 weeks of development and then gradually decreased. The quantitative composition of microorganisms changes with age: the number of lactobacilli and bacilli decreased, accompanied by increased clostridium and other microorganisms that increase the risk of diseases in populations. The study showed that age-related changes in broilers' intestinal microbiota significantly affected their biochemical activity and disease risk. To maintain optimal microbial activity, using probiotic supplements in feed is recommended.

Keywords: Fractal profile, age-related activity and mass accumulation activity of the biosystem of microorganisms, computational neural network.

INTRODUCTION

Broilers' intestinal microbiota is the main biological component of their intestinal microbial biosystem, which initiates and regulates mass accumulation and protective biochemical processes occurring in the birds' bodies. The microbiota of broilers includes normal flora (cellulosolytic bacteria, bacilli, lactobacilli, and bifidobacteria) and pathogenic microflora (*Salmonella enteritidis*, *S. gallinarum*, *S. typhimurium*, *Clostridium perfringens*, *Cl. botulinum*) (Okolelova *et al*., 2023; Vertiprakhov, 2022). The intensity of mass accumulation biochemical processes and the success of countering pathogenic infections depend on the activity of the microbial biosystem. The investigation of the gut microbiota in broiler chickens is conducted through metagenomic sequencing methodologies. This technique entails the collection of fecal samples from which microbial DNA is extracted. Subsequent sequencing facilitates the analysis of conserved genetic markers, such as 16S rRNA, thereby enabling the identification and quantification of microbial diversity within the gastrointestinal tract (Church *et al*., 2020). Zhou *et al*. (2021) examined 16S rRNA sequencing data derived from the duodenum and cecum of male Arbor Acres broilers at various developmental stages: 1, 7, 21, and 35 days of age. Both gut segments shared four similar dominant phyla - Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidota. Firmicutes and Proteobacteria accounted for more than 90% of the total sequences. Among the top 32 genera with abundances greater than 2%, 22 belonged to Firmicutes (Zhou *et al*., 2021). Liao *et al*. (2020) confirmed the dominant status of the Firmicutes phylum. Its abundance was 70-99% in the duodenum, 74-95% in the jejunum, 78-99% in the ileum, and 50-91% in the cecum. Proteobacteria emerged as the second most common phylum across the duodenum, jejunum, and cecum. Notably, the prevalence of Proteobacteria in the cecum was influenced by age, whereas

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no significant age-related variations were observed in the duodenum and jejunum. The highest Proteobacteria abundance in the cecum was found in newly hatched broiler chickens (Liao *et al*., 2020). However, other data demonstrated that in broiler microbial communities collected from the caecum, crop, jejunum, jejunal mucosa and proventriculus mucosa, sequences assigned to the phyla Firmicutes and Actinobacteria were predominant, followed by Bacteroidota in the caecum and Proteobacteria elsewhere. The sequencing of broiler gut showed that Actinobacteria were more prevalent than Proteobacteria. Phyla with low abundance contain Fusobacteriota, Campylobacteriota, Desulfobacteriota, Verrucomicrobiota, Acidobacteriota and Chlorofexi (Kayal *et al*., 2022). The detected families within the phylum Firmicutes include Lactobacillaceae, Enterococcaceae, Lachnospiraceae, Ruminococcaceae, Clostridiaceae, Oscillospiraceae and Peptostreptococcaceae. The family Lactobacillaceae and its genus Lactobacillus exhibited the highest abundance in the duodenum, the jejunum and ileum. The relative abundance of Lactobacillus was minimal at day 1, significantly increased until day 7, decreased at day 21 and rebounded thereafter. The age-related dynamic trend of Escherichia-Shigella mirrored those observed for Lactobacillus. Escherichia-Shigella were found to be more common in feces than in cecum and duodenum samples. Also, the genus Acinetobacter (Proteobacteria) was identified as a biomarker of 21- and 35-day-old broilers in the duodenum. The cecum was colonized by the family Clostridiaceae immediately after hatching, Clostridium_sensu_stricto_1 accounted for 83.50% of the total sequences, but their abundance significantly diminished thereafter. Newly hatched broilers also displayed the greatest prevalence of *Salmonella* (Proteobacteria). By day 7, representatives of the families Lachnospiraceae and Ruminococcaceae became predominant. Microbial diversity and composition of the samples were similar between 21 and 35 days of age - five genera including Alistipes from Bacteroidota, and Blautia, Ruminiclostridium_5, Ruminococcaceae_UCG-014 and [Ruminococcus]_torques_group from Firmicutes were significantly enriched in the samples. However, one genus - Butyricicoccus - was more prominently expressed at 21 days in the cecum. In the duodenum, Butyricicoccus reached its peak abundance at 7 days of age. The highest predominance of the Peptostreptococcaceae family was observed on day 42 (Zhou *et al*., 2021). *Salmonella* is a prevalent intestinal colonizer in poultry, but it poses a significant pathogenic threat to human microbiota, necessitating rigorous control measures within poultry production facilities (Sheets *et al*., 2022). Beyond salmonellosis, the consumption of contaminated poultry meat can also lead to clostridial necrotizing enteritis, with Clostridium perfringens identified as the etiological agent of this condition (Fancher *et al*., 2020). Therefore, stringent monitoring of its presence is imperative to mitigate associated health risks.Although sequencing is a widely used method for studying avian microbiota, it has several drawbacks. The complexity of data analysis and high costs of sequencing make this method less accessible. The frequency-taxonomic profile of broilers' intestinal microbiota can be determined by the molecular genetic TRFLP method (terminal restriction fragment length polymorphism) followed using polymerase chain reaction (PCR). The TRFLP method makes it possible to measure accurately the frequency of occurrence of conservative sections of the genome of microorganisms, and by the size and nucleotide sequence of the isolated fragments, one can identify the corresponding bacterial species (Ilina, 2012; Singh *et al*., 2013).

Frequency-taxonomic profiles of the birds' intestinal microbiota are usually presented in order of increasing size of identification fragments. In our study, we found that the informativeness of molecular genetic data could be increased if taxa were arranged in a profile in order of the decreasing frequency of occurrence of taxa. With this arrangement of taxa, a monotonously decreasing (fractal, FractPr) form of the frequency-taxonomic profile of the birds' intestinal microbiota is formed (Zaikina *et al*., 2022) (Fig. 1). The decreasing character of the FractPr profile of the intestinal microbiota is regularly reproduced and some deviations from the ideal power-law form of the FtactPr profile are possible due to changes in housing conditions and an increase in the age of birds (Kochish *et al*., 2020; Vorobev and Selina, 2021).

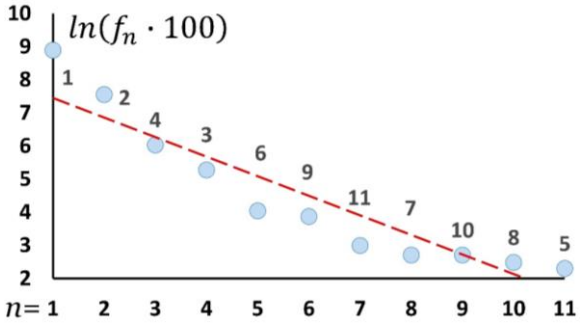

Figure 1. Logarithmic fractal frequency-taxonomic FractPr profile of the intestinal microbiota of broiler chickens from poultry house No. 5 (Table 1). f_n, n are the frequency and ordinal number of the taxon in the FractPr profile. The dotted line represents the ideal logarithmic fractal FractPr profile. The numbers around the circles correspond to the ordinal numbers of the groups of microorganisms in the FractPr profile (Table 1).

The actual FractPr profiles of the intestinal microbiota may differ significantly from the FractPr profile of the ideal power-law form, characterized by a decrease in the frequencies of taxa in the profile according to the power law (Shreder, 2001) (for example 10^{-1}, 10^{-2}, 10^{-3}, ...; dotted line in

Figure 1) and a constant step-by-step decrease in the logarithm of the frequency of taxa in the ideal FractPr profile. A preliminary analysis of FractPr profiles showed that the increase in deviations of the actual FractPr profile from the ideal shape was a consequence of increased exposure of the microbial biosystem to destabilizing external factors and an increase in the age of birds. Based on this, it was proposed to calculate by the magnitude of such deviations the CSImass and CSIfract indexes to which the cognitive rights are delegated to quantitatively and qualitatively represent changes in the age and mass accumulation activity of the biosystem of broilers' intestinal microbiota. The study aims to visualize the mathematically non-formalizable age dependence of mass accumulation activity (CSImass index) on age activity (CSIfract index) of the biosystem of broilers' intestinal microbiota using a two-dimensional Scale matrix. The NONN neural network is supposed to be used to calculate the CSImass and CSIfract indexes based on digital data from FractPr fractal profiles. The CSImass and CSIfract indexes are dimensionless qualitative parameters of the activity of the biosystem of broilers' intestinal microbiota. They cannot be used in classical regression mathematical expressions, since they have no dimension, but CSImass and CSIfract indexes can be used in comparisons based on the multidimensional scaling method (Sutrop, 2001) and the method of constructing a special two-dimensional Scale matrix. The cells of the Scale matrix are filled with the ordinal numbers of the experiment variants (numbers of poultry houses) following the coordinates of the cells specified by the values of the CSImass and CSIfract indexes. As a result, using the Scale matrix, one can visualize the dependence of qualitative indicators (mass accumulation and age-related activity) of broilers' intestinal microbiota biosystem.

To reach this goal, the following actions were performed.

1. The NONN neural network has been developed with the Learning service, which is used for free correction of the CSI procedure. After training, the NONN neural network acquired the ability to correctly convert the FractPr profiles of broilers' intestinal microbiota into the values of the CSIfract and CSImass indexes, representing quantitatively and qualitatively the mass accumulation and age-related activity of the biosystem of broilers' intestinal microbiota.
2. Using the Learning service, correct CSI-procedure algorithms have been found that calculate the CSIfract and CSImass indexes based on digital data from fractal FractPr profiles of broilers' intestinal microbiota.
3. The Scale matrix has been constructed, visualizing the mathematically non-formalized dependence of mass accumulation activity (CSImass index) on age-related activity (CSIfract index) of the biosystem of broilers' intestinal microbiota.

MATERIALS AND METHODS

In 2013, the BIOTROF molecular genetic laboratory used the molecular genetic TRFLP method to determine the frequency and taxonomic profiles of broilers' intestinal microbiota (ages 3 to 37 days, Table 1) from six poultry houses of the poultry farm of Elinar-Broiler LLC (Moscow region, Russia). Identification and quantitative analyses were carried out by the size of the isolated nucleotide fragments using the FragSort Internet database, which let us identify bacterial taxa and determine the frequency-taxonomic profile of broilers' intestinal microbiota.

Table 1. Frequency-taxonomic profiles of broilers' intestinal microbiota of six poultry houses of Elinar-Broiler LLC (%), age of broilers (days) in poultry houses, and CSIfract and CSImass indexes of the biosystem of broilers' intestinal microbiota.

The number of poultry house	1	2	3	4	5	6	Coefficient of correlation with CSIfract
1. Lactobacilli	85.2	3.5	6.5	3.12	73.00	6.5	0.33
2. Unidentified bacteria	6.9	30.0	55.9	11.60	19.10	17.0	0.17
3. Bacilli	4.0	46.0	29.0	13.80	1.96	2.2	0.45
4. Saccharolytic bacteria	1.2	10.8	2.3	16.50	4.17	58.5	-0.74
5. Pseudomonads	0.1	8.8	2.3	39.10	0.10	0.2	-0.18
6. Clostridia	0.3	0.4	0.8	5.90	0.57	7.3	-0.78
7. Enterobacteria	1.7	0.1	2.5	8.40	0.15	0.1	-0.08
8. Actinomycetes	0.1	0.1	0.1	0.52	0.12	6.4	-0.69
9. Bifidobacteria	0.2	0.2	0.2	0.63	0.48	0.2	-0.42
10. Acidobacteria	0.1	0.1	0.1	0.12	0.15	1.2	-0.69
11. Campylobacteria	0.2	0.2	0.2	0.35	0.20	0.4	-0.77
Age of broilers	3.0	8.0*	17.0*	25.00	28.00	37.0	-0.998
CSIfract Index	7.4	6.3	5.8	4.00	3.90	2.5	1.00
CSImass Index	1.7	5.0	6.6	6.30	5.60	4.8	-0.51

*The age of broilers was determined by the NONN neural network

***NONN Neural Network Learning and Computing Algorithm*:** The calculations of the NONN neural network assumed that the increasing deviation of the actual FractPr profile of broilers' intestinal microbiota from the ideal shape was a consequence of the increasing destabilizing biotic and abiotic external effects on the intestinal microbiota and the consequence of an increase in the age of birds. As the birds age, the number of disruptions in the functioning of the biosystem of microorganisms in the birds' intestines increases. Therefore, the deviation of the actual profile of the intestinal microbiota from the ideal form also increases. Taking this into account, it was proposed to calculate the age-related activity (CSIfract index) and mass accumulation (CSImass index) activity of the biosystem of broilers' intestinal microbiota according to the deviation of the actual FractPr profile from the ideal form. To mathematically assess the difference between the actual FractPr profile and the ideal shape and the subsequent calculation using the CSI procedure of the CSIfract and CSImass indexes, we created the computational neural network NONN in an Excel environment (Fig. 2) (Gafarov and Galimyanov, 2018; Zaikina *et al*., 2022). The calculations of the CSI procedure were based on the procedures of cluster and discriminant analysis of digital data. To adjust the algorithm of the CSI procedure, we decided to add the Learning software service to the NONN neural network (Kruglov and Borisov, 2002; Kulakov and Dimitrov, 2018; Pogodaev *et al*., 2021; Schmidhuber, 2015), which allowed us to freely and repeatedly change the algorithm of the CSI procedure and evaluate the correctness of the calculations performed.

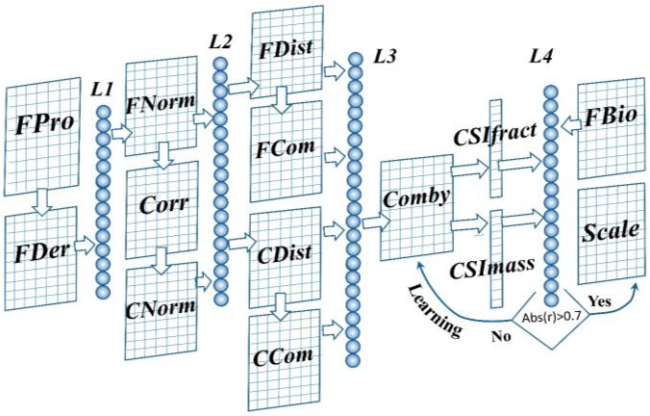

Figure 2. The NONN computational neural network that converts digital data of the FractPr frequency-taxonomic profile of broilers' intestinal microbiota into CSIfract and CSImass indexes. Learning is a learning software service that allows one to adjust the algorithm of the CSI procedure repeatedly and cyclically.

1. The L1 neuron layer transforms the initial frequency-taxonomic profiles of broilers' intestinal microbiota (FPro matrix, Table 1, Fig. 2) into the logarithmic fractal FractPr profile. After arranging the taxon frequencies and obtaining their logarithm, the L1 neuron layer calculates the step-by-step rate of decrease in the logarithm of the taxon frequency in FractPro profiles and records the calculation results in the FDer matrix. Finally, the L1 neuron layer performs line-by-line normalization of the digital data of the FDer matrix using the standard normalization function for numerical data *Normalization()* (Mascarenhas, 2018) and records the normalization results into the FNorm matrix.

$$FNorm = Normalization(FDer) \quad (1)$$

2. The L1 neuron layer calculates the correlation matrix Corr using the FNorm matrix data and the standard *Correlation()* procedure.

$$Corr = Corralation(FNorm) \quad (2)$$

3. The L1 neuron layer calculates the product of FNorm×Corr matrices, performs line-by-line normalization of the received digital data using the standard *Normalization()* procedure, and records the normalization results in the CNorm matrix.

$$CNorm = Normalization(FNorm \times Corr) \quad (3)$$

4. The L2 neuron layer calculates the FDist and CDist Euclidean distance matrices using digital data located in the columns of the FNorm and CNorm matrices and the standard computational procedure *EuclidDistance()* (Everitt *et al*., 2011).

$$FDist_{kl} = EuclidDistance \quad (4)$$
$$(FNorm_{1k}, \dots, FNorm_{Nk}; FNorm_{1l}, \dots, FNorm_{Nl})$$
$$CDist_{kl} = EuclidDistance \quad (5)$$
$$(CNorm_{1k}, \dots, CNorm_{Nk}; CNorm_{1l}, \dots, CNorm_{Nl})$$

where k, l = 1,..., M are the ordinal numbers of columns in the FNorm and CNorm matrices; M=6, N=11 are the number of columns (number of poultry houses) and the number of rows (number of taxa in FractPr profiles) in the FNorm and CNorm matrices; *EuclidDistance()* is the standard procedure for calculating Euclidean distances between columns of FNorm and CNorm matrices.

5. The L2 neuron layer calculates the FCom and CCom matrices using data from the diagonal symmetric matrices FDist, CDist, and the standard procedure for calculating the Eigenvectors of symmetric matrices *EigenVectors()* (Markova and Korchevskaya, 2011).

$$FCom = EigenVectors(DFist) \quad (6)$$
$$CCom = EigenVectors(CDist) \quad (7)$$

6. The L3 neuron layer iterates through the algorithms of the CSI procedure presented in the Comby matrix and converts the digital data of the FNorm, CNorm, FDist, CDist, FCom, CCom matrices into the values of the CSIfract and CSImass indexes. When creating the CSI procedure algorithm package, algebraic expressions were used where the operands of addition, subtraction, multiplication, and division of digital data of the FNorm, CNorm, FDist, CDist, FCom, and CCom matrices were combined.

7. The L4 neuron layer in the learning cycles of the (Widrow

et al., 2013) NONN neural network performs testing of the CSI procedure algorithms by calculating the correlation and regression coefficients of the CSImass and CSIfract indexes with the data of the FBio matrix (Table 1, Fig. 2). When the correlation coefficient of the CSIfract index with the age of broilers reached the value r = -0.998 (Table 1) the program exited the training cycles of the NONN neural network and the following formulas for calculating the values of the CSImass and CSIfract indexes were accepted as correct.

$$CSIfract_k = 5 - Normalization(FDist7_k + FDist8_k) \cdot 4.07 \quad (8)$$

$$CSImass_k = Normalization(FDist1_k) \cdot 3.93 + 5 \quad (9)$$

where $FDist1_k$, $FDist7_k$, *and* $FDist8_k$ are the numeric data of the 1st, 7th, and 8th rows of the FDist matrix; k=1,..., M=6 are the ordinal numbers of columns (poultry houses) in the FDist matrix. The calculated values of the CSIfract and CSImass indexes are shown in Table 1.

Using the calculated values of the CSIfract and CSImass indexes (Table 1) the cells of the Scale matrix (Fig. 3) were filled with the ordinal numbers of the experiment variants (numbers of poultry houses, Table 1). The cells to be filled were selected considering their coordinates, determined by the corresponding values of the CSIfract and CSImass indexes. As a result, using the Scale matrix, we managed to visualize the mathematically non-formalized dependence of mass accumulation activity (CSImass index) on the age-related activity in the biosystem of broilers' intestinal microbiota.

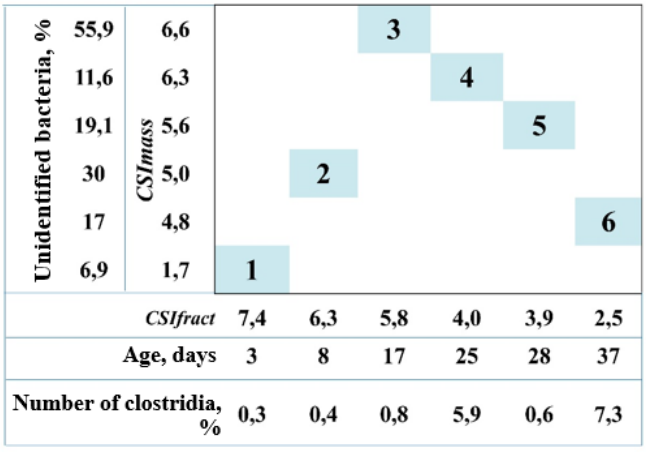

Figure 3. A Scale matrix visualizing the mathematically non-formalized dependence of mass accumulation activity (CSImass index) on age-related activity (CSIfract index) in the biosystem of broilers' intestinal microbiota. The numbers in the matrix cells correspond to the numbers of the poultry houses (Table 1).

DISCUSSION

Age-related changes in the intensity of mass accumulation processes in the body of broilers were assessed using the CSImass index, which was delegated the cognitive rights to represent the mass accumulation activity of broilers' intestinal microbiota biosystem. Using the CSIfract index (transformed digital data of FractPro fractal profiles), we managed to estimate the age of broilers and construct a Scale matrix demonstrating the extreme dependence of the intensity of mass accumulation processes on the age of broilers (Fig. 3). The CSIfract index (activity of the intestinal microbiota biosystem) decreases from 7.4 to 2.5 (Table 1), and this is a consequence of the functional disruptions of microbial biosystem interactions in the intestines of broilers that increase with age. These disruptions are the main reason that at 2-5 weeks of broiler development, the mass accumulation activity of broilers' intestinal microbiota biosystem decreases monotonously (Fig. 3). The correlation coefficients of the numbers of groups of microorganisms of broilers' intestinal microbiota with the CSIfract index (Table 1) demonstrate the features of age-related changes in the quantitative composition of broilers' intestinal microbiota biosystem components. As the age of birds increases, the relative number of groups of saccharolytic bacteria, clostridium, actinomycetes, bifidobacteria, acidobacteria, and campylobacter increases as well (correlation coefficients with the CSIfract index r = -0.78... -0.42; Table 1), and the number of lactobacilli and bacilli groups decreases (correlation coefficients with the CSIfract index r = 0.33, 0.45; Table 1). Unidentified bacteria have the status of microflora that accompanies feed. With age, the number of this group of microorganisms decreases, indicating the displacement of undesirable microorganisms, including pathogenic species, from the intestines of birds by the normal flora. 06Some Clostridium species have a high ability to form toxic or carcinogenic metabolites in the intestines of birds, which provokes the occurrence of intestinal diseases in birds (Laptev *et al*., 2022). Therefore, age-related disruption of microbial biosystem interactions in broiler gut microbiota, leading to an increase in the number of clostridia, may increase the risks of bird disease. The consequence of a decrease in the number of lactobacilli and bacilli is a decrease in the mass storage capacity of the biosystem of the intestinal microflora of broilers. To maintain this activity, probiotic supplements are used in poultry feed (Iyldyrym *et al*., 2024). To compensate for the decrease in the number of beneficial bacteria, it is necessary to use probiotic supplements in broiler feeds (Goncharov *et al*., 2017a;Goncharov *et al*., 2017b).

***Conclusion*:** Neural network processing of fractal profiles of the intestinal microbiota allowed us to determine the indexes of age-related and mass accumulation activity of broilers' intestinal microbiota biosystem. As a result, we found that the

overall biochemical activity of the intestinal microbiota decreased with increasing age of broilers, which is associated with the age-related accumulation of functional disruptions in the biosystem interactions of broilers' intestinal microbiota. Because of this, the mass accumulation activity of the intestinal microbiota biosystem reaches a maximum at 2-3 weeks of birds' development and then gradually decreases. With the age of birds, the ratios of microorganisms in the intestinal biosystem change. The number of lactobacilli and bacilli decreases, and the number of other microorganisms, including clostridia, increases, which indicates a weakening of mass accumulation processes and an increase in the risks of broiler diseases.

***Authors' contribution statement*:** Conceptualization, G.L. and N.S.; Methodology, A.G.; Software, M.S.; Validation, N.V., G.L., and M.S.; Formal Analysis, N.S.; Investigation, N.V. and A.G.; Resources, G.L.; Data Curation, M.S. and A.G.; Writing – Original Draft Preparation, N.V.; Writing – Review & Editing, G.L. and N.S.; Visualization, A.G.; Supervision, G.L.; Project Administration, N.V.; Funding Acquisition, N.V. and G.L.

***Informed consent*:** N/A

***Conflict of interest*:** The authors declare that there is no conflict of interest.

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***SDGs Addressed*:** Zero Hunger, Good Health and Well-Being

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