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Trehalose Supplementation Effects on Growth, Intestinal Morphology, Gut Bacteria, and Footpad Dermatitis of Broiler Chickens Reared at High Density

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This study aimed to measure the effects of trehalose (Tre) supplementation on the growth, intestinal morphology, gut bacteria, and footpad dermatitis (FPD) of broiler chickens reared at different stocking densities (SD). Four hundred newly hatched Ross 308 male chicks were randomly allocated to four groups of eight, following a 2 × 2 factorial arrangement in a randomized complete block design using two SDs (normal, 11; high, 14 birds/m^2) and two diets: basal with and without 0.5% Tre. Tre supplementation was provided during the starter/grower phase, but not the finisher phase. Data were analyzed using a two-way analysis of variance. We observed no significant effects of SD or Tre, individually or combined, on body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) during the starter/grower period. However, high SD decreased both BWG ($P < 0.001$) and FI ($P < 0.05$), and increased FCR ($P < 0.001$), during the finisher period. Whereas Tre reduced FCR ($P < 0.05$) as a main effect, no combined effect was observed on FCR. Over the total period, high SD negatively affected BWG and FCR ($P < 0.001$), and Tre significantly reduced FCR, with its effect unaffected by SD. No significant effects of SD or Tre were observed on jejunal morphology. The ileal abundance of *Clostridium perfringens* ($P > 0.05$) was not affected by high SD but was significantly reduced by Tre. Neither high SD nor Tre altered *Lactobacillus* spp. counts; however, high SD increased FPD lesion scores, whereas Tre had no effect. The study showed that Tre supplementation during the starter/grower period improved FCR during the finisher period, possibly by decreasing the abundance of *C. perfringens* in broiler chickens.

Key words: bacteriocin, *Clostridium perfringens*, feed conversion ratio, *Lactobacillus*, prebiotics

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Introduction

Managing intestinal function, including digestion, absorption, barriers against luminal bacterial infiltration, and gut microbial compositions, has been a significant focus for modern broiler production. However, it has become a more significant concern owing to restrictions on the use of antimicrobial growth promoters aimed at preventing the selection of antimicrobial-resistant bacteria. Given that physiological, pathological, environmental, and dietary factors negatively affect these intestinal functions[1,2], prebiotics, probiotics, and their combination (synbiotics) have been widely used to improve intestinal conditions and growth[3–6].

High stocking density (SD) is a stressor that impairs intestinal function and retards the growth of broiler chickens. High SD causes increased corticosterone secretion, decreased barrier function, intestinal inflammation, and abnormal intestinal microbial compositions[7–13]. Moreover, it leads to the incidence and progression of footpad dermatitis (FPD), described as inflammatory, necrotic lesions on the plantar surface of the footpads[14]. FPD negatively affects welfare, health status, walking, and feeding activity, thereby reducing growth[15].

Trehalose (Tre) is a glucose-glucose disaccharide linked by an

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α, α-1,1-glycoside bond; it is ubiquitous in diverse organisms, including bacteria, yeast, fungi, and invertebrates. Tre has a unique chemical structure that confers cryoprotection and drought tolerance in microorganisms, plants, and insects. It has recently garnered attention for its mitigation of protein aggregation by stimulating cellular autophagy, suppressing hepatic inflammatory cascade, enhancing energy metabolism by browning white adipocytes, and alleviating neurodegenerative disease[16–20]. Tre effects on the gut microbiome have also gained attention, specifically with respect to the human pathogen *Clostridium difficile*[21]. A study using an *in vitro* human colon model showed that Tre supplementation remodeled the gut microbiota to prevent *C. difficile* infection[22]. A recent study using a culture model demonstrated that Tre stimulated the growth of the bacteriocin-producing lactic acid bacteria *Lactococcus lactis* spp. and *Lactococcus* sp[23]. In broiler chickens, Tre supplementation has been reported to alleviate intestinal inflammation and improve intestinal morphology in juvenile chicks and increase the abundance of *Lactobacilli* in *Salmonella typhimurium*-infected chickens, increasing growth[24–26]. Although little is known about the effects of Tre on FPD, the treatments conditioning intestinal microbiota have been reported to partly alleviate high SD-induced stress, microbial status, and behavioral symptoms, improving growth[15].

From these data, we hypothesized that Tre would ameliorate high SD-induced impairment of intestinal function and growth retardation, possibly through prebiotic effects. Thus, we measured the effects of Tre supplementation on growth, intestinal morphology, bacterial populations, and FPD in broiler chickens reared under high SD.

Materials and Methods

Ethics statement

Animal experiments were conducted at Bangkok Animal Research Center (BARC) Co., Ltd. The trial (AB19463A) was approved by the Animal Ethics Committee of the institute and was conducted following the guidelines for using animals for scientific purpose of the National Research Council of Thailand, under the Act of Animals for Scientific Purpose (B.E. 2558). Procedures, documentation, equipment, and records were examined to ensure that the study was conducted under BARC's internal standard operating procedures for animal research.

Animals and experimental design

Four hundred newly hatched Ross 308 male chicks (*Gallus gallus domesticus*) were obtained from a local hatchery. They were allocated to four treatment groups following a 2 × 2 factorial arrangement in a randomized complete block design with two SD (normal and high) and two diets: basal with and without 0.5% Tre (Hayashibara Co., Ltd.). Each pen housed eight birds at 11 and 14 birds/m^2 for normal and high SD, respectively. The groups exhibited similar mean initial body weights. Chicks were reared in floor pens (1 x 1 m) littered with rice hulls and equipped with tubular feeders and three nipple drinkers. A practical corn-soybean meal diet was formulated and provided for each growth period (starter, 0–10 d; grower, 11–24 d; finisher, 25–42 d) (Table 1). This basal diet was supplemented with 0.5% (*w/w*) Tre, only during the starter and grower periods. All diets were processed with a conditioning temperature of 82 °C and further processed to crumble (starter) and pellet (3-mm diameter, grower/finisher). Tre is present in many plants (Botrychium, Selaginella), fungi, and invertebrates, so those were not present in the feed.

Birds were maintained under the lighting and management programs specified by the Ross 308 broiler management manual and were provided *ad libitum* access to water and feed. All birds were vaccinated for Newcastle disease and infectious bronchitis at 7 d of age, and for Gumboro disease at 14 d of age. Total feed consumption per pen was recorded weekly for 0–10, 11–24, 25–35, and 36–42 d ages. Birds were weighed on a pen basis at 1, 10, 24, 35, and 42 d, after which body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were calculated. At 42 d of age, three birds per pen were selected and slaughtered for carcass yield measurements: dressing, breast meat, thigh, drumstick, wing, and abdominal fat.

Intestinal morphology and bacterial number

At 24 d of age, one bird from each pen with a BW close to the pen's mean was euthanized to collect jejunal samples for morphological analysis, villus height (VH), crypt depth (CD), and VH/CD ratio. Approximately 1 cm of each jejunal sample was dissected, fixed in 10% buffered formalin, and embedded in paraffin. The samples were cross-sectioned, placed on glass slides, and stained with hematoxylin and eosin. Villus morphology was imaged and captured using Image-Pro Plus version 5.1. VH and CD were determined as previously described[25]. Ileal contents from each bird were collected for counts of *Lactobacillus* spp. and *C. perfringens*. Approximately 1 g was transferred under a stream of CO_2 into flasks containing 9 mL of a pre-reduced salt medium (0.85% NaCl). The suspension was further homogenized for 2 min in CO_2-flushed plastic bags using a bag mixer, then diluted ten-fold using pre-reduced salt medium as previously described[27]. Presumptive *Lactobacillus* spp. were counted using de Man, Rogosa and Sharpe (MRS) agar with $CaCO_3$. Plates were incubated in an anaerobic cabinet at 35 °C for 24 h. Presumptive *C. perfringens* were counted on tryptose sulfite cycloserine agar plates incubated under anaerobic conditions at 35 °C for 24 h.

FPD quantification

FPD lesion scores for each pen were calculated as previously described[28]. Briefly, the lesion scores were 0, 1, or 2, from absent to severe. Scores were calculated from the percentages of the specimens scored per pen as: Flock FPD score (%) = 100 × [(0.5 × total number of feet with score 1) + (2 × total number of feet with score 2)]/total number of scored feet.

Statistical Analysis

Data are presented as means of eight replicates (growth) or individual birds (all other parameters) and were analyzed using two-way analysis of variance (ANOVA) using a bell curve (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences were considered significant at $P < 0.05$.

Table 1. **Diet compositions**

Ingredient	*Starter* (0-10 d)	*Grower* (11-24 d)	*Finisher* (25-42 d)
Corn	54.62	57.17	59.62
Dehulled soybean meal	37.07	33.93	30.55
Soybean oil	3.33	4.45	5.58
Monodicalcium phosphate	1.940	1.726	1.601
Limestone	1.198	1.095	1.029
Sodium bicarbonate	0.231	0.201	0.204
Salt	0.217	0.240	0.240
Choline chloride	0.111	0.103	0.111
DL-methionine	0.328	0.277	0.269
L-lysine HCl	0.226	0.162	0.157
L-threonine	0.127	0.081	0.069
L-isoleucine	0.044	0.014	0.019
L-valine	0.000	0.000	0.000
Vitamin/mineral premix*	0.200	0.200	0.200
Pellet binder	0.300	0.300	0.300
Coccidiostat	0.050	0.050	0.050
Calculated values			
Metabolizable energy (MJ/kg)	12.56	12.98	13.40
Crude protein (%)	23.0	21.5	20.0

*Per kg: vitamin A, 12,000 IU; vitamin D3, 2,400 IU; vitamin E, 20 mg; vitamin K, 2.45 mg; vitamin B1, 1.9 mg; vitamin B2, 4.99 mg; vitamin B6, 1.94 mg; vitamin B12, 0.02 mg; niacin, 49 mg; calcium pantothenate, 14.78 mg; biotin, 0.05 mg; folic acid, 0.98 mg; copper, 9 mg; ferrous, 38.75 mg; manganese 60 mg; zinc, 45 mg; iodine, 0.75 mg; selenium, 1 mg; antioxidant, 2.5 mg.

Results

Growth and yields

There were no significant effects of SD or Tre, individually or combined, on BWG, FI, and FCR during the starter and grower periods ($P > 0.05$) (Table 2). During Tre supplementation (starter/grower), high SD increased FCR but not significantly ($P = 0.083$). During the finisher period, high SD decreased BWG ($P < 0.001$) and FI ($P < 0.05$), and increased FCR ($P < 0.001$). Tre supplementation reduced FCR ($P < 0.05$), whereas no combined effect was observed on FCR, indicating that the reduction was not specific to high SD. Over the entire experiment, BWG and FCR were negatively affected by high SD ($P < 0.001$); moreover, FI tended to decrease ($P = 0.079$). Tre reduced FCR ($P < 0.05$); however, this effect was not specific to high SD.

The effects of Tre supplementation on percentages of dressing, breast meat, thigh, drumstick, wing, and fat per carcass are shown in Table 3. High SD increased only the wing, whereas no significant effects of SD and Tre were observed on any other parameters.

Intestinal morphology, ileal bacteria, and FPD lesion score

Table 4 shows jejunal morphologies, ileal bacterial abundances, and FPD lesion scores. No significant effects of SD or Tre, individually or combined, were observed on jejunal VH, CD, or VH/CD ratio ($P > 0.05$). The abundance of ileal *C. perfringens* ($P > 0.05$) was not affected by high SD but was decreased by Tre ($P < 0.05$). In contrast, neither high SD nor Tre affected the abundance of *Lactobacillus* spp. FPD lesions were increased by high SD but were not affected by Tre.

Discussion

Stocking broiler chickens at high density is a strategy to maximize production performance per cycle[15]. The density is defined differently in different countries and regions; the permissible SD for broiler chickens in most cases is 33 kg total live weight per square meter, provided proper management[29]. Chickens reared under high SD often experience negative effects, including growth retardation, decreased feed intake and efficiency, increased mortality, and poorer general health. Nutritional strategies to minimize high SD-induced stress have been widely attempted, including pre-, pro-, syn-, and phyto-biotics; vitamins; and amino acids[15]. However, this study is the first to investigate using Tre supplementation as a prebiotic on broiler chickens reared at high SD. We found that high SD negatively affected BWG and FCR during the grower period. Tre supplementation at the starter and grower periods reduced FCR during the finisher period; however, this effect was not specific for high SD. This reduction was not observed during the starter or grower periods, suggesting that the efficacy of Tre may not be mediated pharmacologically. In terms of cost performance, Tre

Table 2. **Effects of Tre supplementation on growth at normal and high SD**

Period	Normal SD		High SD		Pooled	Two-way ANOVA		
Parameter	Control	Tre	Control	Tre	SEM	SD	Diet	SD x Diet
Starter (0-10 d)								
BWG, g	219	220	220	222	2.1	0.577	0.550	0.667
FI, g	222	221	223	224	2.7	0.522	0.887	0.710
FCR	1.01	1.01	1.01	1.01	0.01	0.696	0.524	0.985
Grower (11-24 d)								
BWG, g	1,111	1,122	1,122	1,118	10.6	0.747	0.754	0.500
FI, g	1,312	1,323	1,332	1,328	13.0	0.336	0.818	0.583
FCR	1.18	1.18	1.19	1.19	0.005	0.119	0.881	0.831
Starter/Grower (0-24 d, Tre feeding periods)								
BWG, g	1,331	1,342	1,342	1,340	11.5	0.689	0.690	0.589
FI, g	1,534	1,544	1,555	1,552	14.0	0.312	0.810	0.661
FCR	1.15	1.15	1.16	1.16	0.004	0.083	0.703	0.859
Finisher (25-42 d)								
BWG, g	1,877	1,939	1,765	1,798	29	< 0.001	0.113	0.623
FI, g	3,259	3,321	3,166	3,186	51	0.033	0.424	0.680
FCR	1.74	1.71	1.80	1.77	0.01	< 0.001	0.024	0.998
Total period (0-42 d)								
BWG, g	3,207	3,281	3,106	3,138	33	0.001	0.123	0.533
FI, g	4,793	4,865	4,721	4,738	55	0.079	0.422	0.621
FCR	1.49	1.48	1.52	1.51	0.005	< 0.001	0.034	0.904

Data are presented as means of eight replicates, analyzed using two-way analysis of variance (ANOVA). *Abbreviations*: SD, stocking density; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

Table 3. **Carcass yields at 42 d**

	Normal SD		High SD		Pooled	Two-way ANOVA		
Parameter	Control	Tre	Control	Tre	SEM	SD	Diet	SD x Diet
Dressing, %	77.3	77.1	77.9	77.4	0.30	0.195	0.292	0.665
Breast, %	32.4	32.5	32.4	32.4	0.45	0.882	0.955	0.977
Thigh, %	19.7	19.6	19.6	19.6	0.18	0.764	0.842	0.744
Drumstick, %	13.6	13.7	13.7	13.6	0.14	0.974	0.754	0.642
Wing, %	9.9	10.1	10.3	10.3	0.13	0.049	0.446	0.400
Fat, %	1.4	1.3	1.3	1.4	0.07	0.677	0.594	0.281

Data are presented as means of eight replicates, with values per replicate from three individual birds. Data were analyzed using two-way ANOVA.

supplementation was limited to only the starter/grower diets in this study. Therefore, we conclude that Tre supplementation for a limited period should be considered to improve growth. While we did not specifically investigate the mechanisms underlying the Tre-induced improvement of FCR, a previous investigation has shown that Tre suppresses gene expression associated with intestinal inflammation in chicks[24]. Nutrients and energy are utilized to repair inflammatory damage, thereby increasing FCR. One could hypothesize that the Tre supplementation-induced inflammatory modulation participates in the improvement of FCR; however, this would not explain the slow-acting effects of Tre on it. Thus, another mechanism might improve FCR in broiler chickens.

Our previous findings suggested that Tre improves the intestinal morphology of broiler chickens during the grower phase and that these changes could contribute to subsequent increased growth[25]. This conjecture was originally conceived from two individual animal trials; the first showed improved intestinal morphology but did not show increased growth, whereas the second did not assess morphology but showed increased growth at a later phase. Thus, this idea has yet to be substantiated. Therefore, we examined intestinal morphology during the grower phase. We hypothesized that high SD negatively affected intestinal morphology, which was then alleviated by Tre supplementation. However, we found that high SD did not affect jejunal morphology during the grower phase and that Tre supplementation did

Table 4. **Intestinal morphology, ileal bacterial abundance, and footpad dermatitis**

	Normal SD		High SD		Pooled	Two-way ANOVA		
Parameter	Control	Tre	Control	Tre	SEM	SD	Diet	SD x Diet
Intestinal morphology at 24 d								
VH, μm	1,058	1,144	1,025	1,105	54.8	0.518	0.138	0.956
CD, μm	103	103	97	97	3.8	0.109	0.952	0.987
VH/CD ratio	10.3	11.1	10.5	11.4	0.5	0.553	0.074	0.938
Ileal bacterial number at 24 d (Log CFU/g)								
C. perfringens	2.35	2.20	2.58	1.57	0.28	0.478	0.049	0.136
Lactobacillus spp.	7.48	7.94	7.86	8.09	0.25	0.291	0.174	0.644
Footpad dermatitis at 42 d								
Lesion score	82	63	140	139	18	<0.001	0.570	0.609

Data are presented as means of eight individual samples with one bird selected per pen for intestinal morphology and ileal bacterial abundance. FPD lesion scores were calculated from percentages of the specimens scored per pen in each group. Data were analyzed using two-way ANOVA. *Abbreviations*: SD, stocking density; VH, villus height; CD, crypt depth.

not affect the morphology. Information on intestinal morphologic changes due to high SD at earlier ages is limited. One study showed that high SD (16 birds/m^2 vs. 10 birds/m^2 control) induced morphological changes in the duodenum; however, changes were not observed in the jejunum at 21 d[30]. A study of Arbor Acres broiler breeders showed that high SD (22 birds/m^2 vs. 14 birds/m^2 control) lowered jejunal VH and VH/CD ratios at 21 d[31]. Meanwhile, the effects of high SD on intestinal morphology were inconsistent, even during later phases[9,32]. Therefore, the negative effects of high SD on intestinal morphology may depend on strain, SD, rearing system, or other factors. We did not clarify the reason for the small effect Tre supplementation had on intestinal morphology during the grower phase. It can be hypothesized that the positive effects of Tre on FCR during the finisher phase are independent of the intestinal morphological changes.

FPD is common in chickens reared at high SD[33]. We found that high SD increased FPD scores, on which Tre supplementation had little effect. It has been reported that FPD may not occur due to high SD itself; instead it could be caused by its secondary effects, including a deterioration of litter quality characterized by high moisture and ammonia concentration[34,35]. From these findings, it appears that Tre supplementation does not improve litter quality.

Overgrowth of *C. perfringens*, specifically types A and C, which generate necrotic enteritis B-like (NetB) toxins, causes necrotic enteritis (NE) in younger broiler and breeder chickens[36]. The clinical sign of this disease is high mortality, whereas subclinical cases decrease weight gain and increase FCR, leading to significant economic losses. *C. perfringens* is common in the chicken digestive tract, as well as in soil, dust, feed, and litter. Several predisposing factors that alter the intestinal flora participate in the occurrence of this enterotoxemia. High SD increases the abundance of *C. perfringens* in the cecum and contributes to the development of intestinal disease, possibly through factors such as wet litter[37,38]. However, we found that high SD did not affect the ileal abundance of *C. perfringens*, which might be attributable to salinomycin in the diets, a primary component of coccidiostat that inhibits its multiplication[39]. In this study, Tre supplementation reduced the ileal abundance of *C. perfringens* despite these antibiotic conditions. Furthermore, Tre supplementation did not affect the ileal abundance of *Lactobacillus* spp., which appears to be inconsistent with the findings of a previous study of *Salmonella typhimurium*-infected broiler chickens[26]. *Lactobacillus* has been reported to reduce the cecal colonization of *C. perfringens*[40]. Several species, such as *Lactobacillus acidophilus*, *L. fermentum*, and *L. casei*, prevent *C. perfringens* cecal colonization and inhibit the growth and production of the α-toxin, thereby mitigating the intestinal lesions it causes[41–44]. Therefore, the specific species belonging to the *Lactobacillus* genus might be associated with reduced abundance of pathogenic bacteria. Other species belonging to *Enterococcus* and *Butyricicoccus* might also be associated with the elimination of *C. perfringens*[39]. Moreover, Tre has been reported to stimulate the growth of bacteriocin-producing lactic acid bacteria, as well as enhance their bacteriocin production, which acts against pathogenic bacteria[23]. Therefore, it is possible that enhanced bacteriocin production caused by Tre might be associated with the reduced abundance of *C. perfringens*, serving as another mechanism of suppression. However, further investigation is necessary to clarify the mechanism(s) of action of Tre in the intestinal microbiota.

In conclusion, this study demonstrated that Tre supplementation during the starter and grower periods improved FCR during the finisher period, possibly by reducing the numbers of *C. perfringens* rather than changing the intestinal morphology of the broiler chickens. Further investigations are required to obtain mechanistic insights into the action of Tre and its influence on the growth of broiler chickens.

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Author Contributions

Conceptualization, KM, MK; methodology, TS, CR, SS, KM, MK; performing experiments, TS, CR, SS; data analysis, TS, CR, SS, MK; manuscript preparation/writing, TS, CR, SS, KM, MK; review and editing, KM, MK; supervision, KM, MK. All authors have read and agreed to the published version of the manuscript.

Declaration of Interest

The authors have declared that no competing interests exist.

Conflicts of interest

The authors declare that there are no conflict of interests.

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