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Effect of Stocking Density on Selected Blood Physiological, Biochemical and Enzymatical Variables of Broilers Grown to 3 kg under Antibiotic-free Conditions[1,2]

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ABSTRACT

Background: Reducing stocking densities may play a key role in minimizing difficulties such as physiological welfare when reducing or ending antimicrobials in poultry diets. As the demand for poultry and associated products increases, one of the major concerns centers on the question of whether stocking density influences welfare responses that are characteristic of physiological welfare. This study investigated the effect of stocking density on selected blood physiological variables along with plasma biochemistry variables and enzymes activities of broilers fed antibiotic-free diets grown to 3 kg.

Methods: A total of 888 1-d-old Ross × Ross 708 chicks were randomly distributed into 24 pens based on stocking density treatments assignment. The treatments consisted of 4 densities (29, 33, 39 and 42 kg/m^2) with six replicates. Treatments were blocked within the room to account for any variations in room conditions. Treatment assignments were randomized within each block. Used litter bedding was obtained from commercial farms to mimic commercial conditions and litter microflora. Birds were provided a three phase-feeding program (Starter: 0-14 d, Grower: 15-28 d and Finisher: 29-42 d) antibiotic-free diets. Feed and water provided *ad libitum*. Blood samples were collected from the brachial wing vein of 3 males and 3 females' birds per pen on d 28 and 42 and analyzed immediately for blood physiological variables. Blood plasma samples were analyzed for T_3, T_4, corticosterone, biochemical variables and enzyme activities.

Result: The only effect of stocking density was observed on partial pressure of CO_2 (pCO_2) and uric acid which were within physiological ranges for this species. However, blood glucose and plasma corticosterone concentrations were not affected by stocking density, suggesting no signs of physiological stress. Stocking densities up to 42 kg/m^2 with proper environmental management may be suitable for both poultry integrators and contract growers to enhance broilers production efficiency without compromising the welfare of broilers grown to 3 kg body weight.

Key words: Acid-base-balance, Antibiotic-free-diets, Broilers, Stocking-density, Welfare.

INTRODUCTION

Consumers assume stocking density (SD) to be one of the most crucial factors that influence animal welfare (Vanhonacker *et al*., 2009), but livestock producers on the other hand assume that reduction of stocking density for advanced animal welfare will have a negative impact on farm profitability. This is because as the number of birds per unit of housing space decreases, the cost of labor, housing, fuel and equipment per bird increases (Estevez, 2007). However, it has been documented that extremely high SD in broilers has been linked to growth retardation, increased proportion of downgraded poultry products, stress, bird's disruption, lameness and substantial risk of health-related problems (Sanotra *et al*., 2001; Feddes *et al*., 2002; Thaxton *et al*., 2006).

One of the negative implications of extremely high SD is high moisture content of the litter that enhances microbial activities, leading to increased temperature and ammonia in broiler houses (Bessei, 2006). In addition, extreme SD is a predisposing factor in the pathogenesis of intestinal diseases and the mechanisms underlying these effects on poultry body physiological homeostasis. One of the microbial activities is necrotic enteritis, which is defined as an intestinal infection that has a high economic effect

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on health and welfare of broilers, which pose a threat to public health (Van der Sluis, 2000; Van Immerseel, *et al*., 2004).

Despite the importance of SD as a management practice, no universal SD recommendations exist. The National Chicken Council in the United States bases SD allowances on bird weight, with a range from 32 to 44 kg/m^2. In Canada, it is required that broiler SD not exceed 31kg/m^2 unless certain conditions are met, in which case SD can be increased up to a maximum of 38 kg/m^2. In the European Union (EU), the largest SD is 33 kg/m^2, but this can be increased to 39 kg/m^2 or 42 kg/m^2 if the producer follows specific requirements.

The public health issue of utilizing antibiotics as growth promoters (AGPs) in poultry diets is the claim that AGPs could increase the antimicrobial resistance of bacteria making antibiotics ineffective against foodborne illnesses (Hedman *et al*., 2020). The Food and Drug Administration (FDA) banned the use of antimicrobials as growth promoters in early 2017 (Singer *et al*., 2019). The increasing movement away from AGPs together with increasing market weights and alterations of SD, adjustment of traditional methods of housing management and environmental control is necessary to keep or improve production efficiency. But raising poultry without the use of antibiotics is a new challenge to the producers making the birds prone to a number of bacterial diseases (Dibner and Richards, 2005; Diarra and Malouin, 2014). While the need for poultry and related products increases, escalating production and production efficiencies will be important to the continued feasibility of domestic poultry industries in the United State and around the world.

Blood sample evaluation along with other biochemical determinations are used to assess the health condition of animals, assessing internal organ functions and systemic homeostasis (Kral and Suchy, 2000; Olanrewaju *et al*., 2017). Moreover, variations of acid-base balance are the prompt clinical signs of various diseases in both domestic animals including chicken and humans (Gunnerson, 2005; Seifter and Chang, 2016). In addition, alterations in key selected blood parameters are widely used to figure out various effects of environmental, nutritional and pathological factors (Olanrewaju *et al*., 2017). Concentrations of glucose, T_3, T_4 and corticosterone among others have been suggested to be sensitive indicators of physiological responses in stressed broiler chickens (Puvadolpirod and Thaxton, 2000; Olanrewaju *et al*., 2006). In additions, elevated levels of GGT have been documented as a marker of oxidative stress and subclinical inflammation (Kasapoglu *et al*., 2010). The activities of ALT, AST, LDH and ALP in the broilers are indicators of protein metabolism and hepatic damage or injury (Ognik and Krauze, 2016). It has been documented that green muscle disease, cardiac damage and stress are associated with substantial increase in plasma CK and AST activities (Kong *et al*., 2021; Estarreja *et al*., 2024). Hence, in extension of our recent study (Olanrewaju *et al*., 2024a), this study running concurrently with the same group of birds, investigated the effect of stocking density on selected blood physiological variables along with plasma biochemistry variables and enzymes activities of broilers fed antibiotic-free diets grown to 3 kg. The outcome of this study will provide information crucial to prevent costly restrictions related to the number of birds per unit of housing space (m^2) in poultry industries, while helping them to deliver the best and humane production environments that enhance broilers production efficiency without compromising the welfare of broilers grown to 3 kg body weight.

Materials and Methods

Birds, experimental design and management

This study was carried out at US Department of Agriculture, Agricultural Research Service, Poultry Research Unit in 2024. All procedures relating to the use of live birds in this study were reviewed and approved by the USDA-ARS Animal Care and Use Committee at the Mississippi State location (license number: 19-3). In this study, a total of 888 1-d-old Ross × Ross 708 chicks were randomly distributed into 24 pens based on stocking density treatment assignment. Stocking density treatments of 29, 33, 39 and 42 kg of BW/m^2 were selected based on EU (2007), National Chicken Council (2017) and Global Animal Partnership (2017) recommendations. The birds were reared under identical conditions including diets except for SD. Pens were adjusted to a total area of 34 ft^2 for this study. Commercial stocking densities of 1 ft^2/bird (Dozier *et al*., 2005; Thaxton *et al*., 2006) were typically employed to maintain commercially relevant feeder and drinker space allotments to stimulate typical feeding and drinking behavior observed in commercial practice. Each pen was equipped with one tube feeder and nipple drinkers. Pens air temperature was initially set at 32°C and was decreased according to the schedule in Table 1. Lighting was provided with 5000K LED bulbs. Lighting intensity, photoperiod and room temperature were adjusted according to the schedule in Table 1. Birds were fed antibiotic-free (ABF) diets and offered a three phase-feeding program (Starter: 0-14 d, Grower: 15-28 d and Finisher: 29-42 d) diets on a biweekly change schedule with corn-soy diets according to NRC (1994). Starter feed was provided as crumbles and subsequent feeds were offered as whole pellets. Feed and water were provided *ad libitum*. Litter material was sourced from a commercial farm to mimic commercial conditions and litter microflora.

Experimental treatments

The experimental treatments were 4 densities (29, 33, 39 and 42 kg of BW/m^2), which consisted of 30, 34, 40 and 44 chicks per pen, respectively at placement with six replicates. Treatments were blocked within the room to account for any variations in room conditions. Treatment assignments were randomized within each block.

Blood collections and biochemical analyses

Blood samples (3 ml) were collected between 0800 and 0900 h from the brachial vein of 6 (3 males and 3 female) randomly selected birds/pen on d 28 and 42 of age within 45 sec after birds were caught. Blood samples were analyzed instantaneously using blood gas electrolyte analyzer (ABL90-Flex, Radiometer America, Westlake, OH) as described previously (Olanrewaju *et al*., 2016). This ABL-90 Flex blood analyzer was set to reflect a broiler body temperature of 41.5°C as per the manufacturer's instructions. The mean corpuscular hemoglobin concentration (McHc) in grams per deciliter and arterial oxygen saturation (SaO_2), which is the amount of oxyhemoglobin (O_2Hb) in blood expressed as a percent of the total amount of hemoglobin able to bind oxygen (O_2Hb+deoxyhemoglobin) were determined as described previously (Olanrewaju *et al*., 2016). The needle mounted on each syringe was then removed, a cap was placed over the needle port. The syringes containing the blood samples were placed into ice. The iced samples were transferred to the laboratory and centrifuged at 4,000×g for 20 min at 4°C. Two milliliters of each of the plasma samples from the syringes were stored in 2.5 mL graduated tubes at -20°C for subsequent laboratory chemical analyses. On the day of analyses, plasma samples were brought out from the freezer, thawed and analyzed for corticosterone (CS) using a universal microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) with ELISA reagent assay test kits (EIA-CS Kit, Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's directions. Concentrations of plasma triiodothyronine (T_3) and thyroxine (T_4) concentrations were determined using a universal microplate spectrophotometer (Bio-Tek Instruments Inc.) with ELISA reagent assay test kits from ALPCO Diagnostics (Salem, NH) according to the manufacturer's recommendations. Moreover, plasma concentrations and activities of albumin (ALB), total bilirubin (TBILI), blood urea nitrogen (BUN), creatinine (CREAT), total protein (TP), uric acid (UA), cholesterol (CHOL), low density lipoprtein (LDL-C), high density lipoprotein (HDL), triglycerides (TRIG), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMYL), aspartate aminotransferase (AST), creatine kinase (CK), Gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and lipase (LIP) were evaluated using ACE-AXCEL (Alfa Wassermann Diagnostic Tech, West Caldwell, NJ) through biochemical and enzymatic rate reactions as described previously (Olanrewaju *et al*., 2023b). Most of these blood variables are indicators of internal organ health and systemic homeostasis.

Statistical analysis

The experimental design was a randomized complete block design with room location as the blocking factor. Each 4-stocking density treatment was signified by 6 replicate pens. Pen was considered the experimental unit. Treatments were blocked within the room to account for any variations in room conditions. Treatment assignments were randomized within each block. The main effects of stocking density on physiological parameters along with plasma biochemistry variables and enzymes activities were evaluated by using the mixed model ANOVA with PROC MIXED procedure of SAS software, SAS Institute Inc., Cary, USA, (2013). Least-squared means comparisons on day 28 and 42 were separated with Tukey's Honestly Significant Different Test; significance was considered at $P \leq 0.05$ unless otherwise said. Analyses of variance combined across days were performed to obtain treatment comparisons averaged across days.

RESULTS AND DISCUSSION

Stocking densities in this study were 29, 33, 39 and 42 kg of BW/m^2 with 30, 34, 40 and 44 birds/pen, respectively from d1 to 42 d of age and with six replicates. The earlier study investigated the effect of stocking density on growth performance and carcass characteristics of broilers grown to 3 kg under antibiotic free diets (Olanrewaju *et al*., 2024a). The present study, running concurrently with the same group of birds, investigated the effect of stocking density on selected blood physiological parameters along with plasma biochemistry variables and enzymes activities of broilers fed antibiotic-free diets grown to 3 kg. The data represent the main effects of treatments (stocking densities) over day because there were no effect of treatments and sex for each of the sampling days.

Table 2 shows the main effects of SD on blood gases and acid-base balance. There was only a significant ($P<0.013$) effect of stocking density of 42 kg/m^2 noted on partial pressure of CO_2 (pCO_2), which was within physiological acid-base ranges for broiler chickens (Martin *et al*., 2010). These results agree with the earlier studies (Park *et al*., 2018; Ruby *et al*., 2022; Olanrewaju *et al*., 2023a) who said that SD in broilers had no major effect on hematology, blood gases and acid-base balance. Acid-base status is faced daily by environmental factors such as light, temperature, humidity and air quality among others and they influence respiratory and metabolic activities (Parvin *et al*., 2014; Arowolo *et al*., 2019; Olanrewaju *et al*., 2019).

Table 1: Air temperature and lighting program.

Age	Temperature (C)	Light intensity (lux)	Light program
Placement	32	30	23L:1D
4	31	10	23L:1D
8	29	10	20L:4D
14	27	10	20L:4D
21	24	10	20L:4D
28	21	5	18L:6D
35	18	5	18L:6D
41	18	5	23L:1D

The regular role of all physiological processes in the body depends on maintenance of right acid-base balance where the body controls and allocation of acids and bases to accomplish acid-base homeostasis (Bianca *et al*., 2021). The primary organ systems used in acid-base homeostasis in birds are the lungs and kidneys, supported by the gastrointestinal tract and cardiovascular system that take part in thermoregulatory processes through the adjustment of heat dissipation and oxygen transport (Burggren *et al*., 2016). The 3 systems (buffer, respiratory and renal systems) function interdependently to regulate and keep acid-base balance, while the function of the liver, kidneys and the brain are among the most important elements in body physiological homeostasis.

Table 3 shows the effects of SD on blood electrolytes and anion gap levels. There was no effect of SD on any of the blood electrolytes and anion gap levels, which is consistent with our earlier study (Olanrewaju *et al*., 2023a). Moreover, the influence of SD on selected blood metabolites is shown in Table 4. There was no effect of SD noted on selected blood metabolites which also agrees with our previous study (Olanrewaju *et al*., 2023a).

The influence of SD of boilers fed ABF diets on plasma biochemistry variables and enzymes activities are presented in Table 5. The effect of SD was observed only on uric acid levels, but not on the rest selected plasma enzymes activities in the present study. The present results of the biochemical and enzymatical variables in Table 5 are without statistically significant differences between treatments. This shows from the physiological aspect there was no difference in broiler welfare and internal organs health in the investigated stocking densities. The result also is consistent with other studies who found that SD did not cause physiological changes indicative of stress (Dozier *et al*., 2006; Thaxton *et al*., 2006). In addition, non-significant effects of SD on hemato-biochemical was reported by Gupta *et al*. (2017). Uric acid (UA) has been shown to stimulate the immune responses of animals against the invasion of pathogenic microorganisms (Nery *et al*., 2015). It has also been documented that serum UA

Table 2: Main effects of stocking density of broilers grown to 3 kg body weights on BW, pH, blood gases, sO_2, SaO_2, hematocrit, hemoglobin and McHc along with Mean ± SE.

Treatments	Stocking density (kg/m²)[1]				SEM[3]	P-value
variables[2]	29	33	39	42		
BW (kg)	3.016	3.128	2.929	3.006	0.059	0.160
pH	7.33	7.35	7.36	7.34	0.008	0.199
pCO_2 (mmHg)	45.68^b	45.95^b	45.87^b	47.98^a	2.507	0.013
pO_2 (mmHg)	44.14	44.80	44.43	45.00	0.668	0.803
HCO_3^- (mmHg)	27.76	27.69	27.62	27.69	0.309	0.991
sO_2 %	32.26	34.16	34.40	33.02	1.013	0.446
SaO_2 %	71.61	80.42	80.85	80.02	0.074	0.394
Hb (g/dL)	8.09	7.95	7.88	8.02	0.074	0.923
Hct (%)	24.80	24.35	24.61	24.56	0.234	0.241
McHc (%)	32.71	33.11	32.61	32.42	0.260	0.317

$^{a-b}$Means within a row and effect that lack common superscripts differ significantly (P≤0.05).

[1]29 kg/m²= 30 birds/pen; 33 kg/m² = 34 birds/pen; 39 kg/m² = 40 birds/pen; 42 kg/m² = 44 birds/pen.

[2]BW= Body Weights; pCO_2= Partial pressure of CO_2; pO_2= Partial pressure of O_2; HCO_3= Bicarbonate; SO_2= Saturated O_2; SaO_2= Arterial Saturated O_2; Hb= hemoglobin; Hct= hematocrit; McHc= Mean corpuscular hemoglobin concentration.

[3]Pooled SEM for main effects (n=6).

Table 3: Main effects of stocking density of broilers grown to 3 kg body weights on blood electrolytes and Angap along with Mean ± SE.

Treatments	Stocking density (kg/m²)[1]				SEM[3]	P-value
variables[2]	29	33	39	42		
Ca^{2+} (mEq/L)	2.76	2.74	2.71	2.7	0.040	0.680
Na^+ (mEq/L)	147.90	147.65	147.57	148.04	0.290	0.636
K^+ (mEq/L)	5.31	5.21	5.03	5.18	0.077	0.093
Cl^- (mEq/L)	109.62	109.45	109.56	109.65	0.291	0.960
Anion gap (mEq/L)	20.83	20.65	20.41	20.79	0.287	0.732

$^{a-b}$Means within a row and effect that lack common superscripts differ significantly (P≤0.05).

[1]29 kg/m²= 30 birds/pen; 33 kg/m² = 34 birds/pen; 39 kg/m² = 40 birds/pen; 42 kg/m² = 44 birds/pen.

[2]Ca^{2+}= Calcium; Na^+= Sodium; K^+= Potassium; Cl^-= Chloride; Angap= Anion gap.

[3]Pooled SEM for main effects (n =6).

concentrations can be an indicator of amino acid use in broilers fed amino acid-suficient and amino acid-deficient diets (Donsbough *et al*., 2010). Blood metabolites indices of liver, kidney functions and hematological contents may provide good instances of animal health status and physiological condition (Toghyani *et al*., 2010). Alanine aminotransferase (ALT) and GGT are liver enzymes which are higher at the time of liver damage (hepato-cellular degeneration). Increasing blood glucose levels are termed as an important indicator of stress conditions (Simon, 1984; Gunnerson, 2005). It has been reported that the triglyceride results did not differ significantly in the blood serum of broilers reared at the level density of 11.9 and 17.5 birds/m^2, which indicated birds lack of exposure to stress or metabolic disorder (Sturkie, 1986). The nonsignificant difference of HDL and LDL levels observed in this study

Table 4: Main effects of stocking density of broilers grown to 3 kg body weights on blood GLU, Osmo, CS, T_3 and T_4 along with Mean ± SE.

Treatments variabes[2]	Stocking density (kg/m^2)[1]				SEM[3]	P-value
	29 kg/m^2	33 kg/m^2	39 kg/m^2	42 kg/m^2		
GLU (mg/dL)	243.92	242.83	244.36	244.9	0.593	0.775
Osmo (mOs/kg)	309.36	308.81	308.56	309.57	0.593	602
Lac (mg/dL)	61.24	57.71	55.58	59.71	2.167	0.288
Sbc (mmol/L)	25.4	25.61	25.71	25.45	0.336	0.911
CS (pg/mL)	2507.6	2302.1	2542.9	3299.9	641.27	0.427
T_3 (ng/ml)	3.747	3.750	3.604	3.617	0.136	0.560
T_4 (mg/dl)	1.775	1.825	1.779	1.792	0.071	0.888

[a-b]Means within a row and effect that lack common superscripts differ significantly (P≤0.05).

[1]29 kg/m^2= 30 birds/pen; 33 kg/m^2= 34 birds/pen; 39 kg/m^2= 40 birds/pen; 42 kg/m^2= 44 birds/pen.

[2]GLU= Glucose; Osmo= Osmolality; Lac= Standard Lactate; Sbc= Standard Bicarbonate; CS= Corticosterone; T_3= Triiodothyronine; T_4= Thyroxine.

[3]Pooled SEM for main effects (n=6).

Table 5: Main effects of stocking density of broilers grown to 3 kg body weights on plasma biochemical and enzymatical variables along with Mean ± SE.

Treatments variables	Stocking density (kg/m^2)[1]				SEM[2]	P-value
	29	33	39	42		
Chemistry assays						
Albumin (ALB), g/dL	1.042	1.032	1.061	1.062	0.024	0.753
Bilirubin, OR total (TBILI), mg/dL	0.575	0.591	0.529	0.525	0.047	0.372
Blood urea nitrogen (BUN), mg/dL	0.922	0.878	0.881	0.868	0.058	0.375
Creatinine (CREAT), mg/dL	0.257	0.261	0.255	0.261	0.008	0.678
Total protein (TP), g/dL	2.341	2.249	2.319	2.368	0.067	0.375
Uric acid (UA), mg/dL	6.722[a]	6.564[a]	6.654[a]	6.239[b]	0.182	0.038
Cholesterol (CHOL), mg/dL	105.76	103.98	107.23	107.28	2.679	0.686
Low density lipoprtein (LDL-C), mg/dL	11.095	10.871	11.497	11.802	0.497	0.376
High density lipoprotein (HDL), mg/dL	77.285	76.402	78.445	77.845	1.967	0.471
Triglycerides (TRIG), mg/dL	58.175	54.207	54.802	53.743	2.805	0.348
Enzyme assays						
Alanine aminotransferase (ALT), U/L	0.039	0.031	0.0154	0.012	0.022	0.482
Alkaline phosphatase (ALP), U/L	6248.3	5755.9	6178.5	5238.8	492.55	0.658
Amylase (AMYL), U/L	675.14	595.47	640.90	602.88	34.541	0.597
Aspartate aminotransferase (AST), U/L	142.64	138.78	131.23	139.89	4.243	0.476
Creatine kinase (CK), U/L	3495.6	3401.2	3367.2	3455.9	429.45	0.486
Gamma-glutamyl transferase (GGT), U/L	15.204	15.477	15.258	15.083	0.403	0.465
Lactate dehydrogenase (LDH), U/L	375.44	377.34	365.65	387.21	20.123	0.765
Lipase (LIP), U/L	25.612	20.517	26.405	24.285	3.978	0.578

[a-b]Means with in a row not sharing a common superscript are significantly different (P≤0.05).

[1]29 kg/m^2= 30 birds/pen; 33 kg/m^2= 34 birds/pen; 39 kg/m^2= 40 birds/pen; 42 kg/m^2= 44 birds/pen.

[2]Pooled SEM for main effects (n = 6).

supported the insignificant results that obtained in the cholesterol values in this study. The results of this study showed that the values of lipid biochemical parameters evaluated did not differ within broilers of the same age at different densities. These values reported here were consistent with results reported by Zabir *et al*. (2021) who found no effect of SD on serum cholesterol under different bird densities.

Table 2-5 data display the nonsignificant effect of SD on final BW, selected blood physiological, plasma biochemistry and enzymes variables except pCO_2 and uric acid and were within physiological acid-base ranges for broiler chickens (Martin *et al*., 2010; Al-Nedawi, 2018; Arzour-Lakehal and Boudebza, 2021). The values found for each selected variables (blood physiological variables, plasma biochemistry and enzyme activities) are in broad agreement with those reported in the literature by others (Shailesh *et al*., 2017; Park *et al*., 2018; Thema *et al*., 2022) that would be extremely useful in detecting not only environmental welfare conditions of broilers fed ABF diets, but also metabolic-nutritional disorders of broilers.

CONCLUSION

Based on the results of the present study, blood glucose, plasma corticosterone along with other selected biochemistry and enzyme activities welfare indicators were within blood homeostasis reference of this species and were not affected by SD, showing no signs of physiological stress. The study also adds to the accumulating evidence that stocking densities with ABF diets used in this study do not trigger organs and muscle damage, nor do they cause an increase in stress indicators in broiler chickens. This study revealed that SD up to 42 kg/m^2 with proper environmental management of broilers fed ABF diets may be suitable for both poultry integrators and contract growers to enhance broilers production efficiency without compromising the welfare of broilers grown to 3 kg BW. In addition, this study will help researchers and the commercial poultry industry uncover critical broiler management information that will lead to profitable broiler production.

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Conflict of interest

The authors of the manuscript declare no conflicts of interest exist.

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