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A mixture of Chinese herbs and a commercial probiotic *Bacillus* species improves hemato-immunological, stress, and antioxidant parameters, and expression of HSP70 and HIF-1α mRNA to hypoxia, cold, and heat stress in Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

A 56-day feeding trial was performed to evaluate the effect of herbal-probiotic supplements comprising a mixture of Chinese herbs (CH) (i.e., *Astragalus membranaceus, Angelica sinensis,* and *Crataegus hupehensis*) and or commercially prepared probiotic *Bacillus* species (BS) (i.e., spores of *B. subtilis* and *B.licheniformis*) on the physiological response of Nile tilapia. Fish (50 ± 2 g) were fed six diets in duplicates including a control (CT) enriched with BS [at 10 g kg^{-1}], CH [at 10 g kg^{-1}], CHBS1 [CH at 3 g kg^{-1} and BS at 7 g kg^{-1}], CHBS2 [CH at 5 g kg^{-1} and BS at 5 g kg^{-1}], and CHBS3 [CH at 7 g kg^{-1} and BS at 3 g kg^{-1}] for a period of 56 weeks. At the end of the feeding trial, some hemato-immunological parameters and antioxidant indices, as well as stress-related genes in tilapia, were evaluated; and afterwards exposed to hypoxia for 18 h and to cold and heat each for 2 h. The results showed increased hematocrit, red blood cells, haemoglobin, and white blood cells, in fish blood in most of the herbal-probiotic groups compared to the control group ($P < 0.05$). Lowered cortisol, glucose, as well pyruvate kinase, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, and increased myeloperoxidase, total antioxidant capacity, superoxide dismutase, and catalase activities were observed in the serum of all herbal-probiotic groups over the control group ($P < 0.05$). Besides, all herbal-probiotic groups showed increased expression of heat shock protein 70 and hypoxia-inducible factor 1 alpha mRNAs in the livers of fish ($P < 0.05$). Among the herbal-probiotic groups, tilapia fed with CHBS2 diet showed enhanced values in most of the indices measured under the different stress conditions ($P < 0.05$). These findings suggest CHBS2 supplement as a prophylactic against the suppressive effects of stress in tilapia.

1. Introduction

High rearing densities in aquaculture practices have given rise to a variety of environmental perturbations which include changes in dissolved oxygen (DO) levels or water temperature (Emikpe, 2016; Fawzia et al., 2016). These perturbations of DO and temperature among others have been reported as major stressors that affect physiological function, growth, and survival of cultured fish (Dawood et al., 2017; Elabd et al., 2017).

Evidence indicates that environmental stressors affect many biomarkers in fish. For example, blood parameters such as hematocrit (HCT), red blood cells (RBCs), and haemoglobin (HGB) levels which are essential in oxygen uptake in organisms could be altered by stressful conditions (Bao et al., 2018). Additionally, white blood cells (WBCs) and myeloperoxidase (MPO) which are indicators of an immune response are affected by physiological stress (Harikrishnan et al., 2011b; Awad et al., 2015). Likewise, cortisol (CS), glucose (GC), and pyruvate kinase (PK) in serum/plasma as indicators of energy regulation (Lai et al., 2006;

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Welker et al., 2007; Guardiola et al., 2015) are affected during stressful conditions. These have been implicated to cause elevated activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) in blood/serum to reflect tissue damage (Guardiola et al., 2015; Li et al., 2015; Bao et al., 2018). Additionally, stressful conditions have been reported to suppress the antioxidative response including total antioxidant capacity (T-AOC)(Duan et al., 2017), superoxide dismutase (SOD), and catalase (CAT) (Cheng et al., 2017).

In addition, increase in expression of stress-related genes, for example, chaperone molecules like heat shock protein (HSP) 70 (HSP70) (Zhou et al., 2010) and hypoxia-inducible factor-1α (HIF-1α) (Nikinmaa and Rees, 2005) in most organisms are known to respond to changes in DO levels or water temperature. HSP70 and HIF-1α have been reported to play critical roles in the control of metabolism and cell survival in an attempt to maintain homeostasis during environmental perturbation including warm and cold stress in fish (Mladineo and Block, 2009).

Tilapia, *Oreochromis niloticus* is a commercially important freshwater aquaculture fish that readily adapts to many culture environments (Abarike et al., 2013). Tilapia require a normal DO level of 5–6 mg L^{-1} (Abdel-Tawwab et al., 2014) and water temperature of 28–30 ℃ (Xie et al., 2011). However, hypoxia stress associated with low DO levels has been reported to reduce feed intake, pose physiological stress, weaken the immune response and as well decrease resistance to diseases in tilapia (Abdel-Tawwab et al., 2015). Also, reduced growth, increased physiological stress, and mortality have been reported in tilapia exposed to cold temperature below 12 ℃ and high temperature above 35 ℃ (Sifa et al., 2002; Ndong et al., 2007; Xie et al., 2011; Qiang et al., 2013).

Herbal plants and probiotics are substances that can serve as a suitable alternative therapy to antibiotic therapy in aquaculture (Nayak, 2010; Hai, 2015; Dawood et al., 2018). It has been widely reported that Chinese herbs (CH) such as Angelica sinensis, Astragalus membranaceus, and *Crataegus hupehensis* have been used to prevent or treat various diseases in fish; this is because their incorporation in fish diets have resulted in improved activities of SOD, CAT, ALT, and AST among others in cultured fish (Elabd et al., 2016). Likewise, probiotic species (BS) such as *Bacillus subtilis* and *Bacillus licheniformis* have been reported to enhance growth performance, immune response, and resistance to infectious diseases in many cultured fish (EL-Haroun et al., 2006; Das et al., 2013; Elsabagh et al., 2018). However, there are few research reports on how a combination of herbs and probiotics supplements affect fish growth, immune and stress responses and disease resistance (Harikrishnan et al., 2011a, 2011b; Abarike et al., 2018a).

Notwithstanding the significant losses in aquaculture associated with stressful conditions (Elabd et al., 2017) to the authors' knowledge, the role of herbal-probiotic supplements in mitigating physical stressors such as hypoxia, cold, and heat stressors in tilapia are lacking. Research to identify natural feed additives that can enhance the stress resistance capacity of fish will be beneficial to the development of fish health management in aquaculture (Neissi et al., 2015; Jiang et al., 2017). Therefore, in this study, we determined the effects of diets supplemented with mixture of CH (i.e., a mix of *A. membranaceus, A. sinensis,* and *C. hupehensis*) and BS (i.e., a mix of *B. subtilis* and *B. licheniformis*) on some hemato-immunological parameters, stress and antioxidant response parameters, as well the expression of stress-related genes before and after exposure to hypoxia, cold, and heat stressors of tilapia.

2. Materials and methods

2.1. Experimental diet

CH (i.e. dried roots of *A.membranaceus, A. sinensis,* and *C. hupehensis*) were purchased from the open market in Zhanjiang, China, washed in double distilled water, dried in shade, powdered, and stored at −20 °C for later use. Commercially produced BS comprising of spores of a mix of *B. subtilis* and *B. licheniformis* were obtained from Lubiaduo company in China, and the viability checked before use (see Table 1). A commercial tilapia pelleted diet was purchased from Heng Xing Company, China and used in addition with the CH and BS in preparation of 6 diets to include a control (CT), BS, CH, CHBS1, CHBS2, and CHBS3 (i.e., CHBS means a mix of Chinese herbs and *Bacillus* species. See Table 1 for detailed composition). The inclusion levels of CH and or BS supplements, diet preparation, and storage were based on our previous study (Abarike et al., 2018a).

Table 1
Dietary codes used throughout the study.

Dietary codes	Diets
CT	A commercial pellet diet void of herbs and *Bacillus*
CH	A commercial diet supplemented with herbs (10 g/kg)
BS	A commercial diet supplemented with *Bacillus* (10 g/kg)
CHBS1	A commercial diet supplemented with herbs (3 g/kg) and *Bacillus* (7 g/kg)
CHBS2	A commercial diet supplemented with herbs (5 g/kg) and *Bacillus* (5 g/kg)
CHBS3	A commercial diet supplemented with herbs (7 g/kg) and *Bacillus* (3 g/kg)

Where: CT, Control, BS, *Bacillus* species, CH, Chinese herbs, CHBS1, Chinese herbs and *Bacillus* species CHBS2, Chinese herbs and *Bacillus* species 2, CHBS3, Chinese herbs and *Bacillus* species 3. BS comprises a mix of spores of *Bacillus subtilis* and *Bacillus licheniformis* (1:1 w/w) of viability 2×10^9cfu/g. CH consisted of a mixture of *Astragalus membranaceus Angelica sinensis,* and *Crataegus hupehensis* at a ratio of 1:1:1 w/w.

2.2. Experimental set-up and fish management

Three hundred and sixty (360) healthy *Oreochromis niloticus,* without any symptoms of infection (i.e. abdominal distension, hernia, and haemorrhage) of an average weight of 50 ± 2 g, were obtained from Langye fish farm in Gaozhou city, Guangdong province, China for use in our laboratory. Fish were distributed into 12 plastic tanks (i.e. capacity 400 L containing 300 L of static water, each containing 30 fish). Fish in the tanks were assigned in duplicates for each of the groups (i.e., CT, BS, CH, CHBS1, CHBS2, and CHBS3) and allowed to acclimatize for 1 week. During the time of acclimatization, all fish were fed with control diet twice daily at 2% of the body weight in 2 equal rations with each feeding at 9:00 a.m. and 4:00 p.m. Water quality in the tanks was maintained daily by renewing of 30 % of the water with a maintenance temperature of 28 ± 2.0 °C, pH of 6.8 ± 0.30, and DO of 6.05 ± 0.55 mg l^{-1}. After acclimatisation, fish in the tanks were fed with respective diets for an experimental period of 56 days. During the experimental period, feeding, and water quality were maintained as before (i.e., during acclimatisation).

2.3. Time point I: pre-exposure

After 56 days of feeding, twelve (12) fish were randomly sampled using a scoop net from each group for the collection of blood, serum, and tissue samples (see section 2.5) for determination of the effect of feed on stress indices and expression of stress-related genes before stress initiation (BSI) test.

2.4. Time point II: stress exposure

From the remaining fish in each experimental group, twelve (12) fish were randomly sampled and transferred from the 400 L tanks (i.e., 6 fish from each replicate tank per group) to 100 L tanks (2 tanks per group, each with 6 fish) set for application of different stressors namely hypoxia stress (HYS), cold stress (CDS), and heat stress (HTS). Each stressful condition was introduced as follows:

2.4.1. Hypoxia stress (HYS)

Fish from each experimental group were transferred from 400 L tanks of temperature 28 ± 2 °C of 6.05 ± 0.55 mg L^{-1} DO to 100-L tanks set after shutting off the supplemental aeration and decreasing the water column to 50 L maintained at the same temperature at the time of transfer (Elabd et al., 2017). Fish were sampled (see section 2.5) after 18 h (.i.e., in prior experiment, fish from all groups exhibited hypoxia signs including surfacing, rapid opercula movement, gasping, and lethargy after 18 h) during which time the DO when recorded was found to be 1 ± 0.55 mg L^{-1}.

2.4.2. Cold stress (CDS)

As previously described (Shi et al., 2015), fish from each experimental group were transferred from the 400 L tanks of 28 ± 2 °C, ambient temperature to 100 L (containing 50 L of water) tanks. The water temperature in the 100 L tanks was gradually reduced from 28 ± 2 °C to 12 ± 0.5 °C at a rate of 2 °C h^{-1} period using an automatic cooling system to reduce the water temperature and the stability measured using a multipurpose meter (YSI, USA). Aquarium tanks were continually aerated but no feeding during the test. Fish in the desired temperature (12 ± 0.5 °C) were then sampled (see section 2.5) after 2 h.

2.4.3. Heat stress (HTS)

As previously described (Fawzia et al., 2016), fish from each experimental group were transferred from the 400 L tanks of 28 ± 2 °C, ambient temperature to 100 L tanks (containing 50 L of water). The water temperature in the 100 L tanks was gradually increased from 28 ± 2 °C to 38 ± 0.5 °C at a rate of 2 °C h^{-1} using aquarium heaters and the stability measured using a multiparameter water quality meter (YSI, USA). Aquarium tanks were continually aerated without feeding during the test. Fish in the desired temperature (38 ± 0.5 °C) were then sampled (see section 2.5) after 2 h.

2.5. Sample collection and measurement

Whole blood, serum, and liver samples from 12 fish per group were collected first after 56 days of experimental feeding BSI and after exposure to HYS, CDS, and HTS to determine the activities of some stress indices. Whole blood samples were collected from caudal puncture and divided into two aliquots. The first aliquot of whole blood was used immediately in determining HCT and HGB levels using the Aimstrip® Hb haemoglobin analyser (Cascade health products, Portland, Oregon) according to the manufacturer's instruction. RBCs and WBCs were counted under a light microscope using a hemocytometer as previously described (Sarder et al., 2001). From the second aliquot of blood, serum was collected as previously described (Sirimanapong et al., 2015) and stored at −20 °C for later use. Serum MPO, CS, GC, PK, T-AOC, SOD, CAT, ALT, AST, and LDH were determined using commercial kits from Nanjing Jichang Company, China following the manufacturer's instructions. Liver tissue samples from the same fish were aseptically removed and stored in Eppendorf tubes containing RNA later at 4 °C overnight and later transferred to -80 °C until use in determining the expression of stress-related genes including HSP70 and HIF-1α mRNAs using quantitative real-time polymerase chain reaction (qRT-PCR).

2.6. Gene expression

2.6.1. RNA extraction and cDNA synthesis

Commercial RNA extraction kit (Transgen Biotech Company, China) was used for total RNA extraction following the manufacturer's instruction. The quality of the RNA was measured in 1.0 % agarose electrophoresis and quantitated at 260 nm, OD_{260}/OD_{280} using a nanodrop machine. Transgen cDNA reverse transcription genomic removal kit (Transgen, Biotech, China) was used for total RNA reverse transcription to cDNA following the manufacturer's protocol for subsequent determination of gene expression using qRT-PCR.

Table 2
Primers used for detection of target genes.

Target gene	Sequence (5'-3')	Genebank accession no
β-actin	F-TGACCTCACAGACTACCTCATG R-TGATGTCACGCACGATTTCC	KJ126772.1
HSP 70	F-ACCCAGACCTTCACCACCTA R- GTCCTTGGTCATGGCTCTCT	FJ213839.1
HIF-1α	F-GCAGTGAAGAGGAGGTGATGGA R-TTGCTGAACTTTCGGATGACGA	KY415998.1

Where: HSP70, Heat shock protein 70 and HIF-1α, Hypoxia-inducible factor 1 alpha.

2.6.2. Primer design and real-time PCR (qRT-PCR)

Primer 5 software was used for primer design using sequences of β-actin (i.e., a housekeeping gene), HSP70, and HIF-1α obtained from Genebank (Table 2). qRT-PCR amplification reactions were carried out using C1000 thermal cycler (CFX96 real-time PCR System) as previously described (Abarike et al., 2018c). Polymerase Chain Reaction (PCR) efficiency was analyzed as previously described (Livak and Schmittgen, 2001). Afterwards, the relative expression levels of target genes were analyzed by the $2^{-\triangle\triangle CT}$ method (Schmittgen and Livak, 2008).

2.7. Statistical analysis

The effect of the type of feed (i.e. CT, BS, CH, CHBS1, CHBS2, and CHBS3) and stress conditions (BSI, HYS, CDS, and HTS) on haematology, stress, immune, and antioxidant indices in whole blood/serum as well the expression of stress-related genes in the liver of tilapia were analyzed in two ways. 1) One-way ANOVA in which the type of feed was the main factor treated as the nominal independent variable and 2) two-way ANOVA in which the type of feed and stress conditions were the main factors treated as the nominal independent variables. Post-hoc Turkey HSD test was used to compare means which were considered statistically significant at $P < 0.05$. Data were expressed as a mean ± standard error (SE). All the statistical analyses were done using the SPSS programme version 13 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Fish survival

Fish in both the control and herbal-probiotic groups showed 100 % survival BSI. Fish in the control exhibited weakness showing some sluggish movement but survived the exposure period after been exposed to HYS, CDS, and HTS.

3.2. Effects of hypoxia, cold, and heat stressors on hemato-immunological parameters

3.2.1. HCT, RBCs, and HGB levels

The effect of hypoxia, cold and heat stressors on fish fed both control and herbal-probiotic diets on hematological parameters such as HCT, RBC and HGB are shown in Fig. 1. Among the different stress conditions (i.e., BSI, HYS, CDS, and HTS) in each group, the levels of HCT (Fig. 1 A), RBCs (Fig. 1 B), and HGB (Fig. 1 C), were significantly lower in BSI ($P < 0.05$) and significantly higher after exposure to HYS ($P < 0.05$). Among the groups in the current study, HCT and HGB were lowest in the CT group although similar to the CH group ($P < 0.05$) and highest in the CHBS2 group under the different stress conditions ($P < 0.05$)

3.2.2. WBCs and MPO activities

Fig. 2 illustrates the activities of some immunological parameters of fish after feeding with test diets and exposure to different stress conditions (i.e., BSI, HYS, CDS, and HTS). The results showed significant differences in immune indices in each group exposed to the different stress conditions. It was observed that, WBC (Fig. 2 A) and MPO (Fig. 2

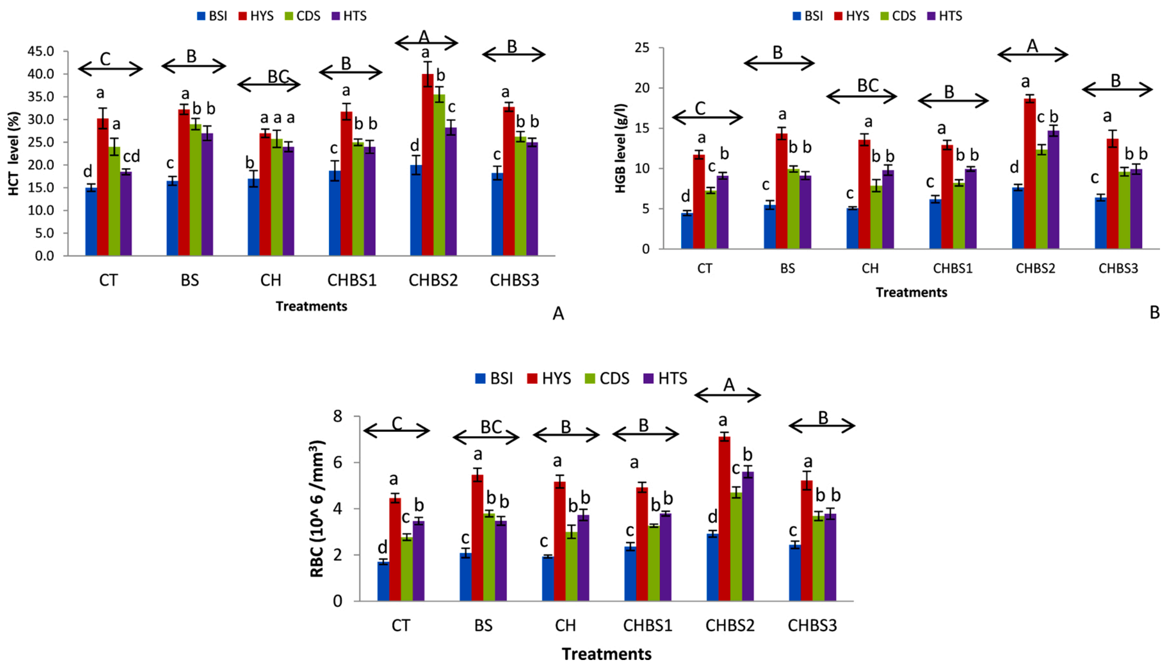

Fig. 1. Comparison (mean ± SE, Turkeys' HSD test, n = 2) of Hematocrit [HCT] (graph A), Hemoglobin [HGB] (graph B), and Red blood cell [RBC] (graph C) of tilapia fed with control (CT), Chinese herbs (CH), *Bacillus* species (BS), or a combination of CHBS (1, 2, and 3) diets, before stress initiation (BSI) and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS) stressors.
MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANT DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, AND CHBS3) under the different stress condition (.i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$). Mean values labeled with lower letters show significant difference in a group under different stress condition ($P < 0.05$). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

B) activities were highest ($P < 0.05$) in the CHBS2 group and lowest ($P < 0.05$) in the CT group. Also, among the treatment groups, WBC and MPO activities were similar ($P > 0.05$) except in the CHBS2 group which demonstrated significantly higher ($P < 0.05$) results.

3.3. Effects of hypoxia, cold, and heat stressors on stress parameters

3.3.1. CS, GS levels, and PK activity

Examination of the effect of control and treatments diets on stress indices in the serum including CS, GC levels, and PK activity are shown in Fig. 3. Among the different stressors, exposure to HYS showed higher CS (Fig. 3 A) and GC (Fig. 3 B) levels ($P < 0.05$) while exposure to CDS demonstrated the highest induction of PK activity (Fig. 3 C) in each group ($P < 0.05$). Also, among the groups in the present study, CS, GC, and PK activities were higher in the CT group compared to the herbal-probiotic groups ($P < 0.05$) among which the CHBS2 group showed significantly lower activities under the different stress conditions (i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$).

3.3.2. AST, ALT, and LDH activities

The effects of control and herbal-probiotic diets under different stress conditions are illustrated in Fig. 4. In each group, these biomarkers of tissue damage BSI were significantly lower compared to after exposure to HYS, CDS, and HTS ($P < 0.05$), among which HYS showed significantly higher inducing effects on AST (Fig. 4 A), ALT (Fig. 4 B), and LDH (Fig. 4 C), activities ($P < 0.05$). Among the groups, lower AST, ALT, and LDH, activities were observed in all herbal-probiotic diets in relation to the different stress conditions (i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$). Although in all herbal-probiotic groups, these indices were significantly lower compared to the control, the most remarked decrease was found in the CHBS2 group ($P < 0.05$).

3.4. Effects of hypoxia, cold, and heat stressors on antioxidant parameters

3.4.1. T-AOC, SOD, and CAT activities

As shown in Fig. 5, lower antioxidant activities were found in the control group compared to herbal-probiotic groups ($P < 0.05$). In each group, higher antioxidant activities were observed after exposure to HYS, CDS, and HTS compared to BSI ($P < 0.05$). Among the herbal-probiotic groups, fish fed with CHBS2 showed higher values of T-AOC (Fig. 5 A), SOD (Fig. 5 B), and CAT (Fig. 5 C) activities. However, statistically, no differences were observed among CHBS2, CH, CHBS1, and CHBS3 groups in all stress conditions (i.e., BSI, HYS, CDS, and HTS) ($P > 0.05$).

3.5. Effects of hypoxia, cold, and heat stressors on the expression of HSP70 and HIF-1α mRNAs

Expression of HSP70 and HIF-1α mRNAs in the liver of tilapia detected before and after exposure to different stressors are shown in Fig. 6. In each herbal-probiotic group increased expression in HSP70 mRNA (Fig. 6 A) and HIF-1α mRNA (Fig. 6 B) were detected BSI but not significant compared to the control ($P > 0.05$). However, after exposure to HYS, CDS, and HTS, significantly increased expression of HSP70 and HIF-1α mRNAs with prominence in the order HYS > CDS > HTS were detected in all the herbal-probiotic groups relative to the control group ($P < 0.05$). Also, all the herbal-probiotic groups demonstrated significant increase in the expression of these genes compared to the control group ($P < 0.05$. Among the herbal-probiotic groups, the CHBS2 group showed the highest expression of HSP70 and HIF-1α mRNAs ($P < 0.05$) while the BS group showed the lowest ($P < 0.05$).

4. 4 Discussions

Hypoxia (Gomes et al., 2009; Abdel-Tawwab and Wafeek, 2017),

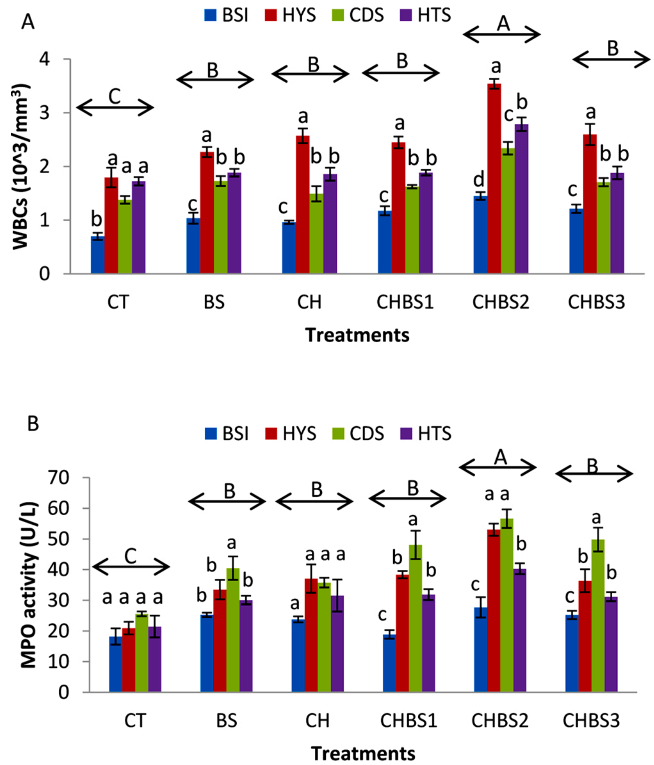

Fig. 2. Comparison (mean ± SE, Turkeys' HSD test, n = 2) of white blood cells [WBCs] (graph A) and myeloperoxidase [MPO] (graph B) in tilapia with control (CT), Chinese herbs (CH), *Bacillus* species (BS) or a combination of CHBS (1, 2, and 3) diets, before stress initiation (BSI) and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS) stressors.
MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANT DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, and CHBS3) under the different stress condition (.i.e., BSI, HYS, CDS, and HTS) ($P <$ 0.05). Mean values labeled with lower letters show significant difference in a group under different stress condition ($P <$ 0.05). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

cold temperature stress (Shi et al., 2015), and heat temperature stress (Duan et al., 2017) lead to stressful conditions in aquatic systems, and have been reported to cause significant losses in cultured fish (Bao et al., 2018). As a result, in recent times the goal in research is to increase the stress resistance capacity of fish to withstand the effects of environmental stress conditions (Varela et al., 2010).

A measure of the levels of some fish blood parameters including HCT, HGB, and RBCs will indicate fish's health status (Harikrishnan et al., 2010). HCT is the volume percentage of RBCs, HGB is the oxygen-carrying protein in the blood (Zimmerman et al., 2017) whereas RBCs transport oxygen in the blood (Longeville and Stingaciu, 2017). Respiratory metabolism in fish during stressful conditions, for example, HYS (Yang et al., 2017), CDS (Barros et al., 2009) and HTS (Bao et al., 2018) can alter the oxygen demand in organisms. In previous studies in tilapia, *Oreochromis niloticus* (Qiang et al., 2013), tench, *Tinca tinca* (De Pedro et al., 2005) and in rainbow trout, Oncorhynchus mykiss significant increases HCT, HGB, and RBSs levels have been recorded following exposure to water temperature and oxygen stressors (Morgan et al., 2008). Also, significant increments in these parameters have been reported in Goldfish, *Carassius auratus* (Harikrishnan et al., 2010) and *Paralichthys olivaceus* (Harikrishnan et al., 2011c) fed with herbal mixtures only and herbal-probiotic mixtures respectively after pathogen stress. In the current study the data demonstrates that tilapia is highly sensitive to HYS compared to CDS or HTS suggesting that HYS has may more severe effects on fish compared to CDS or HTS. The significantly higher in HCT, HGB, and RBC levels following herbal-probiotic treatment suggests that a physiological response in tilapia could be enhanced to cater for increased oxygen demand during stressful conditions. However, from this study best results from herbal-probiotic combinations thus the CHBS2 treatment could provide a good balance of the synergies of properties between of herbs and probiotic mixtures.

It is known that WBCs are involved in cellular immunity (Harikrishnan et al., 2011b), MPO enzymes utilise H_2O_2 to produce hypochlorous acid for the killing of infectious pathogens (Awad et al., 2015). The level of WBCs and MPO activities are critical immune responses associated with physiological stress response in fish (Hernandez et al., 2010; Yokoyama et al., 2006). It has been suggested that in evaluating the effects of herbs and or probiotics, data on these immune indices might provide a broader view of how they contribute to stress alleviation while maintaining an effective immune response (Jiang et al., 2017). Improved MPO activity against pH stress has been reported after feeding *allspice,* to *Pimenta dioica* in *Oreochromis mossambicus* (Yılmaz et al., 2015). The results of this current work particularly after feeding fish with CHBS2 strongly suggest it can enhance the fish immune response by significantly enhancing WBCs and MPO activities to cope with different stressors.

In response to stress, CS, GC, and PK are secreted or synthesised as a physiological response to regulate the use of available energy reserves to satisfy the need for extra energy (Welker et al., 2007; Mahfouz and Sherif, 2015; Guo et al., 2016). However, excessively elevated levels of these indices could have damaging effects on other physiological processes including growth and immunological responses (Michaelidis et al., 2007). Probiotic use in aquaculture has been promising in reducing stress indices in fish. For example, probiotic Efinol®L in cardinal tetra, *Paracheirodon axelrodi* (Schultz) (Gomes et al., 2009) and probiotic Pdp11 in gilthead seabream, *Sparus auratus* (Varela et al., 2010). *Pediococcus acidilactici* in green terror, *Aequidens rivulatus* (Neissi et al., 2015) have been reported to decrease CS and GC levels to density and heat, and pH stress respectively. In the present study, increased CS, resulted in increased GC and PK values. Similar results have been reported by Ming et al. (Ming et al., 2012). Dwelling on the results of the current study, administering CHBS2 can induce a higher anti-stress response mechanism compared to the other groups during stress condition. However, there is the need for further research to understand the mechanism of action of how herbs, probiotics or a combination contribute to ameliorating the pressures of stress stimuli in fish (Gomes et al., 2009; Hernandez et al., 2010; Neissi et al., 2015).

AST, ALT, (Kunjiappan et al., 2015) and LDH (Shi et al., 2015; Cheng et al., 2017) are essential enzymes that support protein metabolism in the liver of fish. However, these enzymes when released significantly into the blood/serum could serve as indications of tissue damage or organ dysfunction attributable to the action of stressors (Feroz and Khan, 2011; Yin et al., 2011). Therefore, the activities of ALT, AST, and LDH in serum are used to monitor fish health (Ming et al., 2012). For instance, significant increases in AST and ALT activities have been reported in the serum of pufferfish (*Takifugu obscurus*) (Cheng et al., 2017), Yellow perch (*Perca flavescens*), (Elabd et al., 2017) and in tilapia (*Oreochromis niloticus*) (Bao et al., 2018) after exposure to cold, physical and heat stressors respectively. Also, the feeding of tilapia with probiotic enriched diets has been reported to show a significant decrease in AST, ALT, and LDH activities (Mohapatra et al., 2013). In the present study, tilapia exposed to HYS, CDS, and HTS showed an increase in AST, ALT, and LDH activities were moderate in the herbal-probiotic groups compared to the control group. The results in the current study suggest that herbal-probiotic supplements might have hepatoprotective ability to prevent liver damage in tilapia under stressful conditions and this can be associated with their ability to significantly enhanced antioxidant defences in fish. Support for this explanation is drawn from earlier reports (Su et al., 2016).

Environmental stress conditions can trigger the excessive development of reactive oxygen species (ROS) causing oxidative injuries (Kammer et al., 2011). Therefore an increase in the activities of

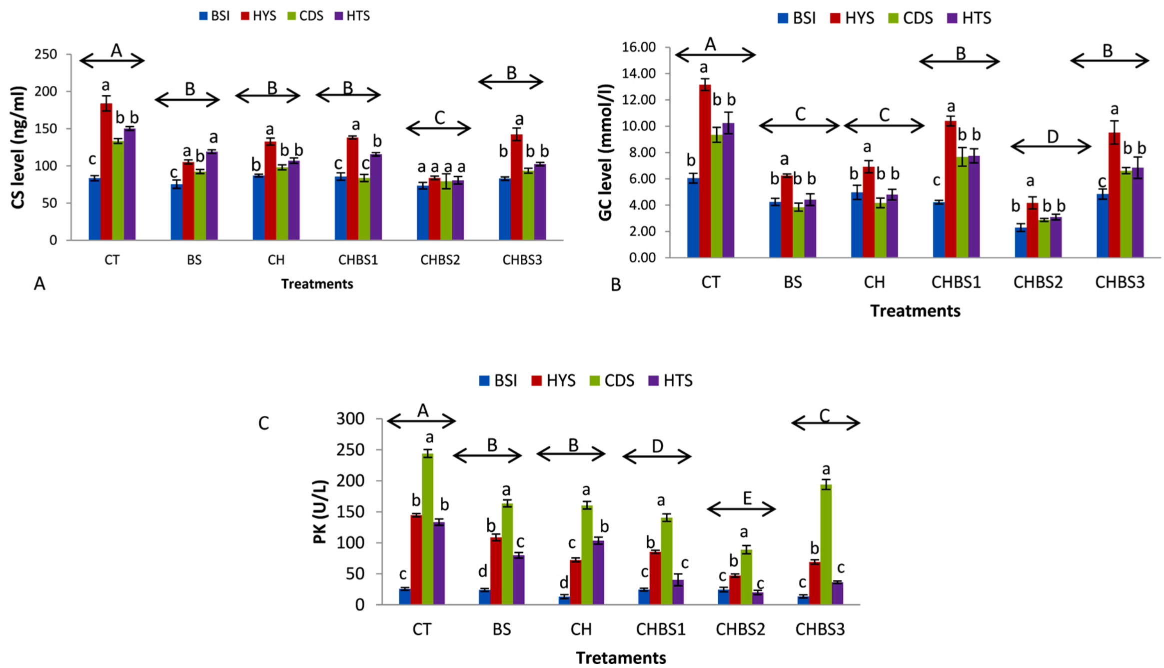

Fig. 3. Comparison (mean ± SE, Turkeys' HSD test, n = 2) of cortisol [CS] (graph A), glucose [GC] (graph B), and pyruvate kinase [PK] (graph C) of tilapia fed with control (CT), Chinese herbs (CH), *Bacillus* species (BS), or a combination of CHBS (1, 2, and 3) diets, before stress initiation (BSI) and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS) stressors.
MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANT DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, and CHBS3) under the different stress condition (.i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$). Mean values labeled with lower letters show significant difference in a group under different stress condition ($P < 0.05$). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

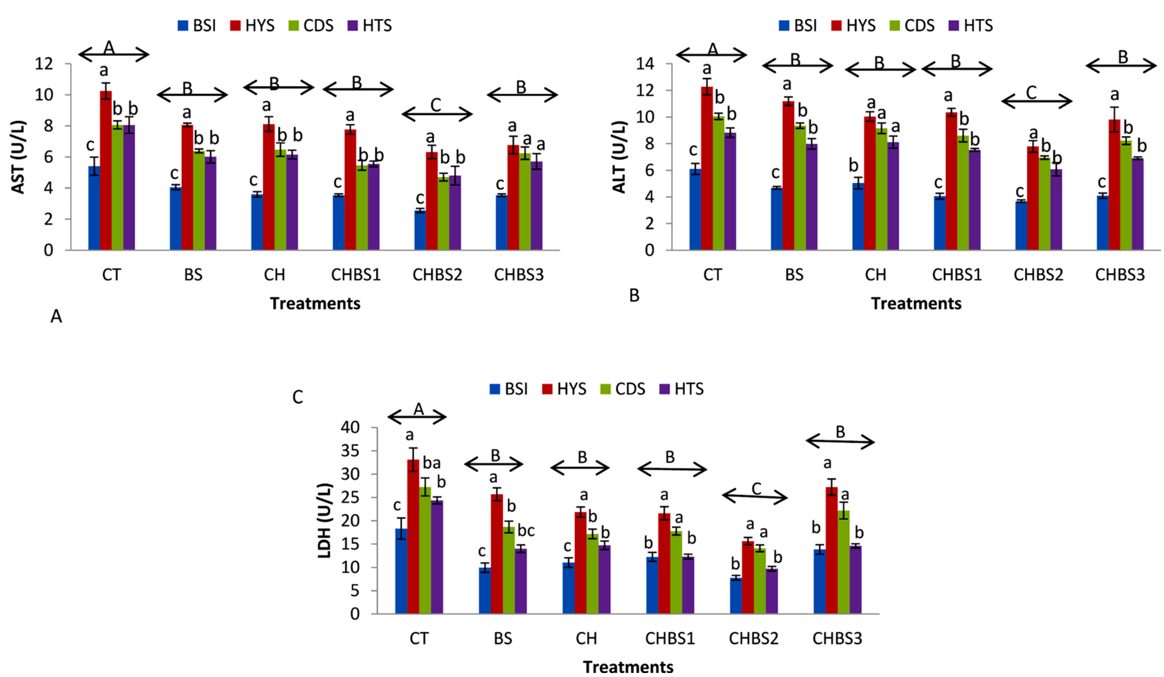

Fig. 4. Comparison (mean ± SE, Turkeys' HSD test, n = 2) of aspartate aminotransferase [AST] (graph A), alanine aminotransferase [ALT] (graph B), and lactate dehydrogenase [LDH] (graph C) in tilapia fed with control (CT), Chinese herbs (CH), *Bacillus* species (BS), or a combination of CHBS (1, 2, and 3) diets, before stress initiation (BSI) and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS) stressors.
MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANT DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, and CHBS3) under the different stress condition (.i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$). Mean values labeled with lower letters show significant difference in a group under different stress condition ($P < 0.05$). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

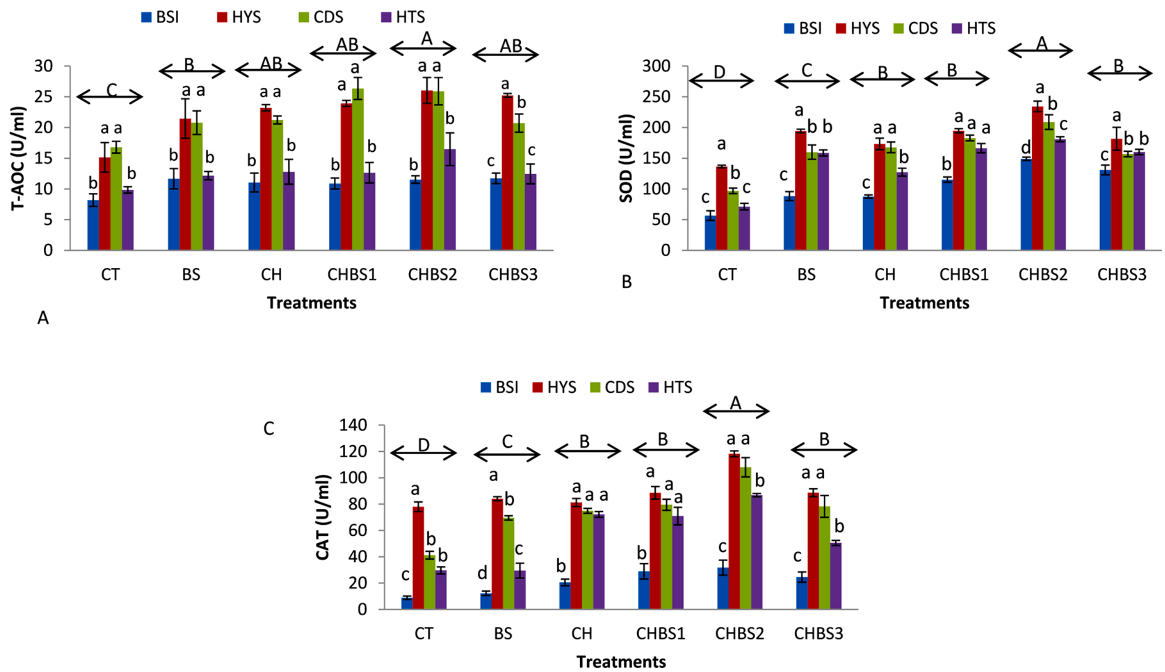

Fig. 5. Comparison (mean ± SE, Turkeys' HSD test, n = 2) of total antioxidant capacity [T-AOC] (graph A), superoxide dismutase [SOD] (graph B), catalase [CAT] (graph C), in tilapia fed with control (CT), Chinese herbs (CH), *Bacillus* species (BS), or a combination of CHBS (1, 2, and 3) diets, before stress initiation (BSI) and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS) stressors.
MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANT DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, and CHBS3) under the different stress condition (.i.e., BSI, HYS, CDS, and HTS) ($P < 0.05$). Mean values labeled with lower letters show significant difference in a group under different stress condition ($P < 0.05$). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

antioxidant enzymes, for example, T-AOC, SOD, and CAT are needed to counteract oxidative stress to maintain anaerobic homeostasis in organisms (Li et al., 2016). T-AOC can prevent the deleterious effects of ROS, superoxide anions are transformed to hydrogen peroxide by SOD, and hydrogen peroxide is removed by the CAT enzyme thereby reducing oxidative damage (Wang et al., 2017). Probiotic *Clostridium butyricum* in diets of kuruma shrimp *Marsupenaeus japonicas* (Duan et al., 2017) and Yellow perch, *Perca flavescens* fed a mixture of *Astragalus membranaceus*, and *Glycyrrhiza glabra* (Elabd et al., 2017) have been reported to enhance antioxidant enzymes activities in stressful condition for example heat stress. In the current study, antioxidant enzymes (i.e. T-AOC, SOD, and CAT) activities in fish fed herbal-probiotic diets suggest that herbal-probiotic supplements particularly CHBS2 can improve the antioxidant capacity of fish before and during environmental stress condition.

Stressful conditions induce the up-regulation of HIF-1α which activates other genes including HSP70 to support hypoxic survival and anaerobic energy production and increase oxygen demand (Nikinmaa and Rees, 2005; Heise et al., 2006, 2007). Also, under stressful conditions, an increase in the transcription of HSP70 is required to interact with other signaling genes to mount a defence against oxidative damage and apoptosis in cells during stressful condition (Shi et al., 2015). Although few studies exist on the effect of temperature on HIF-1α in fish, is has been shown that interaction of HSP70 and HIF-1 α genes during heat or cold acclimation is critical for the maintenance of homeostasis during a stressful condition in the liver of fish (Mladineo and Block, 2009). The liver is an organ for detoxification of harmful substances, and the metabolism of carbohydrates (Xia et al., 2016) as well as a hub for many genes including HSP70 and HIF-1α whose transcription promotes survival during stress in an organism (Wang et al., 2017). In this study, it is evident that HYS, CDS, and HTS induce the expression of HSP70 and HIF-1α ($P < 0.05$) in tilapia. However, higher expression of these genes in the herbal-probiotic fed fish, particularly those fed the CHBS2 which suggest a anti-stress response. Moreso, significant transcription of these genes in the liver suggests herbal-probiotic application especially the CHBS2 could enhance the liver health status of tilapia to different stressors and is in agreement with previous reports in Pacific bluefin tuna (Mladineo and Block, 2009) and Nile tilapia (Mahfouz et al., 2015).

In conclusion, the present research showed that a herbal-probiotic supplements comprising a BS, CH, CHBS1, CHBS2, and CHBS3 diets improved hemato-immunological (HCT, RBCs, HGB, WBCs, and MPO), stress (CS, GC, PK, ALT, AST, and LDH), and antioxidative (T-AOC, SOD, and CAT) indices as well the expression of HSP 70 and HIF-1α under different (HYS, CDS, and HTS) stress conditions. Overall, the application of CHBS2 [CH at 5 g kg^{-1} and BS at 5 g kg^{-1}] diet showed the most pronounced enhancement of these physiological responses suggesting its use as a prophylactic against the suppressive effects of stress in tilapia culture.

Submission declaration and verification

This article to be considered for publication has not been published previously and is not under consideration for release elsewhere.

Authors Statement

Jicheng Jian conceptualized the idea and provided funds for project implementation

Jufen Tang designed the experiment and facilitated the purchase of resources for project implementation

Essien Micheal Sakyi and Felix Kofi, Agbeko Kuebutornye collected field data

Emmanuel Delwin Abarike, analyzed data and drafted the manuscript

Cai Jia proofread the manuscript

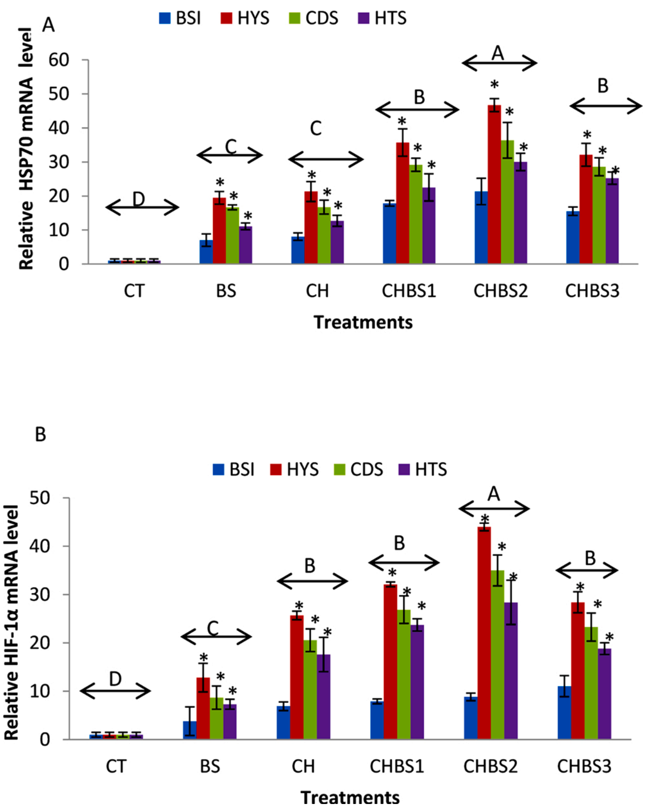

Fig. 6. Expression changes of HSP70 (graph A) and HIF-1α (graph B) mRNA levels in the liver of tilapia fed tilapia fed with control (CT), Chinese herbs (CH), *Bacillus* species (BS), or a combination of CHBS (1, 2, and 3) diets before stress initiation (BSI), and after exposure to hypoxia (HYS), cold (CDS), and heat (HTS). HSP70 and HIF-1α mRNA levels were evaluated using qRT-PCR and expressed relative to the control set to 1.
DATA ARE REPORTED IN AS MEAN ± SE, TURKEYS' HSD, N = 2. MEAN VALUES LABELED WITH UPPER LETTERS SHOW SIGNIFICANCE DIFFERENCE BETWEEN GROUPS (CT, BS, CH, CHBS1, CHBS2, and CHBS3) under different stress condition (.i.e. BSI, HYS, CDS, and HTS) ($P < 0.05$). Mean values in each group labeled with an asterisk (*) means significant difference between stressors in each treated group relative to the control group ($P < 0.05$). (For interpretation of the reference to color in this figure, the reader is referred to the web version of this manuscript).

Authors' contributions

Jian Jicheng and Tang Jufen conceived and designed the experiment, Essien M. Sakyi, Felix Kofi, A.K. collected field data E.D. Abarike, analyzed the data Cai Jia drafted and proofread the manuscript.

Declaration of Competing Interest

No conflicts of interest are there to declare

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