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Puerarin Promotes Overall Health Through Blood Flow and Immune Response within Chinook Salmon (*Oncorhynchus tshawytscha*) Fingerlings

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*An 8-week study was performed to determine the effects of supplementing puerarin, the most biologically active ingredient present in the roots of kudzu (*Puerarin lobata*), in diets of farmed chinook salmon (*Oncorhynchus tshawytscha*) fingerlings. Puerarin was given to study fish through supplementation to commercial fish feed. Administration of puerarin reduced cortisol, proteins, and hematocrit levels while improving spleen somatic indices and respiratory burst response compared to fish fed a control diet without affecting growth, and thereby counteracts the negative consequences of stress induced through handling and experienced by fish reared in aquaculture.*

KEYWORDS *Puerarin, salmon, stress, chronic*

INTRODUCTION

Handling stress may not kill fish, but causes undesirable effects (Gerwick et al. 1999). In fish, the stress response is marked by increase in the levels of plasma cortisol and catecholamine (Bonga 1997; Mustafa et al. 2000). Cortisol is an immunosuppressive agent and may be responsible for increased occurrences of diseases in stressed fish (Barton and Iwama 1991; Leonard and McCormick 1999; Ruane et al. 2000). In fish, acute stressors such as chasing and handling lead to hyperglycemia, which has been associated with strokes

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in both animal and human models (Capes et al. 2001), along with increased susceptibility to infections in fish (Barton and Iwama 1991). Teleost fish have reduced capacities to clear plasma glucose levels and hence are more prone to suffer from stress-induced hyperglycemia (Moon 2001).

Kudzu powder has been used in oriental countries as a natural treatment for cardiovascular diseases (Peng et al. 2009; Tanaka et al. 2011) and has been shown to reduce blood glucose levels in diabetic mice (Prasain et al. 2004; Diaz et al. 2007) and expression levels in the diabetic mice to those of nondiabetic mice within a duration of 3 days (Hu et al. 2003). Chinook salmon (*Oncorhynchus tshawytscha*) elicit very high post-stress plasma cortisol levels, often reaching 400 ng/ml during transportation (Barton 2002). Thus, fingerling chinook salmon, due to its capacity to be highly stressed (Barton and Peter 1982), provided us with a good model organism to determine the effects of puerarin on acute stress.

This study was developed to determine the effects of puerarin, the most biologically active isoflavone present in the roots of kudza (*Pueraria lobata)*, an invasive plant (Peng et al. 2009), on modulating stress within salmonids. We hypothesized that since teleost fish possess diminished capacities to clear blood glucose, the presence of puerarin will help alleviate the symptoms of elevated sugar concentration, signaling the cessation of cortisol secretion, and eventually improve the health of the fish over an 8-week period. We measured the impact of puerarin on physiological parameters such as plasma cortisol, blood glucose, plasma protein levels, packed-cell volume, spleen somatic indices (SSIs), length, and weight to determine condition factor (K) (Barton and Iwama 1991; Bonga 1997; Hadidi et al. 2008; Ruane et al. 2000). The impact of puerarin addition on immune response was determined through the measurement of respiratory burst activity.

MATERIALS AND METHODS

Chinook salmon fingerlings (3 months old) weighing between 2 and 5 grams were obtained from the Bodine State Fish Hatchery (Mishawaka, IN) and transported to the Life Sciences Resource Center (LSRC) at Indiana University–Purdue University Fort Wayne. After being transported into LSRC, the fish were allowed to acclimate to their new conditions for 2 month prior to the start of the study. The fish were fed daily with commercial fish feed (Aquamax Starter Fish Fry 200), which would form the control feed for our study, to satiation while being observed routinely to observe any manifestation of discomfort.

Water temperature was kept within a range of 16°C to 18°C. Dissolved oxygen was kept between 6.5 and 9.3 mg L^{-1}, and the pH between 6 and 9. Ammonia was <0.25 mg L^{-1}, nitrate between 25 and 50 mg L^{-1}, and nitrite at 0.3 mg L^{-1}. All of the aforementioned parameters were within

recommended values (Wedemeyer et al. 1990). Throughout the acclimation and study period, the fish were kept in a room with a diurnal cycle divided into 16 h light and 8 h of darkness.

The salmon were fed commercial Aquamax Starter Frye 200 feed as a control throughout both the acclimation and study periods. Puerarin supplement was purchased from Sigma-Aldrich (St. Louis, MO, USA). To incorporate the puerarin into the control feed, it was dissolved in 95% alcohol at a concentration of 200 mg kg^{-1} (0.02%) and carefully sprayed on the feed in small aliquots (Peng et al. 2009; Wang and Cheng 2005). The feed was then air-dried and stored at 4°C (Mustafa and MacKinnon 1999) within 1 week of the start of the study.

At the start of the study, fish were randomly assigned to one of eight 20-gallon tanks (100 fish per tank) at a density of 34 g l^{-1} in two separate systems to prevent any cross-contamination of feed between the control and puerarin feed groups: The first system of four tanks was fed control feed (C), two of which housed the unstressed (CU) group, while the remaining two tanks housed the stressed (CS) group. Similarly, the second system of four tanks were fed puerarin-supplemented feed (K), two tanks with unstressed (KU) fish and two with stressed fish (KS).

Prior to the distribution of fish into the experimental groups, fish were randomly sampled to assess the health and stress levels of the fish by measuring length and weight (to calculate condition factor, K), blood glucose, hematocrit, plasma protein titre, spleen size (to calculate spleen somatic index, SSI), and respiratory burst. This was sample D0. Samples were then taken at four additional time-points: day 1 (D1), day 14 (W2), day 35 (W5), and at day 56 (W8). At each sampling, six fish were randomly captured (3 fish per group × 2 replicates), euthanized in a 1 liter solution of 200 mg of MS-222 (Sigma-Aldrich, USA) according to (Gerwick et al. 1999; Halloway et al. 2004), and blood was drawn from the caudal vein with a heparinized syringe.

The condition factor (K) was determined by measuring the length and weights of all the sampled fish after euthanization and using the information in the equation:

$$K = \left[\left(\text{weight} \times 100\right)/\left(\text{length}^3\right)\right].$$

Cortisol concentration was determined with the help of an ELISA kit from Enzo LifeSciences (Farmingdale, NY, USA). Plasma cortisol collected from fish sampled were analyzed according to manufacturer's instructions. The measurements are expressed in nanograms per milliliter with a sensitivity of less than 1%. The minimum detectable level of cortisol for the kit was 0.156 ng ml^{-1}.

Blood glucose concentration was determined by placing a drop of collected blood from each fish onto a glucometer strip, which was then placed

in a standard glucometer (Precision Xtra, Abbott Laboratories; Abbott Park, IL, USA). This method for testing blood glucose has been validated for use on fish by Wedemeyer et al. (1990). Following plasma glucose measurement, blood hematocrit levels were measured from the collected blood when loaded into a capillary tube. After loading, the capillary tubes was capped at one end and spun in a microcentrifuge. After spinning, Micro-Hematocrit Capillary Tube Readers (Monoject Scientific, St. Louis, MO, USA) were employed to determine Packed Cell Volume (PCV). Subsequently, plasma protein levels were measured through the usage of refractometer after adding one to two drops of plasma from the spun hematocrit tubes.

Macrophages were isolated for determining macrophage respiratory burst activity by removing the head kidney from each sampled fish and placing them in Leibovitz-15 (L15) medium (Meatech Inc., Manassas, VA, USA) on ice, which was supplemented with 2% fetal calf serum (FCS), penicillin/streptomycin (50 U ml^{-1}), and heparin (10 U ml^{-1}) (Cook et al. 2003; Mustafa et al. 2000). Subsequently, the collected head kidneys were then macerated by passing the organs through a course mesh to separate the cells from larger tissue fibers, which were subsequently placed in L15 on ice. Upon separation, the L15 solutions were spun at 1000 rpm for 15 min. After spinning, the supernatant was discarded and the cells were resuspended with L15 and then counted with the help of a hemocytometer. Following cell counting, different cell solutions were diluted with L15 to ensure a cellular concentration of 2×10^6 ml^{-1} in each tube; subsequently, 100 µl of each cellular solution was placed into predetermined wells in a 96-well plate and allowed to incubate for 2 h. Following incubation, nitroblue tetrazolium (NBT) was mixed with phorbol myristate acetate (PMA) and dissolved in L15 with 0.1% FCS to acquire a concentration of 1 mg/ml. Afterwards, 100 µl of the NBT/PMA solution was added to each well in the 96-well plate being studied and allowed to incubate for 30 min. Following incubation, the wells were washed with sodium perborate (PBS) and the cells were fixed with methanol. Subsequently, KOH and dimethyl sulfoxide (DMSO) were added to each well to start the reaction process. After 30 min of allowing the solutions in the wells to mix, the plates were read in a Packard Spectracount reader (Cole-Parmer, USA) at 600 nm; each well had 2×10^6 cells.

SSI was calculated by removing the spleen from each sampled fish and weighing it on an electrical balance; SSI = (spleen weight/ body weight) × 100 (Pearson and Stevens 1991).

Five hours following the transfer, fish were fed to satiation on the experimental diets according to their respective groups. Shortly after, stress was induced within the stressed groups by chasing, catching, and netting the fish for a period of 5 min and, after 24 h, the fish were again sampled (D1). Subsequently, the fish in the stressed groups were chased for 5 min daily with nets to ensure that the type of stressor was identical throughout the

entire 8-week period of the study. At the end of the study (W8), the data collected through the course of this experiment were analyzed using SigmaPlot 11.0. The means and standard errors of the means were determined for each assay through one-way analysis of variance (ANOVA). In addition to ANOVA, Tukey's tests and Student's simple *t*-tests were performed to determine whether the differences between the samples were significant ($P < 0.05$). The findings of our analyses are presented in the text and graph in the form of means ± standard errors of means (SEM).

RESULTS AND DISCUSSION

Results are displayed graphically in Figures 1–7. After being transferred to new tanks, 24 h was sufficient to return plasma cortisol levels to the basal level, since both CU and KU produced similar concentrations of cortisol. Also, the practice of chasing the stressed groups with nets for 5 min was successful in eliciting elevated plasma cortisol levels. While plasma cortisol concentrations gradually declined throughout the length of the study, plasma glucose concentrations varied from one sampling to another, indicating that puerarin's antihyperglycemic properties work best over a short period of time.

The practice of preparing fish for experimental handling by capturing and transferring them to a small container consisting of anesthesizing or euthanizing agent such as MS-222 (Tricaine methanesulfonate) has deficiencies; since this practice exposes the fish to the stress of being chased, netted, passed through the air, placed into a small, unfamiliar container before the

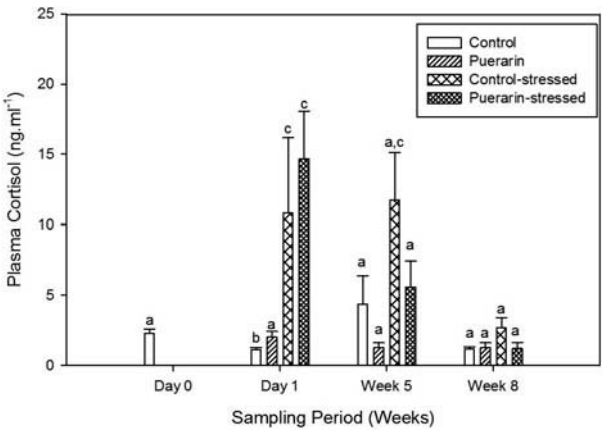

FIGURE 1 Plasma cortisol concentrations (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

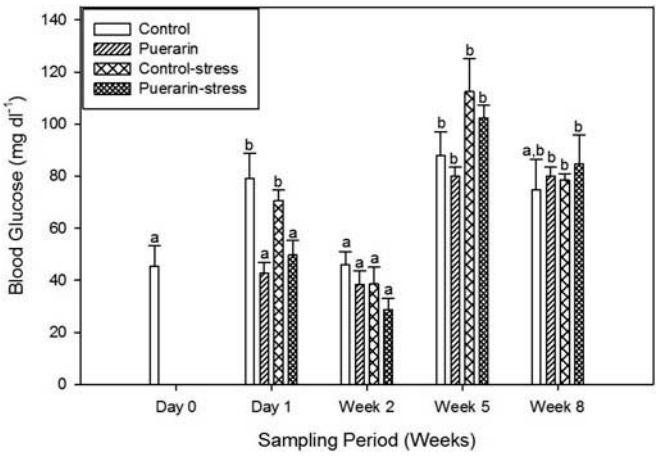

FIGURE 2 Plasma glucose concentrations (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

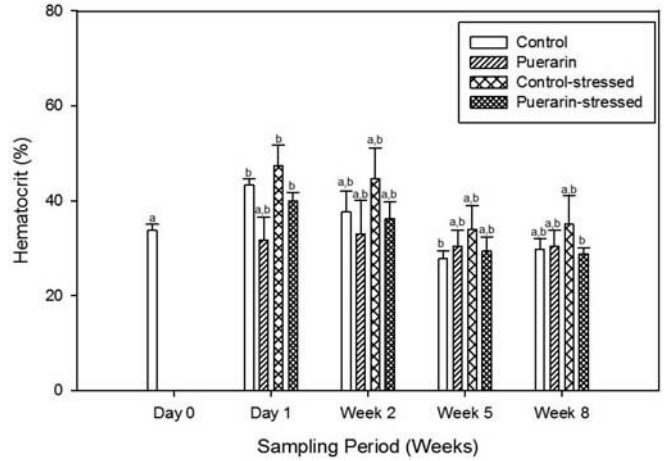

FIGURE 3 Hematocrit or packed cell volume (PCV) (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

anesthetic takes effect (Gerwick et al. 1999). The latter procedure, even when executed proficiently, cannot eliminate the possibility of the activation of sympathetic stress response pathways (Gerwick et al. 1999). Despite the difficulties in creating conditions in the laboratory that mimic chronic stress (Weerd and Komen 1998), our results illustrate that we were able to produce scenarios where the fish were initially highly stressed, which gradually decreased through acclimatization to their stressors as the study progressed.

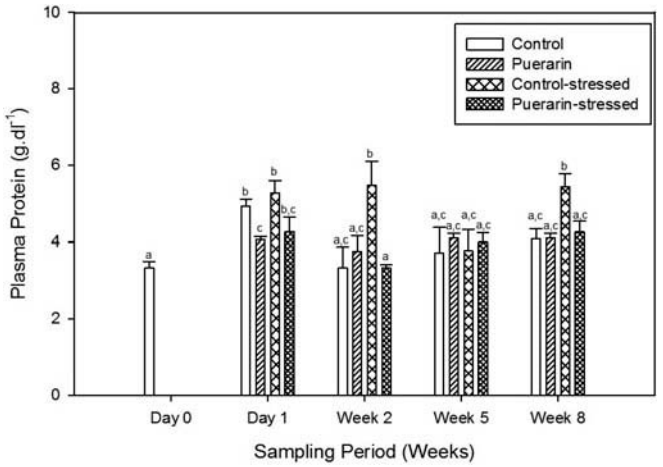

FIGURE 4 Plasma protein concentrations (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

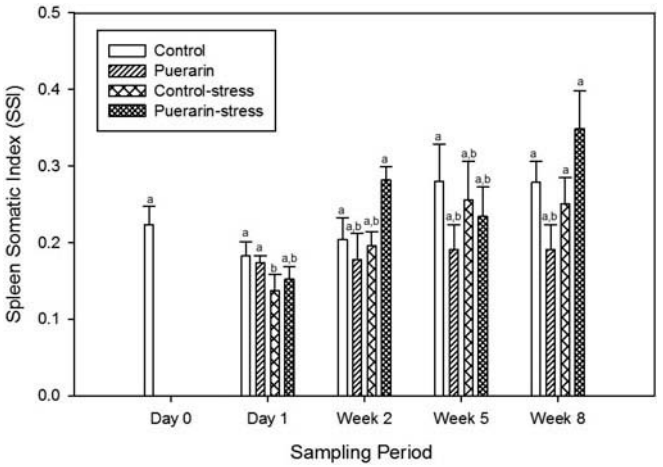

FIGURE 5 Spleen somatic indices (SSIs) (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

Surges in plasma glucose concentrations within stressed fish are hallmark indicators of the stress response within salmonids (Barton and Iwama 1991; Barton 2000; Bonga 1997; Wedemeyer et al. 1990). Elevated plasma glucose levels have been associated with reduced coronary blood flow (Yokoyama et al. 1998). Supplementation of puerarin to commercial fish feed attenuates plasma glucose concentrations to levels similar to the glucose concentrations seen in day 0 samples, which concurs with prior research on glucose homeostasis (Hu et al. 2003).

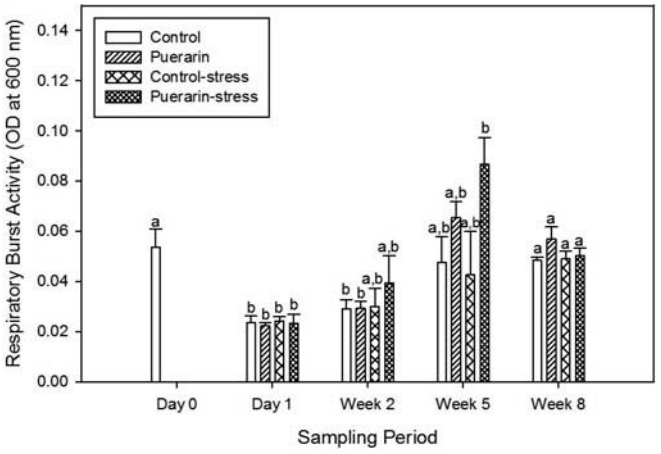

FIGURE 6 Respiratory burst activity (mean ± SEM) in different groups of experimental salmon at separate time points. Means with different letters are significantly different ($P < 0.05$) within each individual sampling group.

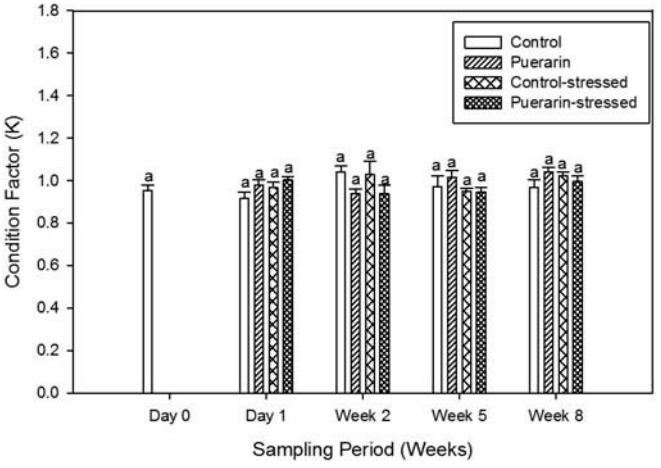

FIGURE 7 Condition factor (K) (mean ± SEM) in different groups of experimental salmon at separate time points. There were no differences that were significantly different ($P < 0.05$) within the individual sampling group.

Although our findings could not illustrate any significant differences within both stressed and unstressed groups fed either control or puerarin feed after 24 h, our study reveals that puerarin-fed fish had lower plasma glucose levels relative to fish fed control feed after day 1. We believe the supplemented puerarin enhances plasma glucose reuptake within the cells of the fish, regardless of stress. These data concur with other studies on the effects of puerarin on insulin-resistant obese mice with reduced glucose tolerance (Meezan et al. 2005; Prasain et al. 2012). The beneficial effects

of enhanced plasma glucose metabolism by puerarin could be observed in other biomarkers of overall health: Puerarin has also been shown to reduce bone loss and blood cholesterol, all while improving blood circulation and overall health (Meezan et al. 2005; Peng et al. 2009; Tanaka et al. 2011; Yeung et al. 2006). Therefore within a 24-h period, we can ascertain that the secretion of cortisol into the blood of our fish led to an elevation of glucose concentration within all four groups. However, while the cortisol levels dropped within both control-feed and puerarin-feed unstressed groups, due to the slower glucose homeostasis within fish (Diaz et al. 2007; Moon 2001), the plasma glucose concentrations remained elevated within the control-feed groups, while returning to their physiologically normal levels within the puerarin-feed groups.

While plasma cortisol and blood glucose are endocrine indicators of stress, packed cell volume (PCV) and plasma protein concentrations are indicative of the cellular response to stress, where PCV is the ratio of the volume occupied by packed blood cells to the volume of whole blood as measured by a hematocrit (Chaplin et al. 1953). The measurement of hematocrit has been used as an indicator for increased risk and development of cardiac disease (Ring et al. 2008). Upon the induction of acute stress, cells produce proteins such as heat shock proteins and chaperones (Iwama et al. 1999), leading to an increase in PCV, and cause reductions in blood plasma volume (Ring et al. 2008). Fish held in aquaculture conditions sometimes possess elevated hematocrit, plasma protein, and red blood cells relative to fish reared in laboratory conditions (Fletcher 1975).

A reduction in blood plasma volume can lead to greater energy expenditure by fish to pump blood to their tissues, therefore leading to elevated plasma glucose levels and in extreme cases, exhaustion (Barton and Iwama 1991; Bonga 1997). Furthermore, stress-induced hyperglycemia can lead to insulin resistance, which can reduce blood flow within skeletal tissues (Laakso et al. 1990). Our results indicate that the presence of puerarin in fish feed reduced both PCV and plasma protein levels, which is in accord with the findings from studies on other animal models (Yeung et al. 2006), in that along with its antihyperglycemic properties, puerarin has been shown to induce vascular relaxation in arteries while enhancing greater blood flow through induction of the synthesis of endothelial nitrous oxide synthase within arteries, and offered protection against hypercholesterolemia within mice (Yeung et al. 2006). Throughout the study, we observed that both hematocrit and plasma protein levels experienced some variations from one sampling date to another; however, we could clearly see the trend of puerarin-fed fish coping relative to their control-fed counterparts.

In addition to endocrine and cellular markers for stress, the sizes of spleens in fish are used as a predictor of disease resistance (Barton and Iwama 1991; Bonga 1997). The spleen is responsible for filtering blood and removing damaged or dying cells and foreign organisms (Bohnsack

and Brown 1986; Hadidi et al. 2008; Tablin et al. 2002). In salmonids, the spleen produces the majority of antibodies and helps form immunological memory (Hadidi et al. 2008). A smaller spleen is indicative of stress and reduced disease resistance, as there is less blood circulating through the spleen (Barton and Iwama 1991; Bonga 1997; Hadidi et al. 2008). In line with prior research, our results indicate that the addition of puerarin assists in enhanced blood flow, which helps in reduced shrinking of the spleen over an 8-week period within the puerarin-stressed fish. Only at the 5-week point of our study do we see that the control-fed fish possessing superior SSIs relative to puerarin-fed fish; however, their differences were not significant, and the puerarin-stressed fish still possessed improved SSIs relative to puerarin-unstressed groups, demonstrating the efficacy of puerarin in stressful environments.

Respiratory burst activity decreased and gradually increased until week 5 of the experiment, where at weeks 2 and 5, KS exhibited superior respiratory burst activity. Increased reduction of NBT through the generation of oxygen radicals by monocytes or macrophages is a marker for establishment for nonspecific defense mechanisms within fish (Babior 1984; Solem et al. 1995; Cook et al. 2003). The enhanced blood flow with the help of puerarin can be postulated to reduce the necessity of fish to expend large quantities of energy required to mount an immune response (Poisot et al. 2009). The reduction in energy expenditure to pump blood and immune cells to any site of infection would mean that the fish would invest that energy in producing more immune cells (Barton and Iwama 1991; Bonga 1997).

In conclusion, we were successful in creating a study that generated stressful conditions, under which puerarin, concurring with prior studies on other animals, was able to promote overall well-being of the fish as it enhanced greater systemic circulation while attenuating plasma cortisol and glucose levels and improving respiratory burst and SSIs.

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Effects of Puerarin on the Reduction of Glucose and Promotion Overall Health in Acutely Stressed Chinook Salmon (Oncorhynchus Tshawytscha)

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EFFECTS OF PUERARIN ON THE REDUCTION OF GLUCOSE AND PROMOTION OF OVERALL HEALTH IN ACUTELY STRESSED CHINOOK SALMON, *ONCORHYNCHUS TSHAWYTSCHA* (ACTINOPTERYGII: SALMONIFORMES: SALMONIDAE)

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Hossain M.R., Blumenthal E., Mustafa A. 2013 Effects of puerarin on the reduction of glucose and promotion of overall health in acutely stressed Chinook salmon, *Oncorhynchus tshawytscha* (Actinopterygii: Salmoniformes: Salmonidae). Acta Ichthyol. Piscat. 43 (2): 85–93.

Background. Farmed fish reared in aquaculture systems are exposed to physiological stressors from biological, chemical, and physical sources. The consequences of these stressors affect the productivity of farming outcomes by reducing immune response, growth, and development within fish. A 24-h study was performed to determine the effects of supplementing puerarin—the most biologically active ingredient present in the roots of kudzu (*Pueraria lobata*)—within farmed fish on acute handling stress.
Materials and methods. Fingerlings of Chinook salmon, *Oncorhynchus tshawytscha*, were maintained at 16–18ºC in 10 US gallon (37.8 L) glass tanks, supplied with recirculated and aerated dechlorinated water. Puerarin was given to fish through supplementation to commercial fish feed pellets. Acute handling stress was induced through transferring the fish from their housing tanks into 8 designated tanks at 0000 h. Subsequently, at 5 h post-transfer all the fish were fed to satiation, following which the stressed groups were chased with nets.
Results. The results indicated that administration of puerarin through feed supplementation reduces plasma glucose, hematocrit, plasma protein, and improves spleen somatic indices after 24 h, and thereby counteracting the negative consequences of acute stress induced through handling.
Conclusion. The results suggest that supplementation of puerarin to commercial fish feed improved overall fish health through enhancing systemic circulation while attenuating plasma glucose. Being one of the first studies to study the effects of puerarin on fish, our results are in agreement with prior research with puerarin on other animal models.

Keywords: salmon, aquaculture, stress, puerarin

INTRODUCTION

In the realm of aquaculture, the loss of productivity through infection and subsequent reductions in growth and development is a major problem which requires innovative methods for generating solutions. There are no commercially available vaccines against many aquatic pathogens, and further exacerbating the problem, the use of antibiotics for treatment of the fish is not preferred due to the risk of developing antibiotic resistance within the bacteria, harming non-target species, and the potential of developing zoonotic parasites which can infect human populations from farmed sources (Hadidi et al. 2008). Further complicating the situation is the fact that in aquaculture, fish are raised in tanks with thousands of other fish under crowded conditions, which can negatively affect feeding behavior, along with impacting other normal physiological functions such as growth, immune response, reproductive prowess (Barton and Iwama 1991). According to the life-history theory, each organism must allocate its energy within several necessary activities: survival, reproduction, and somatic maintenance (Poisot et al. 2009). Since each organism has a limited amount of energy remaining after performing all the necessary activities at its disposal, a pathogenic infection can easily disrupt the homeostatic functions within poikilotherms such as fish. This is because mounting an effective immune response is a highly energetically expensive process (Poisot et al. 2009). Thus, the manifestation of stress in farmed fish through common industry practices such as crowding or handling for vaccination, grading, transportation, etc. in aquaculture makes farmed fish more susceptible to parasitic infections as they possess reduced capacities to mount effective immune responses (Barton and Iwama 1991, Ruane et al. 1999).

Within the aquaculture industry, the protocols for handling fish are well-documented acute stressors, which

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lead to stress levels which are tolerated despite causing undesirable effects (Gerwick et al. 1999). In fish, the stress response is marked by the stimulation of the hypothalamus-pituitary-interrenal (HPI) axis, which leads to an increment in the levels of plasma cortisol and the sympathetic-catecholamine cell axis which elevates catecholamine levels (Wendelaar Bonga 1997, Mustafa et al. 2000). Cortisol is an immunosuppressive agent, and increased occurrences of diseases have been observed within stressed fish (Barton and Iwama 1991, Leonard and McCormick 1999, Ruane et al. 1999). In addition to a suppressed immune response through cortisol, acute stress has been characterized by hyperglycemia, which has been associated with complications such as strokes in both animal and human models (Capes et al. 2001), along with increased susceptibility to infections in fish (Barton and Iwama 1991). Teleost fish have been well documented for their reduced capacities to clear plasma glucose levels, and hence are more prone to suffer from stress-induced hyperglycemia (Moon 2001).

In mammalian tissues, the glucose transporter (GLUT)-4 is the primary glucose transporter that is expressed in insulin-sensitive issues such as adipose tissue, skeletal muscle, and heart (Díaz et al. 2007). This transporter performs an integral task in the homeostasis of glucose by allowing insulin to function via the increase in glucose uptake within the insulin-responsive tissues (Díaz et al. 2007). Under normal cellular conditions, GLUT-4 is present within intracellular stores until it is stimulated by insulin, which causes the transporter protein to be translocated from the cytoplasm to the plasma membrane, thus allowing glucose to enter into the cells stimulated by insulin (Díaz et al. 2007). Hence, GLUT-4 has been considered a crucial component in avoiding plasma hyperglycemia, such as during the postprandial period, or during times of stress (Díaz et al. 2007). Furthermore, Díaz et al. (2007) reveal that while rainbow trout muscle tissue does possess a GLUT-4 transporter which is similar in structure and function to the GLUT-4 transporter proteins found in the skeletal muscle tissues present in mice, the marine homolog of GLUT-4 is less efficient than the murine version in clearing plasma glucose.

Therefore, this study was developed to determine the effects of puerarin, the most biologically active isoflavone present in the roots of kudzu (*Pueraria lobata)* (see Prasain et al. 2004), which is an invasive plant (Peng et al. 2009), on modulating acute stress within salmonids. Since salmonids are known for their migration patterns upstream, a practice that has been reported to induce stress within fish (Leonard and McCormick 1999). While kudzu powder has been used in oriental countries as a natural treatment for cardiovascular diseases (Yan et al. 2006), puerarin has been shown to reduce skeletal tissue blood glucose levels in diabetic mice over a period of 1 day; moreover, puerarin helped restore GLUT-4 mRNA expression levels in the diabetic mice to those of non-diabetic mice within a duration of 3 days (Hsu et al. 2003). Since rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), possessed GLUT-4 transporter proteins, we chose chinook salmon (also called king salmon), *Oncorhynchus tshawytscha* (Walbaum, 1792), which belongs to the same genus as the rainbow trout, and is a species which is farmed. In addition, chinook salmon has been shown to elicit very high post-stress plasma cortisol levels; often reaching 400 ng · mL^{-1} during upstream migration in the wild (Barton 2002). Performing stress response studies under laboratory conditions are difficult since most often the fish are acquired from hatcheries where fish are reared with thousands of other fish. Therefore, it is harder to induce stress through crowding as the fish are accustomed to be in close proximity to each other since birth. Thus, fingerling chinook salmon, due to their capacity to be stressed, provided us with a good model organism to determine the effects of puerarin on acute stress, which can be difficult to elicit amongst other fish species (Barton 2002). In addition, we hypothesized that since crowding is ubiquitous in aquaculture, the major disruptive practice of handling or chasing for transportation or vaccination is more unsettling as they weaken the animals to a greater extent. Therefore, we decided to induce stress through handling, since we could also be certain of the generation of stress within the fish.

We hypothesized that since teleost fish possess diminished capacities to clear their blood-glucose levels, the presence of puerarin will help alleviate the symptoms of elevated sugar concentrations within the organisms. Subsequently, this will allow the fish to regain normal homeostatic levels which will signal the cessation of other stimulators of the stress response such as cortisol. The aim of this study was to determine the impact of puerarin on physiological parameters such as plasma cortisol, blood-glucose, plasma protein levels, packed-cell volume, and spleen somatic indices (SSIs), which has been studied in multiple fish-stress studies (Barton and Iwama 1991, Wendelaar Bonga 1997, Ruane et al. 1999, Hadidi et al. 2008).

MATERIALS AND METHODS

Fish acquisition and maintenance. Chinook salmon fingerlings (3 months old) weighing between 2 to 5 g were obtained from the Bodine State Fish Hatchery (Mishawaka, IN) and transported to the Life Sciences Resource Center (LSRC) at Indiana University–Purdue University Fort Wayne. The fish were housed in twenty-four 10 US gallon (37.8 L) tanks containing dechlorinated water which was filtered and oxygenated by Millennium 2000 filters (Aquarium Systems, OH). The dechlorination stage consisted of filling large bins with city water; bins were left uncovered under light for approximately one week with aeration. Through this process chlorine was slowly evaporated from the supplied water in the bins, which was subsequently used to fill the fish tanks. After being transported into LSRC, the fish were allowed to acclimate to their new conditions for two months. The fish were fed daily with commercial fish feed (Aquamax Starter Fish Fry 200)—which would form the control feed for our study—to satiation, while being observed routinely to prevent any manifestation of any disease.

The water temperature was monitored once daily during the acclimation period and twice daily within the acute stress study. The fish were housed in a room where the ambient temperature did not exceed 18ºC and the water temperature for the fish was kept between 16–18ºC. In addition to water temperature, water chemistry parameters such as dissolved oxygen of the water were kept in between 6.5 and 9.3 mg · L^{-1}, and the pH in between 6 and 9. In addition, the ammonia was less than 0.25 mg · L^{-1}, the nitrate in within 25–50 mg · L^{-1}, and the nitrite at 0.3 mg · L^{-1}. All of the aforementioned parameters were within the prescribed values from Wedemeyer et al. (1990). Throughout the acclimation and study period, the fish were kept in a room with a diurnal cycle which was divided into 16 h light and 8 h of darkness.

During the study, the control fish were fed control feed, while the puerarin-feed fish were fed Aquamax Starter Fry 200 fish feed which was supplemented with puerarin prior to the start of the study. Puerarin supplement (catalogue # P5555) was purchased from Sigma-Aldrich (St. Louis, MO). To incorporate puerarin into the control feed, the isoflavone was dissolved in a solution of reagent grade 95% alcohol due to puerarin's high solubility in alcohol (Wang and Cheng 2005). The dissolved solution of puerarin was then carefully sprayed on the feed in small aliquots, and the feed was air-dried and stored at 4ºC (Mustafa and MacKinnon 1999) to make a concentration of 200 mg · kg^{-1} (0.02%). The concentration was deemed appropriate since prior studies on mice by Peng et al. (2009) used feed which gave mice 200 mg kudzu powder which consisted of 68 mg of puerarin. Our feed consisted of 200 mg of puerarin, which is approximately three times greater than Peng et al. (2009), since we wanted to ensure that the fish were getting enough puerarin, as there is wastage when puerarin is thrown in the water. To minimize the loss of puerarin through evaporation, the feed was prepared within one week prior to the start of the study in June.

Experimental design. On the day of the start of the study at 0000 h, stress was induced by randomly transferring all the fish to eight tanks located in two separate systems: the first system contained four tanks: two of which housed the control unstressed groups while the remaining two tanks housed the control stressed group. Similarly, the second system also consisted of four tanks which were fed puerarin-supplemented feed: two tanks housed fish which were not stressed, while the remaining two consisted of fish which were stressed. The separation of the systems was done to prevent any cross-contamination of feed between the control and puerarin feed groups. This is because the systems used for this study recycled the majority of the water that ran through the tanks. Prior to the random distribution of fish into the experimental groups, 6 fish were randomly selected from the 24 tanks and were sampled for physiological and immunological parameters and accounted for our results from day 0 (CFU D0). Five h following the transfer, the fish were fed feeds according to their respective groups. Shortly after the fish were fed to satiation, stress was induced within the stressed groups by chasing, catching, and netting the fish with a net for a period of 5 min to induce acute handling stress. Subsequently, the fish were sampled 24 h after being transferred to their respective tanks.

Two samplings were performed for this study: at 0000 h and 2400 h. All the sampled fish were selected randomly and caught efficiently with the least amount of chasing with a net. Following capture, the fish were euthanized in a 1 L solution of 200 mg of MS-222 (tricane methanosulfonate; Sigma-Aldrich; St. Louis, MO) (Gerwick et al. 1999, Halloway et al. 2004). Subsequently, the health and stress levels of the fish were determined by the measuring the length, weight, condition factor (K), blood glucose, hematocrit, plasma protein levels, and spleen sizes to calculate spleen somatic indices (SSIs).

The 4 experimental groups in this experiment were categorized as control feed unstressed (CFU), control feed stressed (CFS), puerarin/kudzu feed unstressed (KFU), and puerarin/kudzu feed stressed (KFS). Since the study was performed over a 24-h period, the control feed unstressed fish at the start of the experiment or at 0000 h were categorized as CFU D0, while the remaining groups were sampled after 24 h or 1 day, which are classified as D1. Therefore, the 4 sampled groups at day 1 were labeled as CFU D1, CFS D1, KFU D1, and KFS D1.

Blood collection. The fish were sampled by drawing blood from the caudal vein with the help of heparinized syringes to prevent clotting. The needles were inserted into the midline of the fish while aiming for the center of the vertebral body. The extracted blood samples were then utilized for determining the plasma cortisol, plasma glucose, hematocrit, and total protein levels.

Plasma cortisol and glucose concentration measurements. Cortisol concentration was determined with the help of the goat antibody kit using plasma cortisol collected from fish sampled. The kit was purchased from Enzo Life Sciences, Farmingdale, NY. The measurements are expressed in ng · $mL^{-1}$with a sensitivity of less than 1 percentage point. The minimum detectable level of cortisol for the kit was 0.156 ng · mL^{-1}.

Plasma glucose concentration was determined by placing a drop of collected blood from each fish onto a glucometer strip which was then placed in a standard glucometer (Precision Xtra, Abbott Laboratories; Abbott Park, IL). This method for testing blood glucose has been validated for use on fish by Wedemeyer et al. (1990).

Blood hematocrit and plasma protein levels. Following plasma glucose measurement, blood hematocrit levels were measured from the collected blood when loaded into a capillary tube. After loading, the capillary tubes was capped with at one end and spun in micro-centrifuge. After spinning, Micro-Hematocrit Capillary Tube Readers (Monoject Scientific, St. Louis, MO) were employed to determine Packed Cell Volume (PCV). Subsequently, plasma protein levels were measured through the usage of refractometer after adding one to two drops from the spun hematocrit tubes.

Spleen somatic indices (SSI). SSI were generated when the spleen was removed from each sampled fish and weighed on an electrical scale. Upon weighing, the SSI was calculated through the usage of the formula: SSI = (spleen weight/ body weight) × 100 (Pearson and Stevens 1991).

Statistical analyses. The data collected through the course of this experiment were analyzed using SigmaPlot 11.0 software. The means and standard errors of the means were determined for each assay through one-way analysis of variance (ANOVA). Subsequently Tukey's test and Student's simple *t*-test were performed to determine whether the differences between the samples were significant ($P < 0.05$). The findings of our analyses are presented in the text and graphs in the form of means ± standard errors of means (SEM).

Ethical issues. Fish were kept at optimal aquatic conditions set for this species throughout the duration of the experiment and cared for according to the guidelines of the Purdue University Animal Care and Use Committee.

RESULTS

Over 24-h period, the plasma cortisol concentrations of the unstressed groups within both feed groups receded to levels similar to those of from day 0. On 0000 h (D0), the cortisol concentration for control feed unstressed (CFU) was (2.29 ± 0.31 ng · mL^{-1}), which was similar to that of puerarin feed unstressed (KFU) (2.01 ± 0.41 ng · mL^{-1}) after 24 h (D1), while control feed unstressed at day 1 (CFU D1) underwent a cortisol reduction at (1.14 ± 0.41 ng · mL^{-1}) which was significantly different ($P < 0.05$) from both CFU D0 and KFU D1 (Fig. 1). Whereas both unstressed groups had their plasma cortisol levels revert back to basal levels after 24 h, both stressed groups (control feed stressed (CFS) and kudzu feed stressed (KFS)) experienced increases in circulating concentrations of cortisol. The stressed groups fed control diet (CFS D1) elicited cortisol levels (10.83 ± 5.34 ng · mL^{-1}) which were approximately ten times greater than unstressed control feed. Conversely, puerarin fed stressed group (KFS D1) produced cortisol levels (14.67 ± 3.42 ng · mL^{-1}) which was approximately seven times greater than that of KFU D1 and significantly different ($P < 0.05$) from CFU D1. Therefore, the experimental conditions were successful in manifesting stress, which can be inferred from the elevated levels of cortisol within the stressed groups relative to the unstressed groups.

The blood glucose concentrations within the four groups varied depending on the type of feed, rather than the type of stress treatment being administered. Whereas differences in plasma cortisol concentrations could be distinguished based on the cumulative nature of the stressors applied, blood glucose levels were similar within the stressed and non-stressed groups (Fig. 2). However, the differences between the basal values from 0000 h (CFU D0) (45.33 ± 8.09 mg · dL^{-1}) were significantly different ($P < 0.05$) from those of CFU D1 (79 ± 9.7 mg · dL^{-1}) and CFS D1 (70 ± 3.93 mg · dL^{-1}) after 24 h. Therefore, the plasma glucose concentrations within both stressed (CFS D1) and non-stressed (CFU D1) control feed groups were significantly elevated relative to the fish from day 0 (CFU D0). Similarly, the plasma glucose concentrations for KFU D1 (42.67 ± 4.26 mg · dL^{-1}) was significantly lower ($P < 0.05$) than both CFU D1 and CFS D1. However, there were no significant differences between CFU D1 and CFS D1. Conversely, KFS D1 (49.67 ± 5.97 mg · dL^{-1}) was significantly lower ($P < 0.05$) than CFS D1. Meanwhile, the difference between CFU D1 and KFS D1was approximately 37 percentage points, and there were no significant differences between KFU D1 and KFS D1. Therefore, there were significant differences between control-feed unstressed and puerarin-feed stressed and unstressed groups.

Hematocrit or packed cell volumes (PCVs) were calculated to be elevated across all four groups after 24 h (Fig. 3). On day 0, the basal value (CFU D0) was ($33.75\% \pm 1.25\%$), which was significantly lower ($P < 0.05$) than both CFU D1 ($43.33\% \pm 1.20\%$) and CFS D1 ($47.33\% \pm 4.49\%$) after 24 h. There was no significant difference between CFU D1 and CFS D1. Therefore, both control feed groups produced higher PCVs than that of CFU D0. In addition, CFU D0 was also significantly lower ($P < 0.05$) than KFS D1 ($40.00\% \pm 1.73\%$). While there is no significant difference between KFU D1 ($31.67\% \pm 4.91\%$) and KFS D1, the former was 20 percentage points lower than CFU and 33 percentage points lower than CFS.

Plasma protein levels were elevated within all four groups on day 1 relative to the levels from day 0 (CFU D0) (3.33 ± 0.17 g · dL^{-1}), which was significantly lower ($P < 0.05$) than both CFU D1 (4.93 ± 0.18 g · dL^{-1}) and CFS D1 (5.27 ± 0.33 g · dL^{-1}) (Fig. 4). Therefore, both control feed groups: stressed and unstressed, had greater plasma protein in their blood. Similarly, CFU D0 had significantly lower ($P < 0.05$) plasma protein concentration than KFU D1 (4.07 ± 0.07 g · dL^{-1}), which was significantly lower ($P < 0.05$) than both CFU and CFS at day 1. Therefore, the supplementation of puerarin lowered plasma protein concentrations within the unstressed group. There was no significant difference between KFS D1 (4.27 ± 0.37 g · dL^{-1}) and KFU D1, and KFS D1 about 6 percentage points higher than KFU D1, while being approximately 15 percentage points lower than CFU D1 and approximately 23 percentage points lower than CFS D1. Therefore, fish fed puerarin had improved markers which can be used to predict improved blood flow within fish.

Our findings indicate that the transfer of salmon into specific groups was a stressor, since spleen sizes were reduced in all four groups relative to the values obtained from day 0. However, the spleen sizes were further reduced within both stressed groups relative to both control groups (CFU D1 and CFS D1) after 24 h; conversely, spleen sizes were slightly bigger within puerarin feed stressed groups (Fig. 5). The SSIs for CFU D0 (0.24 ± 0.019) was significantly higher ($P < 0.05$) than CFS D1 (0.12 ± 0.018) and KFS D1 (0.15 ± 0.016). The difference between KFU D1 (0.17 ± 0.008) and KFS D1 was 12 percentage points while the difference between CFU D1 (0.18 ± 0.016) and CFS D1 was 31 percentage points. Overall, SSIs were

approximately 20 percentage points lower within control feed unstressed (CFU) and puerarin feed unstressed (KFU) from D1 relative to control feed unstressed at day 0 (CFU D0). Therefore, the cumulative effects of moving into different tanks and the subsequent chasing with nets produced similar shrinkage of spleens within the fish. However, the puerarin feed stressed group appears to have coped marginally better with the stressful stimuli than control feed stressed group.

DISCUSSION

The production of cortisol and its subsequent release from interrenal cells (adrenal cortex homologue in fish) within the HPI axis takes several minutes, and research has shown that proper execution of protocol is sufficient in allowing the measurement of resting levels of cortisol within fish (Barton 2002). However, the practice of preparing fish for experimental handling by capturing and transferring them to a small container consisting of anesthetizing or

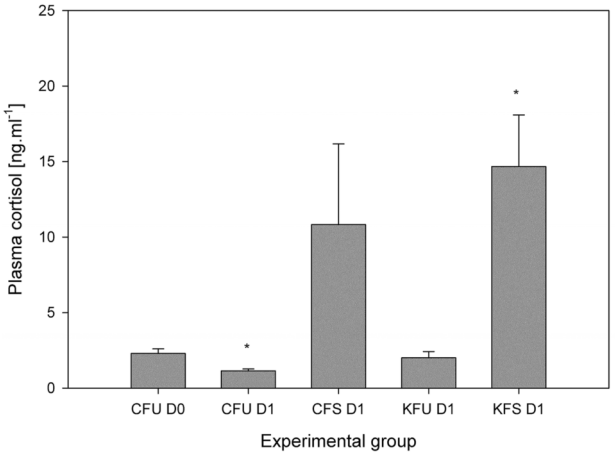

Fig. 1. Plasma cortisol concentrations (mean ± SEM) in different groups of experimental fish (Chinook salmon, *Oncorhynchus tshawytscha*); CFU = control feed, not stressed; CFS = control feed, stressed; KFU = puerarin feed, not stressed; KFS = puerarin feed, stressed; D0 = 0 h per day 0; D1= 24 h per day 1; *significantly different ($P < 0.05$) from CFU D0 (Control feed not stressed group at day 0)

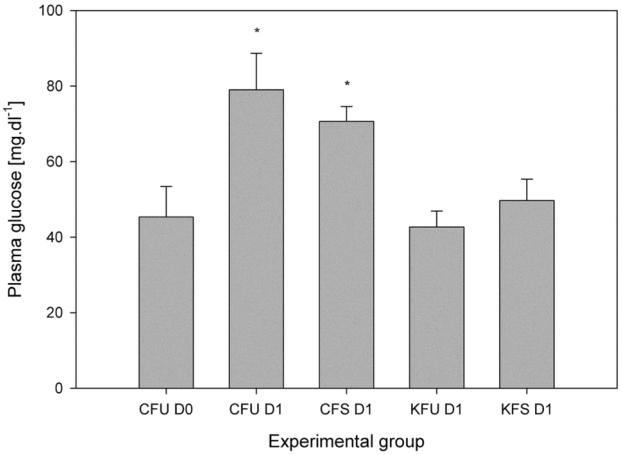

Fig. 2. Plasma glucose concentrations (mean ± SEM) in different groups of experimental fish (Chinook salmon, *Oncorhynchus tshawytscha*); CFU = control feed, not stressed; CFS = control feed, stressed; KFU = puerarin feed, not stressed; KFS = puerarin feed, stressed; D0 = 0 h per day 0; D1= 24 h per day 1; *significantly different ($P < 0.05$) from CFU D0 (Control feed not stressed group at day 0)

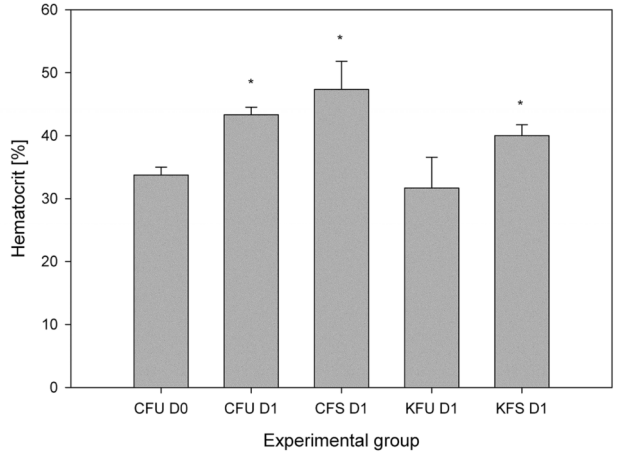

Fig. 3. Hematocrit or Packed Cell Volume (PCV) (mean ± SEM) in different groups of experimental fish (Chinook salmon, *Oncorhynchus tshawytscha*); CFU = control feed, not stressed; CFS = control feed, stressed; KFU = puerarin feed, not stressed; KFS = puerarin feed, stressed; D0 = 0 h per day 0; D1= 24 h per day 1; *significantly different ($P < 0.05$) from CFU D0 (Control feed not stressed group at day 0)

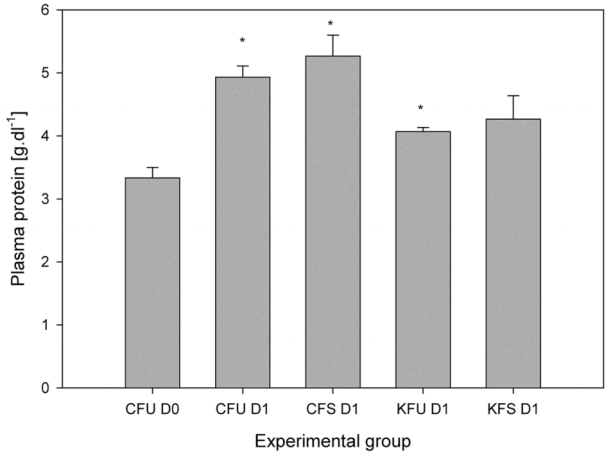

Fig. 4. Plasma protein concentrations (mean ± SEM) in different groups of experimental fish (Chinook salmon, *Oncorhynchus tshawytscha*); CFU = control feed, not stressed; CFS = control feed, stressed; KFU = puerarin feed, not stressed; KFS = puerarin feed, stressed; D0 = 0 h per day 0; D1= 24 h per day 1; *significantly different ($P < 0.05$) from CFU D0 (Control feed not stressed group at day 0)

euthanizing agent such as MS-222 (tricaine methanesulfonate) has deficiencies; since this practice exposes the fish to the stress of being chased, netted, passed through the air, placed into a small, unfamiliar container before the anesthetic takes effect (Gerwick et al. 1999). The latter procedure, even when executed proficiently, cannot eliminate the possibility of the activation of sympathetic stress response pathways (Gerwick et al. 1999). Thereby, increasing the chances of variation within the sampled fish and reducing the possibility of acquiring the most accurate data. On the other hand, one can counteract such an obstacle by sampling more fish; however, the chances of induction of the stress response within both the stressed and unstressed groups increases each time attempts are made to capture more fish. Therefore, near the end of a long sampling run, most of the fish will be stressed and have elicited sympathetic activation; thus, producing inaccurate results (Holloway et al. 2004). Therefore, the sample sizes were kept relatively low in order to reduce the chances of sympathetic activation. Such restrictions highlight the necessity of the development of new techniques in the practice of capturing fish for sampling.

After being transferred to new tanks, 24 h was sufficient for returning plasma cortisol levels to the basal level from day 0, since both CFU D1 and KFU D1 produced similar concentrations of cortisol, which is in agreement from prior research studies (Barton and Peter 1982, Barton 2002). Also, the practice of chasing the stressed groups with nets for 5 min was successful in eliciting activation of the HPI axis as both CFS D1 and KFS D1 produced similarly elevated plasma cortisol levels after 24 h. In addition to the potential of variations resulting from the capture protocol, other factors such as heritability or the environment, such as the time of the day, can lead to variations within sampled fish (Bry 1982). Furthermore, due to the dynamic nature of the stress response, multiple physiological parameters are measured due to the mutually exclusive nature of these indicators. The measurement of elevated plasma cortisol concentrations does not necessarily mean that the plasma glucose levels will also be elevated (Barton 2002). Therefore, while our findings illustrate that plasma cortisol levels were attenuated within both unstressed groups, and elevated within both stressed groups, one cannot, however, use our results as a definite indicator for cortisol modulation through both control and puerarin-supplemented feed.

Whereas plasma cortisol is often used as an indicator of the primary stress response (Barton and Iwama 1991, Wendelaar Bonga 1997), the determination of plasma glucose is a useful tool for determining the secondary stress response due to its ease of implementation and widespread usage within aquaculturists (Wedemeyer et al. 1990, Barton 2000). Despite daily fluctuations in plasma glucose levels within salmonids (Barton 2000, 2002), explaining the variations within the two control groups on day 1; surges in plasma glucose concentrations within stressed fish are often used as hallmark indicators of the stress response within salmonids (Wedemeyer et al. 1990,

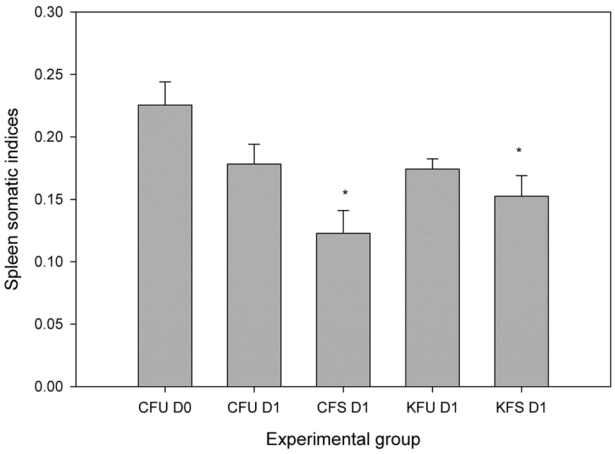

Fig. 5. Spleen somatic indices (SSI) (mean ± SEM) in different groups of experimental fish (Chinook salmon, *Oncorhynchus tshawytscha*); CFU = control feed, not stressed; CFS = control feed, stressed; KFU = puerarin feed, not stressed; KFS = puerarin feed, stressed; D0 = 0 h per day 0; D1= 24 h per day 1; *significantly different ($P < 0.05$) from CFU D0 (Control feed not stressed group at day 0)

Barton and Iwama 1991, Wendelaar Bonga 1997). In addition, prior research with puerarin has shown that puerarin improves glucose tolerance and acts as an antihyperglycemic within mice (Meezan et al. 2005). Furthermore, puerarin has been shown to induce increased cellular reuptake of glucose into cells grown in vitro (Hsu et al. 2003). Therefore, our findings, which indicate that the supplementation of puerarin to commercial fish feed attenuates plasma glucose concentrations to levels similar to the glucose concentrations seen in day 0 samples is concurrent with prior research on glucose homeostasis (Hsu et al. 2003). Although our findings could not illustrate any significant differences within both stressed- and unstressed groups fed either control or puerarin feed, our study reveals that puerarin-fed fish had lower plasma glucose levels relative to fish fed control feed. We believe the supplemented puerarin enhances plasma glucose reuptake within the cells of the fish; regardless of stress. This data is concurrent with other studies on the effects of puerarin on insulin-resistant obese mice with reduced glucose tolerance, where puerarin enhanced plasma glucose clearance and reduced plasma insulin levels-reducing the chances of developing insulin resistance within peripheral tissues (Meezan et al. 2005, Prasain et al. 2012). The beneficial effects of enhanced plasma glucose metabolism by puerarin could be observed in other biomarkers of overall health. Since puerarin has also been shown to reduce bone loss and blood cholesterol, all while improving blood circulation and overall health (Meezan et al. 2005, Yan et al. 2006, Peng et al. 2009, Tanaka et al. 2011). Therefore within a 24-h period, we can ascertain that the secretion of cortisol into the blood of our fish led to an elevation of glucose concentration within all four groups. However, while the

cortisol levels dropped within both control-feed and puerarin-feed unstressed groups, due to the slower glucose homeostasis within fish (Moon 2001, Díaz et al. 2007), the plasma glucose concentrations remained elevated within the control-feed groups, while returning to their physiologically normal levels within the puerarin-feed groups.

While plasma cortisol and glucose are endocrine indicators of stress, hematocrit or packed cell volume (PCV) and plasma protein concentrations are indicative of the cellular response to stress, where PCV is the ratio of the volume occupied by packed blood cells to the volume of whole blood as measured by a hematocrit (Chaplin et al. 1953). The measurement of hematocrit has been used as an indicator for increased risk and development of cardiac disease (Ring et al. 2008). Upon the induction of acute stress, cells produce proteins such as heat shock proteins and chaperones (Iwama et al. 1999). In addition, the presence of acute mental stress can lead to an increase in PCV and combined with elevated plasma proteins, can cause reductions in blood plasma volume (Ring et al. 2008). A reduction in blood plasma volume will lead to greater energy expenditure by fish to pump blood to their tissues, therefore leading to elevated plasma glucose levels and in extreme cases, exhaustion (Barton and Iwama 1991, Wendelaar Bonga 1997). Furthermore, stress induced hyperglycemia can lead to insulin-resistance which can reduce blood flow within skeletal tissues (Laakso et al. 1990). Our results indicate that the presence of puerarin in fish feed reduced both PCV and plasma protein levels, which is in accord with the findings from studies on other animal models (Yan et al. 2006), in that along with its antihyperglycemic properties, puerarin has been shown to induce vascular relaxation in arteries while enhancing greater blood flow through induction of the synthesis of endothelial nitrous oxide synthase within arteries, and offered protection against hypercholesterolemia within mice (Yeung et al. 2006). In addition, elevated plasma glucose levels have been associated with reduced coronary blood flow (Yokoyoma et al. 1998). Therefore, combining our results with prior findings, puerarin promotes improved blood flow and cardiovascular function, both by relaxing the arterial walls and helping maintain a healthy blood volume, and improves overall health of fish under stress.

In addition to endocrine and cellular markers for stress, the sizes of spleens in fish are used as a predictor of disease resistance (Barton and Iwama 1991, Wendelaar Bonga 1997). In mammals, the spleen is responsible for filtering blood and removing damaged or dying cells which are not fit for circulation (Hadidi et al. 2008). The spleen is specialized for this task since it possesses a dense interlocking web of splenic cords, which contains macrophages which remove any foreign organisms very efficiently when circulating blood runs through (Bohnsack and Brown 1986, Tablin et al. 2002). In case of salmonids, the spleen produces the majority of antibodies and helps form immunological memory (Hadidi et al. 2008). Therefore, the spleen is an important organ, and a smaller spleen is indicative of stress and reduced disease resistance, as there is less blood circulating through the spleen, as it is diverted to skeletal tissues for fight or flight response, and there is reduced macrophage activation to counteract any infection (Barton and Iwama 1991, Wendelaar Bonga 1997, Hadidi et al. 2008). In line with prior research, our results indicate that the addition of puerarin assists in enhanced blood flow, which helps in reduced shrinking of the spleen. Therefore, with increased blood flow, macrophages in the spleen will have greater exposure to any pathogens; thereby reducing the need for generating greater numbers of macrophages (an energy extensive process), and henceforth, conserving energy (Barton and Iwama 1991, Wendelaar Bonga 1997, Hadidi et al. 2008).

In conclusion, we were successful in creating a study which generated stressful conditions, under which puerarin, concurring with prior studies, was able to promote overall well being of the fish as it enhanced greater systemic circulation while attenuating plasma glucose. We consider this study informative in illustrating the possibilities with supplementing puerarin with aquaculture feed to improve circulation within fish. However, due to genetic and environmental variations, future studies need to be conducted to determine the cellular interaction with puerarin within fish cells. In addition, subsequent studies plan on evaluating the long-term effects of puerarin-feed on salmonids under stress.

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RESEARCH PAPER

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Microplastic surface properties affect bacterial colonization in freshwater

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Microplastics are a global concern in aquatic ecology and are readily colonized by bacteria in the environment. There is a lack of information on bacterial colonization of eroded and un-eroded microplastics in freshwater. In this study, six types of microplastics were incubated for 8 weeks in microcosms with water from Lake Erie. Microcosms were inoculated with one of three species: *Acinetobacter* (*A.*) *calcoaceticus, Burkholderia* (*B.*) *cepacia*, and *Escherichia* (*E.*) *coli*. These bacterial species are ubiquitous in water bodies associated with human populations. Bacterial surface coverage was determined using electron and fluorescent microscopy. Quantifications of EPS and surface roughness were performed by confocal microscopy and measuring contact angles (θ_w) of water droplets on microplastics, respectively. Analyses revealed surface coverage differed among bacterial species and plastic types after 8 weeks. As the study progressed, *E. coli* remained the most abundant while *A. calcoaceticus* gradually decreased on most surfaces. Analyses of microcosms revealed polypropylene disks had lower bacterial abundance. Conversely, eroded polypropylene disks had highest bacterial abundance, indicating importance of surface roughness (lower θ_w values) and surface physicochemical properties of microplastics in bacterial colonization. Our results demonstrated that bacterial colonization of microplastics is affected by both the physicochemical properties of microplastics and the physiological properties of colonizing bacteria.

KEYWORDS
bacteria, colonization, freshwater, microplastics, surface roughness

1 | INTRODUCTION

Microplastics are plastic debris with diameters of less than 5 mm [1–3] and there are two forms: primary and secondary [3–5]. Primary microplastics, such as microbeads in facial scrubs and other personal care products, are designed and manufactured to be less than 5 mm in diameter [1,3–5]. Secondary microplastics are generated from larger plastic debris that breaks down from exposure to environmental conditions, such as freezing-thawing, solar ultraviolet radiation, water currents, etc. [3–6]. The majority of

Abbreviations: ANOVA, analysis of variance; ATCC, American Type Culture Collection; CLSM, confocal laser scanning microscopy; DAPI, 4′,6′-diamidino-2-phenylindole; EPS, extracellular polymeric substances; HDPE, high density polyethylene; HSD, honest significant difference; LDPE, low density polyethylene; LDPE-E, low density polyethylene-eroded; PP, polypropylene; PP-E, polypropylene-eroded; PS, polystyrene; UV, ultra violet; WGA, wheat germ agglutinin.

microplastics in marine ecosystems originate from waste disposal, including via wastewater (such as from personal care products, fibers from washing synthetic fabrics, etc.) and runoff from landfills [6–9].

Microplastics are abundant in the Laurentian Great Lakes (Lakes Erie and Ontario have ~47,000 and 204,000 pieces/km^2, respectively) [7,8,38]; some locations within the Great Lakes have higher concentrations of microplastics than ocean gyres [8]. Because microplastics are abundant, stable, and inert they serve as a substratum for bacterial colonization in aquatic ecosystems [2,3,5,11–13]. Planktonic bacteria colonize microplastics in aquatic ecosystems and form biofilms, and compositions of attached bacterial communities vary among different plastic types [2].

Plastics are manufactured with different physicochemical surface properties based on their intended applications [1,14,15]. Microplastics with relatively smooth and inert surface morphologies are more hydrophobic and colonized less by bacteria, while rougher and charged surfaces are more hydrophilic and promote colonization [15,16]. Of the roughly 20 different types of consumer plastics, the most ubiquitous are polyethylene (PE), polypropylene (PP), and polystyrene (PS) [2,17]. Subtypes of PE [high density (HD) and low density (LD)], PP, and PS possess unique surface physicochemical properties that influence bacterial colonization [14,15,18]. Polyethylene is hydrophobic when dry, but wet PE surfaces are charged, which promotes bacterial adhesion [15,19]. Moreover, HDPE and LDPE possess different surface physicochemical properties and exposure to surface stressors causes HDPE surfaces to become rougher than LDPE [14]. Polypropylene is a methylated form of PE and more hydrophobic than PE, while LDPE and HDPE are chemically different based on the amount of PE chains packed into the polymers [15,20]. Conversely, PS consists of complex aromatic phenyl groups, making it more hydrophobic than PP [20]. After exposure to the environment, it is difficult to distinguish between HDPE and LDPE, but eroded PE debris remains relatively rougher and more hydrophilic than PP [15].

Plastic surfaces are designed to be smooth, hydrophobic, and not adsorb chemicals for their intended applications [9]. However, once discarded, exposure to solar and ultraviolet radiation, freezing, and thawing erodes plastic surfaces causing them to become pitted and acquire rougher surface morphologies, reducing hydrophobicity [2,3,5]. Increased surface roughness enhances adsorption of nutrients and surface area for bacterial colonization [3,5]. Alterations in surface morphologies resulting from environmental exposure vary among plastic types. For example, in marine ecosystems, PE becomes negatively charged while PP remains relatively inert [15]. Ultimately, these environmental-induced changes in surface properties likely impact bacterial colonization.

Production of EPS (extracellular polymeric substances) occurs in the form of matrices composed of hydrophobic compounds [21]. Matrices of EPS consist of hydrophilic channels which allow flow of water within hydrophobic compounds [16]. These hydrophobic compounds enable bacteria to generate hydrophobic environments on substrata which aid in colonization [16,18,22]. Production of EPS also reduces ionic repulsive forces between bacteria and substrate surfaces, and enhances attraction via Van der Waals' forces after overcoming ionic repulsive forces [18,22]. Components of EPS include polysaccharides, proteins, glycoproteins, and glycolipids, whose production is influenced by substratum properties and environmental conditions [16,23,24].

The majority of studies on bacterial colonization of microplastics have been performed in marine ecosystems [3,5]. Yet, in spite of dramatic differences in environmental conditions and bacterial communities between fresh and salt water [2,5], there is a lack of information on bacterial colonization of microplastics in freshwater ecosystems [3,9]. Therefore, in the present study, colonization of microplastics by bacterial species ubiquitous in freshwater biofilms [19,25] was investigated in water from Lake Erie (OH, USA). Because plastic type and exposure of the plastic to environmental conditions were hypothesized to influence bacterial colonization, plastics with various properties and that had been eroded in the natural environment were compared.

2 | MATERIALS AND METHODS

2.1 | Description of microplastics

All plastic types used in this study were identified using resin identification codes (#1–7) written underneath plastic pieces. In this study, we compared six types of plastics: four were recently discarded post-consumer plastics {HDPE (#2), LDPE (#4), PP (#5), and PS (#6)} that had not been exposed to the environment and two were eroded (E), beached plastics (LDPE-E and PP-E) that were collected from a Lake Erie beach at Edgewater Park in Cleveland, OH (USA). Sufficient quantities of eroded PS and HDPE that could be confidently identified were not obtainable. Beached plastic debris were gently cleaned (with dilute soap) and rinsed before use. All plastic pieces were handled with care to ensure none of their surfaces were scratched or altered while setting up experiments.

Microplastic disks in this study were generated from larger plastic debris. A hole-punch was used to generate flat plastic disks that were 4.5 mm diameter. Flat microplastic disks were used as they are suitable for microscopy and contact angle measurements. Microplastic disks were sterilized by placing them under a UV lamp (Fisherbrand; Pittsburgh, PA, USA) for 5 min on both sides. After

sterilization, aseptic technique was followed. Ten disks of each plastic type were placed in glass bottles that were loosely capped and contained 175 ml autoclaved water from Lake Erie (collected at Edgewater Park).

2.2 | Microplastic microcosms

Each microcosm contained one of the six tested plastic types, amended with one of three bacterial species; treatments were performed in triplicate. Microcosms were stationary when incubated at a temperature of 24 °C for 8 weeks. According to the National Weather Service (NWS), the average surface temperatures of Lake Erie during the summer months (June–August) ranged from 19 to 26 °C. Hence, a temperature of 24 °C (room temperature) was optimal for incubation.

The microcosms were incubated under artificial light. Bacterial species used in this study were: *Acinetobacter calcoaceticus* (ATCC 23055), *Escherichia coli* (ATCC 11775), and *Burkholderia cepacia* (ATCC 25416). All bacterial species are heterotrophic and belong to the phylum Proteobacteria. All bacterial species used in this study were originally obtained from ATCC, and matched their respective properties. These bacteria differ in the number of flagella they possess: *A. calcoaceticus* is non-flagellated, *B. cepacia* is monotrichously flagellated, and *E. coli* is peritrichously flagellated. Moreover, *A. calcoaceticus* can act as bridging bacterium in biofilms on a variety of surfaces and causes surface modification during colonization on some substrates [19,25]. Likewise, surfaces colonized by *B. cepacia* have reduced contact angles with water (θ_w) [19,25].

Bacteria were grown (in 8 g L^{-1} of Difco Nutrient Broth [Franklin Lakes, NJ, USA] with shaking at 35 °C) until they reached 1×10^9 cells/ml (an optical density of ~2 at 600 nm). Subsequently, 1 ml of each bacterial culture was added to a given microcosm. Bacteria were allowed to colonize the microplastics in the microcosms for 8 weeks, and were sampled at weeks 0, 4, and 8 and bacterial abundance, contact angles using water (θ_w), and EPS thickness were measured. Microcosms consisting of microplastic disks with no bacteria were also generated to act as controls.

2.3 | Bacterial abundance measurements

Bacterial abundance or surface coverage on microplastics was examined by fluorescence and scanning electron microscopy (SEM). Fluorescence microscopy was performed on three disks from each microcosm by placing them in 200 µl of glycerol and fixing cells with 200 µl of 3% paraformaldehyde. Subsequently, microplastics were stained with DAPI (4′, 6-diamidino-2-phenylindole) (Sigma–Aldrich, St. Louis, MO, USA) in the dark for 30 s. Bacteria were then visualized under an Olympus iX81 fluorescent microscope (Olympus Corporation; Center Valley, PA, USA) by capturing three images of the microplastics across three different regions on the microplastics. Numbers of bacteria in each region were counted and used to determine abundance.

For SEM imaging, surfaces of three disks from each microcosm were coated with gold nanoparticles for 5 min via a physical vapor deposition using a coating system to neutralize any surface charge and to fix bacterial cells [26]. Upon coating, samples were visualized using a Quanta 450 model (ThermoFisher Scientific, Hillsboro, OR, USA) e-beam lithography electron microscope. Imaging of microplastic disks in this study was difficult as they were environmental samples and the surfaces were not uniform, and generated image interference in the form of surface charge. Multiple images of bacteria were taken at 3, 10, and 20 µm across three different regions on the microplastics. Greater magnifications were difficult to observe, since microplastic disks used in this study consisted of environmental samples, which were not lab grade and generated signal interference at greater magnifications. Moreover, greater physical vapor deposition (5 min, typically 3 min are used for lab grade materials by the SEM facility) was required to eliminate interference from surface charge. Greater physical vapor deposition coated the surfaces of the disks and bacteria with greater amounts of gold. As a result, smaller bacterial cell wall components such as flagella and pili were harder to observe in this study. Subsequently, images were scanned at 20,000× magnification in triplicate, the areas of each image formed grids. The areas of grids were measured in micrometer squared (μm^2) using scale bars on images as seen in Ref. [27]. Bacterial counts from each image were averaged and converted to cells per millimeter squared (cells/mm^2).

2.4 | Surface roughness measurements

Contact angles for water (θ_w) were calculated by using the drop contact angle method for water (nanopure) [25] using Rame-Hart 250 goniometer (Succasumna, NJ, USA) by pipetting 5.0 µl of nuclease-free water on the surface of each microplastic disk and forming a water droplet. Subsequently, DROPImage Advanced (Rame-Hart, Succasumna, NJ, USA) software was used to calculate both left and right contact angles of the water droplet on each disk. Twenty repeated measurements were performed for each sample in triplicate at room temperature (24 °C ± 2). The values of the left and right angles were averaged.

2.5 | EPS measurements

Quantification of EPS thickness was performed using confocal laser scanning microscopy (CLSM). Three microplastic disks from each microcosm were placed on circular cover slips in small petri dishes. The disks were immersed in

200 µl of glycerol and cells on the disks were fixed with 20 µl of 3% paraformaldehyde. Upon fixing, 20 µl of Alexa-Fluor conjugated wheat germ agglutinin (WGA) was added to the disks. Alexa-Fluor conjugated WGA binds to N-acetylglucosamine residues in biofilms and fluoresces at 488 nm wavelength [28]. Microscopy was performed using an Olympus FV1000 microscope (Olympus Corporation, Center Valley, PA, USA). Images were captured at three different locations from each microplastic. EPS was subsequently calculated using ImageJ software on images captured with Olympus FV1000 microscope as described in Ref. [29].

2.6 | Statistical analyses

Data were analyzed using the open source program R as seen in Ref. [2]. Two-way ANOVAs were performed using plastic type and bacterial species as the treatments. Subsequently, Tukey Honest Significant Difference (HSD) tests were performed.

3 | RESULTS

3.1 | Bacterial abundance

Bacterial colonization of microplastic disks and erosion of surfaces were apparent when samples were observed with SEM (Fig. 1). Bacteria were not observed on uninoculated disks using both SEM and DAPI staining. Qualitatively, eroded disks had greater surface roughness at week 0 than recently discarded disks. However by week 8, surface roughness gradually increased on recently discarded disks, while no discernible changes in surface roughness occurred on eroded disks. Bacterial abundance based on SEM (Fig. 2) revealed significant interactions among plastic type and bacterial species at weeks 4 (species: p: < 0.001, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$) and 8 (species: $p < 0.01$, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$). Specifically, at week 4, abundance of *A. calcoaceticus* and *E. coli* was approximately fourfold higher than *B. cepacia* on LDPE (*E. coli*: $\sim 1430 \pm 385$ bacteria per mm^2) and LDPE-E (*E. coli*: $\sim 1450 \pm 366$ bacteria per mm^2) disks. Specifically, *E. coli* successfully colonized PS disks ($\sim 925 \pm 71$ bacteria per mm^2) and was approximately three times more numerous than other bacterial species. At week 8, LDPE disks were colonized by *E. coli* at approximately five times ($\sim 7000 \pm 1273$ bacteria per mm^2) greater abundance than *B. cepacia*. In contrast, at the same time, no *A. calcoaceticus* bacteria were observed on LDPE.

Bacterial abundance measured with DAPI staining generated results which were similar to results observed with SEM (Fig. 3), as there were significant differences at week 4 (species: $p < 0.05$, plastic: $p < 0.001$, species by plastic interaction $p < 0.05$) and week 8 (species: $p < 0.001$, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$). There was an overall decrease on LDPE and LDPE-E disks after 8 weeks, while PS disks had an increase in *B. cepacia* and *E. coli* abundance. On PP disks, *B. cepacia* abundance was approximately 30 times greater ($\sim 3540 \pm 254$ bacteria per mm^2) than other species, while abundance of *A.*

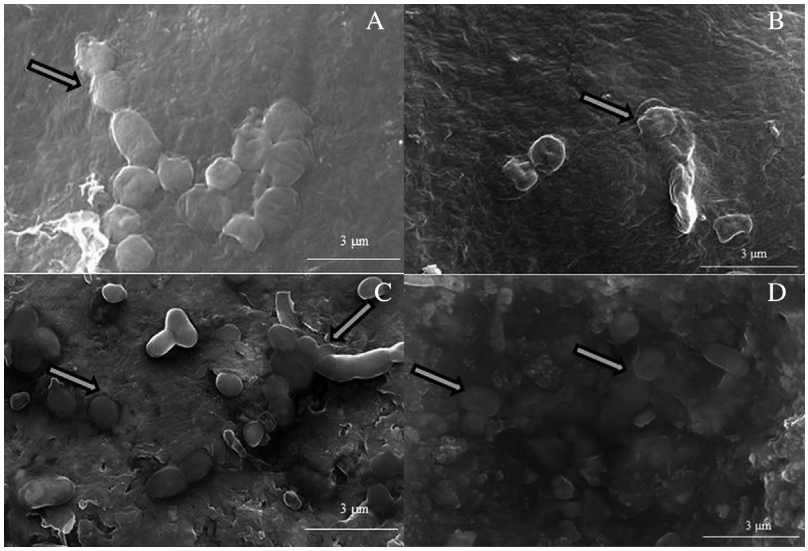

FIGURE 1 Bacterial surface coverage at weeks 4 and 8 on microplastic disks. SEM images are (A) *A. calcoaceticus* on PS at week 4, (B) *B. cepacia* on LDPE at week 8, (C) *A. calcoaceticus* on LDPE-E at week 8, (D) *B. cepacia* on LDPE-E at week 8

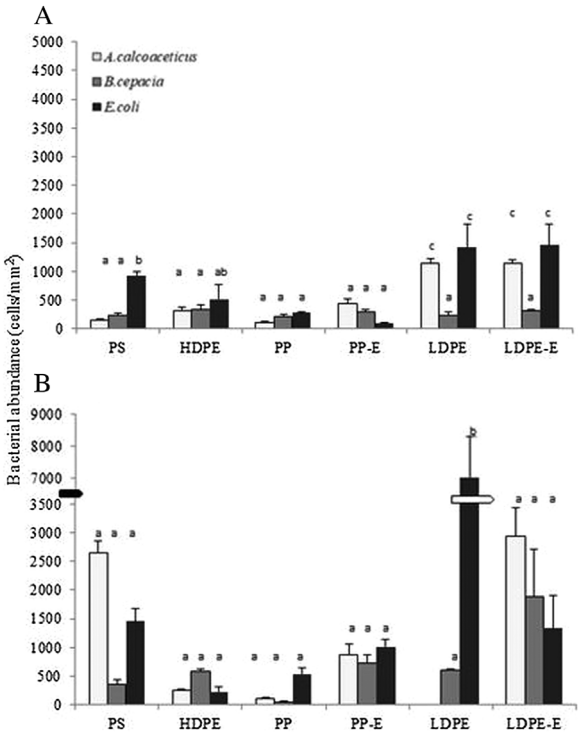

FIGURE 2 Bacterial abundance using SEM at week 4 (A) and week 8 (B). Values are means of (cells/mm^2) plus standard errors

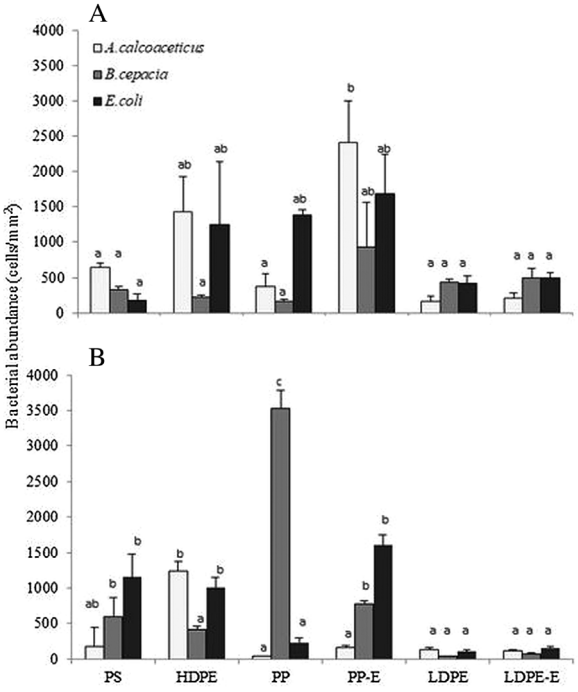

FIGURE 3 Bacterial abundance under fluorescence microscopy using DAPI staining at week 4 (A) and week 8 (B). Values are means of (cells/mm^2) plus standard errors

calcoaceticus on PP-E disks decreased by approximately 12-fold (week 4: ~2470 ± 578 to week 8: 166 ± 27 bacteria per mm^2) after 8 weeks.

3.2 | Surface roughness

Mean contact angles of water (θ_w) gradually decreased (lower θ_w values are indicative of hydrophilic surfaces) for all microplastic types; uninoculated disks (Table 1) had lower θ_w than those observed at weeks 4 and 8 (Fig. 4). At week 0, the θ_w for different microplastic disks were: PS 63.20 ± 0.089, HDPE 81.6 ± 0.98, PP 76.33 ± 0.057 degrees, eroded PP 63.66 ± 3.90, LDPE 82.60 ± 0.83, and eroded LDPE 78.36 ± 2.25. As bacteria colonized microplastic disks over the next 8 weeks, there were statistically significant interactions between species and plastic type at both weeks 4 (species: $p < 0.1$, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$) and week 8 (species: $p < 0.03$, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$). The θ_w for PS (47.48 ± 4.02 with *A. calcoaceticus*, 21. 85 ± 0.14 with *B. cepacia*, and 25.01 ± 2.30 with *E. coli*), PP-E (37.13 ± 3.12 with *A. calcoaceticus*, 52.63 ± 6.71 with *B. cepacia*, and 29.90 ± 1.39 with *E. coli*), LDPE (42.31 ± 8.46 with *A. calcoaceticus*, 27.1 ± 2.06 with *B. cepacia*, and 38.2 ± 0.2 with *E. coli*), and LDPE-E (26.26 ± 4.36 with *A. calcoaceticus*, 41.68 ± 5.04 with *B. cepacia*, and 47.56 ± 7.65 with *E. coli*) disks decreased through the 8 weeks of the study and their final θ_w values were lower than HDPE and PP disks.

3.3 | EPS thickness

Thickness of EPS (Fig. 5) varied among plastics and significant species and plastic interactions were observed at week 4 (bacterial species: $p < 0.1$, plastic: $p < 0.001$, bacterial species by plastic interaction $p < 0.001$) and week 8 (bacterial species: $p < 0.001$, plastic: $p < 0.001$, species by plastic interaction $p < 0.001$). Most microplastic disks had higher EPS thickness at week 4 relative to week 8. At week 4, HDPE colonized by *E. coli* and PP-E disks colonized by

TABLE 1 Contact angle (θ_w) measurements on microplastics at week 0

Plastic	Contact angle (θ_w)
PS	63.2 ± 0.05 a
HDPE	81.6 ± 0.98 b
PP	76.33 ± 0.03 bc
PP-E	63.66 ± 2.25 a
LDPE	82.6 ± 0.48 b
LDPE-E	78.36 ± 1.30 b

Values are means ±standard errors.

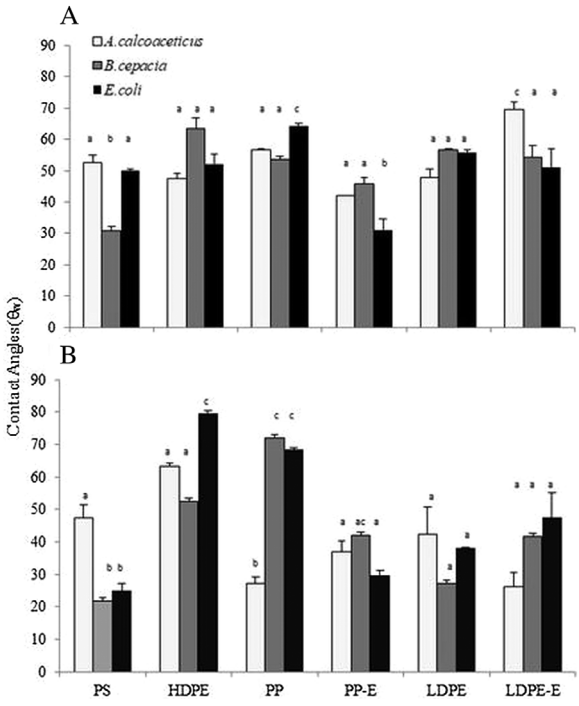

FIGURE 4 Analyses of contact angles on water (θ_w) at week 4 (A) and week 8 (B). Values are means of (θ_w) plus standard errors

A. calcoaceticus had higher EPS thickness than other microplastic surfaces. By week 8, the aforementioned microplastic disk and bacteria combinations had declined, while LDPE-E colonized by *E. coli* underwent a significant increase in EPS thickness by week 8.

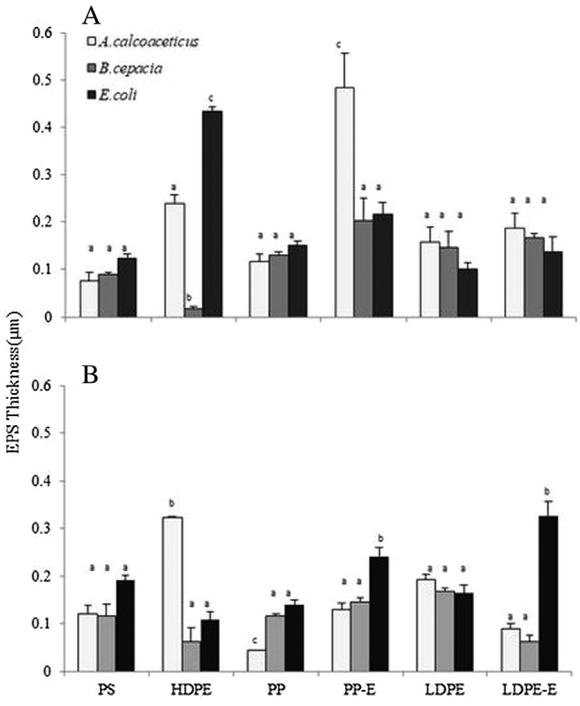

FIGURE 5 Analyses of average EPS thickness at week 4 (A) and week 8 (B). Values are means plus standard errors

4 | DISCUSSION

Plastics have microscopic flaws, such as grooves and pits, inadvertently introduced during manufacturing [14,15,18]. Exposure to environmental conditions expands these flaws and increases susceptibility to bacterial attachment [3,5,15]. In the present study, colonization by bacteria depended on plastic type and bacterial species. Differences among plastic types varied among species examined but, in general, greater bacterial abundance was observed on eroded plastics and on LDPE compared to HDPE and PS in this study.

Microplastics are diverse in their structural chemistry [14,15] and properties relevant for bacterial attachment [2,3]. Thus, plastics examined in this study differ in surface physicochemical properties [15,18,30]. Specifically, PS is a ubiquitous form of plastic which is known for its inert and hydrophobic nature, and is similar to PP [15]. In contrast, PE has a net negative charge in seawater which hinders bacterial attachment, while a net positive charge enhances bacterial attachment, but impedes biofilm formation [15,16,31]. Within the PE subtypes, differences may not be apparent after exposure to the environment [15].

As microplastics age in the environment, their surface roughness increases, which allows adsorption of ions and changes their physicochemical properties [2,15,16]. Exposure to solar radiation, high temperatures, and oxidation can release ester and ketone groups in plastics, which alters surface charge from neutral to a net negative charge on some microplastics, including PE and its subtypes [5,14,15]. Moreover, multiple factors such as the structural chemistry of different microplastics, length of exposure to the environment, and types of environmental conditions affect physicochemical properties of microplastics [3,5,15]. In aqueous environments, the surfaces of eroded microplastics adsorb organic and inorganic molecules which create ideal conditions for bacterial colonization [3]. Thus, as expected, both types of eroded microplastics examined in this study had relatively higher bacterial abundance after 8 weeks compare to un-eroded plastics. Moreover, PP-E disks had similar bacterial abundance to both LDPE and LDPE-E disks, which was higher than PP, HDPE, and PS. Collectively, these results demonstrate the importance of plastic physicochemical properties to bacterial colonization. Surfaces of PE and PS are negatively charged, while PP has been shown to be neutral [15,20]. The cell walls of the bacteria used in this study are negatively charged [20].

In addition to physicochemical properties of microplastics, physiological properties of bacterial species play important roles in adhesion to microplastics [18]. Bacterial adhesion is a complicated process and often diverges from the adhesion models seen in literature [16,32]. Most substrates are usually shielded by organic and inorganic compounds which adsorb on their surfaces prior to bacterial attachment [16,33]. Bacteria

use nanofibers to penetrate and overcome any repulsive forces from surfaces [16]. Nanofibers vary in length from several nanometers to several micrometers based on the bacterial species [16]. Bacterial nanofibers such as pili and fimbriae are important in adhesion to substrate surfaces [16]; flagella are important to probe smooth surfaces for bacterial attachment [16,18].

Bacteria used in this study are heterotrophic proteobacteria which differ in the number of flagella they possess [19,25]. *Escherichia coli* has been used as a model organism for bacterial attachment on multiple surfaces and are peritrichous [16,34,35]. Meanwhile, *A. calcoaceticus* lacks flagella but is a bridging bacterium that can auto-aggregate and form co-aggregates with multiple bacterial species in freshwater [19]. The bacterium is also capable of colonizing multiple surfaces in drinking water, a medium which is relatively deprived of organic and inorganic nutrients relative to freshwater and seawater. Thus, *A. calcoaceticus* has a strategy of aggregating with other bacterial species and forming biofilms to colonize surfaces to compensate for the lack of flagella [19,25]. *Burkolderia cepacia* are monotrichously flagellated and modify anthropogenic surfaces they colonize [19,20,25]. Our results showed that after 8 weeks, *E. coli* and *B. cepacia* were relatively more abundant on most surfaces than *A. calcoaceticus*. Abundance of *A. calcoaceticus* was generally higher at week 4 than week 8 in this study. Moreover, EPS thickness was higher on *A. calcoaceticus* colonized HDPE and PP-E at week 4 than week 8. Production of EPS differs among bacterial species; however, colonization is affected by the hydrophobic nature and surface roughness of microplastics [20]. Bacterial biofilm and EPS production varies according to surface hydrophobicity of plastics and other anthropogenic materials [20]. Our study demonstrates that bacterial EPS thickness was species-specific but was also affected by physicochemical properties of different microplastics. After 8 weeks, the θ_w values for all microplastic disks gradually reduced from the values measured at week 0. These results, combined with analyses of SEM images, suggested bacterial colonization increased surface roughness of microplastic disks over a span of 8 weeks. Prior studies have shown that *A. calcoaceticus* colonized PS at a slow rate and formed weak biofilms, which is in agreement with data from our experiments [36]. Electron microscopy images, combined with EPS measurements, suggest bacterial colonization produced biofilms that gradually increased over 8 weeks. In the present study, *A. calcoaceticus* generated θ_w values that were in between 25 and 55° on metallic- and glass-like surfaces as seen in Refs. [36–38]. However, the θ_w values observed on *A. calcoaceticus* colonized surfaces were measured after 2 h in Ref. [38]; whereas, the results in this study showed a gradual decline in θ_w values on *A. calcoaceticus* colonized surfaces over time. After 8 weeks, the θ_w values on all disks were lower than the values measured at week 0. Hence exposure to bacteria and the aquatic environment inside microcosms led to a decline in θ_w values in this study.

In conclusion, this study showed that physicochemical properties of microplastics (such as hydrophobicity and surface roughness) affect bacterial colonization. Over time, microplastic disks increased in surface roughness which affected bacterial abundance and EPS thickness. Moreover, eroded microplastic disks behaved differently from recently discarded microplastics. This study also demonstrated that the physiological properties of different bacterial species affected colonization of microplastics.

Results from this study raise questions about the roles of conditioning films on microplastic disks and their effects of bacterial colonization. Surfaces of eroded microplastic disks are likely primed for bacterial colonization through exposure to chemical ions in aqueous environments. Differences in colonization and surface modifications by the bacteria examined suggest that properties of the plastic impact composition of the plastic-attached community in freshwater ecosystems and that this, in turn, may alter the physical and chemical states of the plastics. Given the large quantity of plastics that are present in water bodies, these adherent bacterial communities may impact ecosystem functions.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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