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Effects of vitamin A added to diet in different amounts on oxidative stress, antioxidant defence of soft shelled freshwater *crayfish Pontastacus leptodactylus* (Esch, 1823)

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It was detected in this study the effects of vitamin A added to diet in different amounts on oxidative stress (malondialdehyde (MDA)), antioxidant enzyme activities (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px)) and GSH level in hepatopancreas and gills tissues of freshwater crayfish *Pontastacus leptodactylus* during soft shelled. Three different doses of vitamin A (100 mg kg^{-1} (VA1), 150 mg kg^{-1} (VA2), 200 mg kg^{-1} (VA3)) were used for this purpose. Tissue samples of the crayfish were taken immediately after they shed their shells, within one or two hours. After 74 day, the level of MDA was was higher in gills and in hepatopancreas of crayfish in C group according to NC group, SOD and GSH-PX were also same. But GSH was lower. After being fed antioxidant diets, there was a decrease in the MDA, SOD, GSH-PX and GSH values in the hepatopancreas and gill tissues of crayfish in these three groups compared to the control group.

This study was planned to give a concrete answer to the question of whether VA reduces or prolongs oxidative stress during molting of crayfish. At the end of the study, it was concluded that VA significantly reduced oxidatis stress in soft-shelled crayfish.

Key words: *Pontastacus leptodactylus, VA, Oxidative stress, Enzyme.*

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1. Introduction

Vitamin A (retinol) encompasses three distinct chemical com pounds, each serving specific functions in the body: retinol, an alcohol; retinal, an aldehyde; and retinoic acid [1]. As a vital micronutrient for animal growth and health, retinol plays a crucial role in livestock production. This fat-soluble vitamin is essential for various metabolic activities, including vision, immune system regulation, and cell differen tiation [2]. Vitamin A possesses antioxidant properties by directly scavenging reactive oxygen species, boosting antioxidant enzyme activity, and promoting antioxidant defence mechanisms [3].

The reactive oxygen species (ROS), natural by-products of aerobic metabolism, play an essential role in the physiological control of cell function in biological system. These highly deleterious compounds can rapidly react to

form other molecules such as peroxyl and hydroperoxyl radicals. To cope with the continuous generation of ROS in biological systems, there are adequate enzymatic and non-enzymatic antioxidant mechanisms. When the balance between the generation and the neutralization of ROS by antioxidant mechanisms within an organism is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs [4]. Lipid peroxidation, the biggest indicator of OS,

is a non-enzymatic chain reaction based on oxidation of mainly unsaturated fatty acids and is associated with the presence of ROS. It leads to the creation of lipid peroxides and other intermediates. The most common of these intermediates are malondialdehyde (MDA) and 4-hydroxynonenal [4,5]. The defence mechanisms against ROS consist of low molecular weight antioxidants glutathione (GSH), vitamin E, vitamin A and antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase and catalase. Fish and crustaceans that have rich source of polyunsaturated fatty acid lipids are more susceptible to the attack of ROS when compared with other aerobic organisms. For these reasons, excessive rise in ROS has also led to a decline in nutritional quality of these organisms [5,6] Many studies have reported that ROS increase in the several conditions such as exercise, pollution, infection, moulting, reproduction and starvation [5-8].

In crayfish, the body consists of two parts: cephalothorax (head + thorax) and abdomen [9]. The whole body is covered by a non-growing cuticle. As in other crustaceans, growth in crayfish is possible only by molting. The molting period is a process that includes the growth of internal organs and tissues, the formation of gastroliths and the periodic change of the exoskeleton (the animal's outer shell) [10,11]. This process affects the cell biology, physiology and behavior of crustaceans [12,13]. The molting process and frequency can be changed by factors such as photoperiod, temperature, density, hydrological conditions, stress, reproduction and nutrition [4,6]. Crayfish usually molt approximately 6-7 times in the first year, 5 times in the second year, 2 times in the third year, and once in the maturation period for females and twice for males [9]. There are some substances that are necessary for the nutrition of crustaceans during all developmental periods as well as during shell shedding periods, which cannot be synthesized in the body and must be taken from outside [4,14,15]. Some of these substances belong to the vitamin E, C, A and carotenoid group. Vitamin E is a fat-soluble, heat-resistant, strong antisterile vitamin [6]. Vitamin C is a metabolically strong reductant, and is related to the formation and preservation of intercellular material [16,17]. Carotenoids are color pigments, which make living things look attractive and create their meat color [18]. One of the most important known features of these three groups is that they are antioxidants or free radical scavengers [15-19].

The narrow-clawed crayfish, *Pontastacus leptodactylus*, used in this study is of great commercial importance and has a high demand in the Europe. Although A. leptodactylus is the native crayfish species in Turkey, domestic consumption of this species was very low. For this reason, it should be exported regularly to Europe. But the total annual harvest of the crayfish reduced dramatically because of overfishing, pollution and crayfish plague (*Aphanomyces astaci)* [20,21] Due to the importance of vitamin A, the aim of this study was to determine the effects of vitamin A added to diet in different amounts on oxidative stress (malondialdehyde (MDA)), antioxidant enzyme activities (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px)) and GSH level in hepatopancreas and gills tissues of freshwater crayfish *Pontastacus leptodactylus* during soft shelled.

2. Experimental

This study was carried out between July 10 and September 22 (74 days) at aquarium laboratory of Fırat University Aquaculture Faculty, Elazığ, Turkey. The crayfish used in the present study was provided from Keban Dam Lake population of *P. leptodactylus*.

Crayfish were housed in 12 glass aquariums (25 x 25 x 110 cm). Plastic pipes (15-20 cm in length and 7 cm in diameter) were provided as shelters for the crayfish. Adequate aeration was provided for each aquarium by a simple air pump. *P. leptodactylus* were acclimatised to temperature and flow conditions and starved for one week to standardize their nutritional conditions and to ensure that they were in good health prior to the start of the experiment. Samples of 15 crayfish were randomly selected **f**or analysis at the start of the experiment to provide baseline information on biochemical analysis. Triplicate groups of crayfish (12 individuals per group) were randomly assigned to each feeding treatment on July 10. The length (mm) and weight (g) were recorded for each crayfish. Crayfish were fed 2 % of their total wet weight daily, divided into three separate feedings.

Between September 12-22, a sample of 9 crayfish from each of the four dietary treatments was randomly selected for analysis. For biochemical assays, the hepatopancreas and gill tissues in the crayfish were removed and were stored at –80 ^{0}C until used [20].

During the trial, mean dissolved oxygen 7,03±0.32 mg/L; mean pH was 7.54±0.66 and water temperature were 15,42±2,24°C.

The crude protein content was analysed by Kjeldahl's method; the Gross energy was calculated based on physiological fuel values of 9 kcal g^{-1} for lipid and 4 kcal g^{-1} for protein and carbohydrate; the dry matter was determined after the sample was dried at 105 °C for 6 hours; the ash content was determined after 24 h at 550 °C in the furnace; the lipid was analysed by an ether extraction method [22] The ingredients for each diet were thoroughly mixed, before adding water, in a commercial food mixer, cold-pelleted by forcing through 3-mm holes using a

laboratory pellet mill, air-dried at 55 ºC for up to 24 h, and then stored in a refrigerator at -20 ºC until further use [23] The practical control diet used in this study (Table 1) was modified after Barım [20]. The control diet was formulated to contain approximately 38.86 % crude protein on a dry-weight basis and 3.32 kcal/g gross energy. Diet VA values were arranged according to Barım and Sahin [22] (VA1 (100 mg kg^{-1} VA), VA2 (150 mg kg^{-1} VA), VA3 (200 mg kg^{-1} VA)).

Tissue specimens were weighed, rinsed with ice-cold deionised water, cut into small pieces and then dried on a filter paper. The tissue samples were homogenized in an Eveljem-Potter homogeniser (Dupont Instruments, Sorwall Homogenizer, USA). The homogenates were centrifuged at 1000 g for 15 min at + 4 °C and the supernatants were used for MDA, SOD, GSH assays. The homogenates were centrifuged at 18.000 g for 30 min at +4 °C and the supernatants were used for the GSH-Px activity assay [24].

Table 1. Composition and proximate analysis of the control diet [21].

Ingredient	Percent of dry weight
Fish (anchovy) meal	35.78
Soybean meal	38.64
Wheat flour	19.30
Sunflower oil	4.00
Dicalcium phosphate	1.00
Sodium phosphate	0.40
Avilamycine[1]	0.10
Antioxidant [2]	0.10
Vitamin premix [3]	0.50
Mineral premix [4]	0.18

(1) Kavilamycine
(2) Antioxidant (mg/kg dry diet): butylated hydroxytoluene 12.5.
(3) Vitamin premix (IU or mg/kg): vitamin A 2,000,000 IU, vitamin D_3 200,000 IU, vitamin E 20,000 IU, vitamin K 3,000 mg, vitamin B_1 1,000 mg, vitamin B_2 3,000 mg, Niacin 30,000 mg, Calcium D-Pantothenate 10,000 mg, vitamin B_6 2,000 mg, vitamin B_{12} 4 mg, Folic Acid 600 mg, D-Biotin 200 mg, Choline Chloride 100,000 mg and vitamin C 60,000 mg.
(4) Mineral premix (mg/kg dry diet): Mn 80, Fe 35, Zn 50, Cu 5, I 2, Co 0,4, Se 0,15.

MDA level assay: The level of MDA as a marker of lipid peroxidation was measured according to the method of Ohkawa et al. [25] on the basis of the reaction with thiobarbituric acid (TBA). The formed MDA created a pink complex with TBA and the absorbance was read at 532 nm. The MDA level of tissues was expressed as nmol g^{-1} tissue.

SOD activity assay: The SOD activity was determined according to the Sun et al.[26] method. that is based on the principle that xanthine reacts with xanthineoxidase to generate superoxide radicals that react with nitroblue tetrazolium to form a coloured formazan dye. To analyse the SOD activity. 600 µL of the SOD reaction mixture containing 0.1 mM EDTA. 0.1 mM xanthine. 25 µmol L^{-1} of nitroblue tetrazolium and 50 mg of bovine serum albumin was added to 125 µL of the supernatant. Then. 25 µL of 9.9 nM xanthine oxidase solution was added to each tube. The amount of formazan found by measuring the absorbance at 560 nm using a spectrophotometer. The results of SOD activity are provided as U mL^{-1}.

GSH-Px activity assay: The level of GSH-Px was determined using the procedure described by Beutler [27]. which records the disappearance of NADPH through its absorbance at 340 nm. The procedure of analysis performed was based on the oxidation of GSH by GSH-Px coupled with the disappearance of NADPH by glutathione reductase measured at 37°C. The absorbance at 340 nm was placed on record over a period of 5 min. The activity was then calculated from the slope of the lines as µM of NADPH oxidized per minute.

GSH level assay: Glutathione concentration was determined by a kinetic assay using a dithionitrobenzoic acid (DTNB) recycling method described by Ellman [28]. One millilitre of the sample was deproteinated by the addition of a solution containing 0.2 g of Na_2EDTA. 1.67 g of metaphosphoric acid and 30 g of NaCl in distilled water. DTNB and Na_2HPO_4 were added to the supernatants and cleared by centrifugation (10 min. 3000 g/min). The GSH level was measured based on its reaction with DTNB to yield a yellow chromophore. which was measured spectrophotometrically at 412 nm.

Statistical procedures: All results are expressed as mean ± S.E. The data were analyzed with an Independent-Sample T Test and Duncan Test. SPSS 12.0 for Windows was utilized for statistical analysis. The level of significance was set at $p < 0.05$.

3. Results and Discussion

The mean carapace length of crayfish among the experimental groups (control, trout diet) and within the replicates of each dietary treatments were not significantly different (p>0.05 for each cases) at the beginning of the experiment. The mean carapace length of crayfish was 38.22±1.16 mm for diet group.

Vitamin A, as an essential micronutrient with a broad range of physiological functions, has long been recognized for its potent antioxidant properties. Pioneering research conducted by Monaghan and Schmitt in 1932 revealed that retinol could inhibit the oxygen uptake of linoleic acid in vitro for

several hours at low concentrations. Further more, an in vitro peroxidation system has ranked the antiox idant activities of retinoids in the following order: retinol > retinal > retinyl palmitate > retinoic acid. It was reported that retinol and its analogs may have stronger antioxidant properties than certain well-known antioxidants, including α-toco pherol. However, the mechanism underlying the antioxidant activity of vitamin A in vivo remains unclear, and the relationship between its structure and function has not been fully elucidated until now. In recent years, there has been increasing interest in the relationship between vitamin A status and oxidative stress in animals [3].

Lipid peroxidation which is the result of interactions of lipid radicals and/or formation of nonradicals species by ROO^{*} is used to be a valuable indicator of the oxidative damage of cellular companents [4,29]. Because of this reason, in the present study was investigated levels of MDA, as a secondary lipid peroxidation product. The results obtained in our study reveal that changes in antioxidant defence and OS levels of crayfish were generally higher in the hepatopancreas compared with gills. These findings are in agreement with a previous observation that was made by Paital and Chainy [30] who found that the OS physiology markers (SOD, CAT, GSH-Px, GR) were higher in hepatopancreas in comparison to gills of *S. serrata* in all seasons. Similarly, Verlecar et al. [31] determined that digestive gland is specific tissues in seasonal variation of ROS level such as H_2O_2 and lipid proxidation of *P. viridis*. The present study showed that the level of MDA, SOD and GSH-PX was was higher in hepatopancreas of crayfish in C group according to NC group (Table2). It is known that crustacea hepatopancreas, the main digestive gland, contains fat-soluble vitamins, regulates the metabolism of the body and exhibits high oxygen consumption [8,32]. Thus, the generation of O_2^{-*} and H_2O_2 can be comparatively more in this organ than other organs.

The results of our study illustrated that the level of MDA was higher 423.52% in gills and 300.00% in hepatopancreas of crayfish in C group according to NC group. In addition to, in this study was determined; a- the level of SOD was higher in gills (101.926%) and hepatopancreas (108.93%) of crayfish in C group than crayfish in NC group, b- the level of GSH-PX was was higher 110.09% in gills and 82.74% in hepatopancreas of crayfish in C group according to NC group, c- the amounts in hepatopancreas and gills tissues of GSH were lower (57.66%, 59.11%, respectivelly) in C group compared to NC group. It can be attributed to the softness of the shell. Moulting influences all aspects of crustacean biology (cellular metabolism, physiology and behaviour). Metabolism is elevated because organic reserves such as mineral deposits, glucose, α-chitin-protein, gastrolith matrix protein, glycoprotein and ecdysteroids in tissues (especially hepatopancreas) and hemolymph are conversion and release [10,33,]. Aiken and Waddy [10] reported that tissue metabolism can elevate oxygen consumption by as much as 1900% during premoult. Yudkovski et al. [34] determined that during late premoult stage occured up-regulation of genes and three additional gene changes effecting oxidative stress in gastrolith disc. This increase in MDA levels could be related to direct damage to biological molecules and tissues of excessive free radical generation due to an increase of the physiological activity caused primarily by the varying metabolic activity during moulting because those described above.

Table 2. Comparison of the mean concentration of the tested malondialdehyde (MDA (nmol g-1 tissue)), superoxide dismutase (SOD (U ml-1)), glutathione peroxidase ((GSH-Px (U g-1)), reduced glutathione ((GSH) (μmol mL^{-1})) in hepatopancreas (H) and gills (G) tissues (T) of crayfish (*P. leptodactylus*)) in the negative control (NC) and control © diet group and the vitamin A diet groups (VA1 (100 mg kg^{-1}), VA2 (150 mg kg^{-1}), VA3 (200 mg kg^{-1}), $P_{(NC-C)}$: Comparison of the negative control and control diet group (x,y), $P_{(C-VE-VC-VE+VC)}$: The control © diet group, the vitamin A diet groups (VA1 (100 mg kg^{-1}), VA2 (150 mg kg^{-1}), VA3 (200 mg kg^{-1}) among the tissues (a,b,c,d). Significance between groups was shown as asterisk (**p < .01, ***p < .001).

P	T	NC	C	VA1	VA2	VA3
MDA	H	1.79±0.26^{x}	7.16±0.70$^{y/a}$	4.61±0.73^{b}	4.06±0.70^{b}	4.34±0.90^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			***			
	G	1.02±0.08^{x}	5.34±0.59$^{y/a}$	3.81±0.64^{b}	4.04±0.62^{b}	3.68±0.72^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			**			
SOD	H	4.14±0.23^{x}	8.65±0.90$^{y/a}$	3.41±0.42^{b}	3.53±0.51^{b}	3.58±0.38^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			***			
	G	5.19±0.12^{x}	10.48±0.55$^{y/a}$	5.85±0.65^{b}	5.74±0.44^{b}	4.90±1.18^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			***			
GSH-Px	H	242.36±14.29^{x}	442.89±18.79$^{y/a}$	239.41±18.86^{b}	235.92±24.28^{b}	254.47±24.08^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			***			
	G	115.36±15.72^{x}	242.36±14.29$^{y/a}$	104.48±5.54^{b}	106.86±7.66^{b}	99.17±4.72^{b}
$P_{(NC-C)}$		***				
$P_{(C-VE-VC-VE+VC)}$			***			
GSH	H	44.08±3.68^{x}	18.66±1.39$^{y/a}$	11.84±1.34^{b}	12.35±1,37^{b}	11.36±1.56^{b}

P $_{(NC-C)}$		***				
P$_{(C-VE-VC-VE+VC)}$			***			
	G	26.46± 4.33^{x}	10.82 ±1.49^{y} /a	7.11± 0.56^{b}	5.63± 0.54^{c}	4.37± 0.49^{d}
P $_{(NC-C)}$		***				
P$_{(C-VE-VC-VE+VC)}$			***			

The balance between the production and accumulation of ROS during moulting of decapod crustaceans is affected by ecdysteroids in the endocrine control. Ghanawi and Saoud [33] reported that ecdysteroids synthesized by the Y-organ and under negative regulatory control of moult-inhibiting hormone (MIH) are primarily involved in regulating the moulting process (ecdysis) of crustaceans. It has been emphasized that in level of ecdysteroid occurs also the increases or decreases in periods of moulting. Ecdysteroid responsive moult-related genes. Some of these ecdysteroid-responsive genes, particularly in the hepatopancreas, revealed differentially expressed genes encoding metabolic and transport enzymes. Epidemiological studies proved genetic variation in the endogenous and exogenous antioxidant defense systems may affect enzymatic antioxidant activity. It was determined that the damages of the mitochondrial membranes and protein structure occurred with effect of variation in the genes coding antioxidant enzymes formed by changes in level of ecdysteroid can, at its turn, enhance ROS [35,36]. In our study, remarkable increases and decreases in levels of oxidant and antioxidants in the tissues while the shell is soft was determined. For example, in this study, it was determined that the MDA level decreased in the hepatopancreas (35.61%, 43.29%, 39.38% respectivelly) and gills (28.25%, 24.34%, 31.08% respectively) of crayfish in VA1, VA2 and VA3 group compared to C group. These fluctiations in levels of analysed parameters can most likely be ascribed to fail in manage and prevent of ROS species in the mitochondria with to be broken of exposure level of enzymes to target cells because of changes in level of ecdysteroid [33,35,36]. SOD, CAT and GSH-Px enzymes, used as a biomarker of ROS production, are the first line of defence against oxidative stress. SOD catalyses the transformation of $O_2^{-\ *}$ to H_2O_2 and water [37].

In the present study, the SOD level in the hepatopancreas and the gills tissues was lower in VA1 (60.57%, 44.17% respectively), VA2 (59.19%, 45.22% respectively) and VA3 (58.61%, 53.24% respectively) groups compared to C group. Increased SOD level during high temperature, gonadal development and breeding period has also been determined in *P. perna* by Wilhelm Filho et al. [38], in *P. viridis* by Verlecar et al. [31] and in *S. glanis* by Bayir et al. [8]. Barim-Oz et al. [39] was determined that the SOD activity in hepatopancreas was higher in moulting and breeding periods according to other months. High level of H_2O_2, change cell physiology through the production of OH^{*} by Fenton reaction, in this period were also observed [31]. For this reason, increase of SOD may be associated to neutralise the overproduction of O_2^{-} anions and H_2O_2 due to peroxidation. However, the activity of SOD was generally higher in gills according to hepatopancreas. It may indicates an increasing need to destroy O_2^{-} in tissues during metabolic activity [40].

GSH-Px is mainly involved in the removal of organic peroxides. Hence, GSH-Px is considered to play an very important role in protecting membranes from damage due to LPO [4,29,41]. In the study conducted by Barim-Oz et al. [41], it was suppressed that GSH-Px activity in crayfish was higher in hepatopancreas, and gills in moulting and mating months according to previous months. This observation is in good agremeent with an earlier report [4,29,38,39] which the production of $O_2^{-\ *}$ radicals increase during metabolic activities. The increased GSH-Px activity in the hepatopancreas protected the organ from the formation of lipid peroxides by reducing H_2O_2 levels, which in turn attenuated OH^{-*} generation. It was reported that H_2O_2 is neutralized by two different enzymes present in the cellular system, they are GSH-P_x and CAT. Each differs in its affinity for H_2O_2, and intracellular H_2O_2 concentration is one of the factors in deciding which of these enzymes will be functional since each has a different Km value [42] Furthermore, CAT only removes H_2O_2 at low concentrations, while GSH-P_x is responsible for the neutralization of both inorganic and organic hydroperoxides. Antioxidant substances added to food during metabolic development reduce stress and also affect enzyme levels in the body [7,16,29,43,44]. As paralel of our study, Nahrgang et al. [45] also determined that in *M. edulis*, during gonadal development and spawning season, the level of CAT and GSH-Px was higher those found in the rest of the year. Moreover, Barim Oz and Yılmaz [39] was also determined that GSH-Px level in hepatopancreas and gills tissues of *A. leptodactylus* increased during moulting period. The present study showed that the concentration of GSH-PX in the hepatopancreas (45.94%, 46.73%, 42.54% respectively) and in the gills (56.89%, 55.90%, 59.08% respectively) was lower at the VA1, VA2 and VA3 group compared to the C group. As mentioned earlier, high level of GSH-Px might not be sufficient to reduce OS in molting period as evidenced by high level of MDA.

GSH, the non-enzymatic antioxidants, is a primary reductant and is the most abundant thiol-containing substance of low molecular weight in the cells. In this way, it serves multiple functions in protecting tissues from oxidative damage and keeping the intracellular environment in the reduced state. In addition, this enzyme reduces hydrogen- and organic-

peroxides *via* a reaction catalyzed by GSH-Px; it serves as a scavenger of OH^* and $^1O_2$ [4,29]. In this study determined that the level of GSH in in the hepatopancreas (36.45%, 32.73%, 39.12% respectively) and in the gills (34.28%, 47.96% , 59.61% respectively) was lower at the VA1, VA2 and VA3 group according to the C group. This idea was corroborated by the observations of Wilhelh Filho et al. [38], who described that the concentration of the GSH in *P. perna* decreased in increase of oxidative stress The decrease in this enzyme activities were likely responses to an increased utilization of GSH. Furthermore, severe oxidative stress may suppress GSH levels due to the impairment of moulting and reproduction mechanisms in *A. leptodactylus* [21,39,41]. The decrease in the GSH level may be caused by the utilisation of nonenzymatic antioxidants to control the enormous of free radicals produced in the metabolism against soft Shell.

4. Conclusion

In conclusion, vitamin A status and oxidative stress are closely intertwined in animal production. Vitamin A deficiency can increase oxidative stress and cause health issues. It is essential to ensure that animals have sufficient vitamin A status to prevent health problems and improve productivity. This study was planned to research the level of oxidative stress during the period when the crayfish shell is soft and to investigate the effectiveness of antioxidant substances (VA) during this period. At the end of the study, it was determined that oxidative stress was very high when the crayfish had just changed its shell. It was also determined that it was necessary to add antioxidant substances to the feed during this period.

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