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Dietary Supplementation with Recombinant Lactoferrampin-Lactoferricin Improves Growth Performance and Affects Serum Parameters in Piglets

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Abstract: Up to thirty piglets with an average live body weight of 5.9±0.5 kg were challenged using enterotoxigenic *Escherichia coli* and randomly assigned to three treatment groups fed a corn-soybean meal-based diet containing either no addition (control group), 0.1 g kg^{-1} of lactoferrampin-lactoferricin (lactoferrampin-lactoferricin group) or 0.1 g kg^{-1} of chlortetracycline (chlortetracycline group) for 21 days. Compared with the control group, dietary supplementation with lactoferrampin-lactoferricin or chlortetracycline increased the body weight and daily weight gain and decreased the feed to gain ratio, diarrhea rate and serum IgM and IgG levels ($p<0.05$). The serum levels of IL-1β, IL-6, TNF-α, diamine oxidase and endothelin-1 were not significantly different among all treatment groups ($p>0.05$). Compared with the control group, dietary supplementation with lactoferrampin-lactoferricin changed the serum concentrations of 10 amino acids ($p<0.05$); dietary supplementation with chlortetracycline only changed the serum histidine concentration ($p<0.05$). These results indicate that lactoferrampin-lactoferricin and chlortetracycline have similar effects on piglets weaned at 21 days of age which is expected to have practical applications in the livestock industry.

Key words: Antimicrobial peptide, bovine lactoferrampin-lactoferricin, growth performance, piglet, China

INTRODUCTION

Antibiotics act as growth promoters by reducing pathogenic bacteria and modifying the gut microflora of animals (Radostits *et al*., 1994; Dibner and Richards, 2005). However, the widespread use of dietary antibiotics is playing a significant role in the emergence of resistant bacteria in edible animal products (Kumar *et al*., 2005; Tang *et al*., 2009; Khan *et al*., 2005; De Vega *et al*., 2005; Frederick, 2011; Geidam *et al*., 2012; Taddele *et al*., 2012; Salihu *et al*., 2012). Resistant bacteria in animals can be transmitted to humans through the consumption of meat from close or direct contact with animals and through the environment (De Haan *et al*., 2010; Van Cleef *et al*., 2010). Thus, the use of antibiotics may have long-term effects on humans and environmental health. With the ban of antibiotics in the European Union (Castanon, 2007) a global trend toward restrictions on the use of dietary antibiotics has emerged. Therefore, the development of novel control strategies for pathogenic bacteria is essential and urgent. Antimicrobial peptides including bovine lactoferrampin and lactoferricin are a major group of promising novel alternatives to antibiotics based on their effectiveness, safety and enormous diversity (Parisien *et al*., 2008; Sang and Blecha, 2008). Lactoferrampin and lactoferricin are two antimicrobial peptides located in the N1-domain of bovine lactoferrin (Kuwata *et al*., 1998; Masschalck *et al*., 2001; Van der Kraan *et al*., 2004; Haney *et al*., 2007; Haney *et al*., 2009). Lactoferrampin and lactoferricin have broad antimicrobial spectra which include antifungal, antiviral and antitumor activity (Van der Kraan *et al*., 2006). The fusion of lactoferrampin and lactoferricin broadens their antimicrobial spectra *in vitro* (Tang *et al*., 2009, 2010). Moreover, Bolscher *et al*. (2009) reported that the bactericidal activity of the LF chimera is drastically stronger than its constituent peptides (lactoferrampin and lactoferricin) which is attributed to its stronger interaction

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with negatively charged model membranes relative to the constituent peptides and more easily induces the leaky fusion of liposomes (Bolscher *et al*., 2009; Xu *et al*., 2010; Adao *et al*., 2011). Therefore, fused bovine lactoferrampin-lactoferricin may serve as an attractive candidate for the development of antimicrobial preparations in the poultry and livestock industry. However, the side effects of dietary supplementation with lactoferrampin-lactoferricin on growth and health parameters in weaned piglets are limited. The current study reports such effects through dietary supplementation using the fusion peptide lactoferrampin-lactoferricin obtained through the methylotrophic yeast, *Pichia pastoris* (Tang *et al*., 2012) which has recently been utilized widely as a heterologous gene expression system. In the current study, chlortetracycline, an antibiotic popularly used in pig feed was selected as the antibiotic control treatment.

The primary objective of the current study is to determine the effect of dietary supplementation with the antimicrobial peptide bovine lactoferrampin-lactoferricin compared with chlortetracycline on growth performance and serum parameters in piglets weaned at 21 days of age and challenged with Enterotoxigenic *E. coli* (ETEC).

MATERIALS AND METHODS

Materials: The antimicrobial peptide lactoferrampin-lactoferricin was provided by the Institute of Subtropical Agriculture (Chinese Academy of Sciences, Beijing, China). It was obtained through the expression of the lactoferrampin-lactoferricin gene in the expression host *P. pastoris* (KM71) XS10 (Tang *et al*., 2012). ETEC 0149, 0141 and 0164 were provided by the China Institute of Veterinary Drug Control (Beijing, China).

Animal housing: A total of 30 pigs (Landrace x Yorkshire, 5.9±0.5 kg) were selected from 6 L from the Hunan Ground Biological Science and Technology Co., Ltd. (Changsha City, China) and weaned at 21 days of age. After weaning, the pigs were randomly assigned to one of the three treatment groups in a completely randomized design and were placed in individual cages (length x width x height: 0.5×0.3×0.4 m) in an environmentally controlled room (32°C). Each of the three groups of pigs was provided one of the following diets: diet supplemented without lactoferrampin-lactoferricin and chlortetracycline (control group) diet supplemented with 0.1 g kg^{-1} lactoferrampin-lactoferricin (lactoferrampin-lactoferricin group) and diet supplemented with 0.1 g kg^{-1} chlortetracycline (chlortetracycline group). The pigs had free access to water. They were fed a commercial post-weaning diet (Table 1). Lactoferrampin-lactoferricin and chlortetracycline were mixed with a vitamin premix and then added to the diet.

The diet was provided three times daily (08:00, 12:00 and 18:00). None of the pigs had a medical history of gastrointestinal and metabolic diseases. In addition, the pigs had not received any special diet, herbal supplement, probiotic or antibiotic in the entire experimental time before sampling. During the 1st 2 days, all pigs had *ad libitum* access to the diet to eliminate or reduce side effects such as weaning stress and were acclimated to the diet change from sow milk to solid feed. The piglets were challenged using the ETEC mixture of three serotypes (0149, 0141 and 0164) at 24 days of age. Each ETEC strain was cultured in tryptic soy broth (Shanghai Sangon Biological Engineering Technology and Service Co,. Ltd. China) for 12 h which were then mixed and given to each pig as a single oral dose (10^9 cells) as previously described (Davisa *et al*., 2007). In the subsequent 21 days of the experimental period, all pigs had *ad libitum* access to the diet. All components of the feeding system were cleaned thoroughly prior to each feeding. Feed consumption was recorded and the pigs were weighed on day 21 to calculate the Average Daily Gain (ADG), Average Daily Feed Intake (ADFI) and the feed conversion (g/g). Fresh manure samples were collected daily to determine the moisture content (Chen *et al*., 2008). The samples with moisture contents >70% were considered diarrhea. The rate of diarrhea for the piglets was calculated as described by a previous study (Ou *et al*., 2007). Briefly, the rate of diarrhea was

Table 1: Dietary ingredients and nutrient levels in basal diet

Dietary ingredients	Content (%)
Corn	56.0
Soybean meal	21.0
Wheat bran	6.0
Fish meal	5.0
Soybean oil	2.5
Dried porcine solubles	2.5
Sucrose	1.0
Glucose	1.0
Lysine hydrochloride	0.3
Threonine	0.8
Salt	0.3
Limestone	0.8
Calcium hydrophosphate	1.8
Premix*	1.0
Analyzed values of nutrient levels	
Digestible energy (MJ kg^{-1})	14.4
Crude protein (%)	20.0
Lysine (%)	1.4
Calcium (%)	0.8
Phosphorus (%)	0.7

*Supplied per kg diet: 10.0 mg copper; 100.0 mg iron; 0.3 mg sodium; 100.0 mg zinc; 10.0 mg manganese; 386.0 IU cholecalciferol; 3086.0 IU retinyl acetate; 15.4 IU all-rac-α-atocopheryl a cetate; 2.3 mg menadione; 3.9 mg riboflavin; 15.4 mg D-pantothenic acid; 23.0 mg niacin; 77.0 mg choline; 0.15 mg selenium; 1.3 mg thiamin and 15.4 μg cyanocobalamin

calculated as (number of piglets with diarrhea x number of days of diarrhea)/(total number of piglets x number of days of the experiment) ×100% (Hampson, 1986).

Blood sampling: On day 21, whole blood (10 mL) was drawn from the jugular vein using a injector without anticoagulant and was stored at 4°C for 12 h. Half of the blood sample (5 mL) was centrifuged at 3000×g for 10 min and the supernatant serum was collected to determine the IgG and IgM values and the levels of Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Nitric Oide (NO), D-lactate, Diamine Oxidase (DAO) and Endothelin-1 (ET-1). The remaining 5 mL blood sample was centrifuged at 1000×g for 5 min to separate the serum. The latter was stored at -20°C for amino acid analysis.

Analysis of serum immune and biochemical indices: The serum IgG and IgM levels were determined using CX4 Auto-Blood Biochemical Analyzer (Beckman Coulter, USA). Serum cytokine including IL-1β, IL-6, TNF-α, NO, D-lactate, DAO and ET-1 were determined using the respective commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits (R and D Systems, Wiesbaden-Nordenstadt, Germany) following the instructions of the manufacturer.

Analysis of serum amino acids: Serum samples were prepared using ethylenediaminetetraacetic acid as an anticoagulant and mixed with two volumes of 5% (w/w) trichloroacetic acid. The serum samples were centrifuged immediately (4°C, 15 min, 8000×g) to remove the proteins as precipitate. The samples obtained were filtered through an Ultrafree-MC centrifugal filter (Millipore, Billerica, MA). All samples were kept at 4°C to minimize chemical reactions of thiol metabolites. The serum Amino Acids (AA) concentrations were measured via an automatic amino acid analyzer (L-8800; Hitachi, Tokyo, Japan) as previously described by Boudry *et al.* (2004) and Wu (2009).

Statistical analysis: All data except for the diarrhea percentage are presented as means±SD. The data were subjected to one-way ANOVA using the SPSS 15.0 program for Windows (SPSS Inc., Chicago IL, USA). The experimental unit for all statistical procedures was an individual pig and Fisher's least significant difference was performed to identify differences among the groups. The significance level for all tests was set to $p<0.05$.

RESULTS AND DISCUSSION

Feed intake, growth performance and diarrhea percentage: The initial Body Weight (BW) on the 21st day of age was the same ($p>0.05$) among the three groups (Table 2). Compared with the control group, dietary supplementation with lactoferrampin-lactoferricin increased ($p<0.05$) the final BW and ADG of the piglets by 13.3 and 29.3%, respectively and decreased the feed conversion by 11.5% ($p<0.05$). Dietary supplementation with chlortetracycline increased the BW and ADG ($p<0.05$) by 12.8 and 26.2%, respectively and decreased ($p<0.05$) feed conversion by 15.3%. However, ADFI was not significantly different ($p>0.05$) among the three groups. Compared with the control group, supplementation with lactoferrampin-lactoferricin and chlortetracycline decreased the diarrhea rate by 44.0 and 51.6%, respectively.

Serum IgG, IgM and cytokine levels: Compared with the control group, dietary supplementation with lactoferrampin-lactoferricin and chlortetracycline deceased ($p<0.05$) the serum IgG and IgM levels (Table 3). However, dietary supplementation with lactoferrampin-lactoferricin and chlortetracycline had no significant effect on the serum IL-1β, TNF-α and IL-6 levels ($p>0.05$).

Serum DAO, d-lactate, ET-1 and NO levels: The DAO and ET-1 concentrations were not significantly different ($p>0.05$) in any of the treatment group (Table 4). Dietary supplementation with lactoferrampin-lactoferricin induced higher d-lactate than the control group (Table 4) however,

Table 2: Effects of dietary lactoferrampin-lactoferricin or chlortetracycline supplementation for 21 days on the growth performance and diarrhea rate of weaned pigs

Variables	Control	Lactoferrampin-lactoferricin	Chlortetracycline
Initial body weight (kg)	5.9±0.5	5.9±0.5	5.9±0.5
Final body weight (kg)	12.1±1.3[a]	13.7±1.3[b]	13.6±1.2[b]
ADG (g day^{-1})	287.5±51.0[a]	371.8±55.1[b]	366.7±55.9[b]
ADFI (g day^{-1})	522.9±89.5	602.1±75.5	560.0±110.5
Feed conversion	1.8±0.1[a]	1.6±0.1[b]	1.6±0.2[b]
Diarrhea rate (%)	18.7	10.5	9.1

ADG: Average Daily Gain, ADFI: Average Daily Feed Intake; [a, b]Values with different letters within each row are significantly different ($p<0.05$)

Table 3: Serum levels of IgG, IgM and cytokines in experimental animal groups

Serum levels	Control	Lactoferrampin-lactoferricin	Chlortetracycline
IgG (g L^{-1})	188.7±21.0[a]	144.4±13.3[b]	159.3±17.1[b]
IgM (g L^{-1})	7.5±1.50[a]	5.5±0.80[b]	5.1±0.40[b]
IL-1β (ng L^{-1})	183.9±5.80	188.3±2.70	187.5±5.50
IL-6 (ng L^{-1})	25.9±0.50	26.5±1.10	26.2±0.40
TNF-α (ng L^{-1})	126.5±5.00	128.4±9.40	127.2±4.20

IL-1β: Interleukin-1β, IL-6: Interleukin-6, TNF-α: Tumor Necrosis Factor-α; [a, b]Values with different letters within each row are significantly different ($p<0.05$)

Table 4: Serum levels of D-lactate, DAO, ET-1 and NO in experimental animal groups

Serum levels	Control	Lactoferrampin-lactoferricin	Chlortetracycline
DAO (U L^{-1})	7.2±0.60	7.8±1.00	8.3±0.10
D-Lactate (μg L^{-1})	463.8±25.6^{a}	436.0±23.6^{b}	472.6±36.7^{a}
ET-1(μg L^{-1})	55.0±4.30	53.5±3.10	55.5±1.90
NO (μmol L^{-1})	11.0±0.60^{a}	14.1±2.10^{b}	17.1±0.70^{c}

DAO: Diamine Oxidase, ET-1: Endothelin-1, NO: Nitric Oxide; $^{a-c}$Values with different letters within each row are significantly different ($p<0.05$)

Table 5: Serum levels of amino acids and NH_3 in experimental animal groups

Serum levels	Control	Lactoferrampin-lactoferricin	Chlortetracycline
Alanine	105.8±17.70^{a}	75.7±16.2^{b}	81.5±7.60^{ab}
Aspartic acid	11.8±3.100	12.1±4.30	12.3±1.10
Cysteine	38.4±12.90^{a}	83.7±2.80^{b}	53.0±17.5^{a}
Glutamic acid	85.4±21.20	72.6±12.4	65.5±8.60
Glycine	109.1±25.80	121.1±24.3	144.2±19.2
Proline	146.0±40.30^{a}	87.8±23.4^{b}	134.4±20.0^{ab}
Serine	61.8±22.10^{a}	26.48±6.8^{b}	83.0±23.0^{a}
Tyrosine	39.8±7.000^{a}	16.2±2.80^{b}	36.8±3.60^{a}
Argnine*	19.2±7.500^{a}	33.6±6.80^{b}	27.7±6.50^{ab}
Histidine*	13.87±3.20^{a}	52.4±4.40^{b}	20.0±4.70^{c}
Isoleucine*	30.4±9.600	35.0±9.20	28.8±9.10
Leucine*	57.5±14.70^{a}	21.8±4.50^{b}	49.2±4.90^{a}
Lysine*	25.0±3.700	20.6±6.00	14.7±3.70
Methionine*	29.7±1.800^{a}	21.4±3.60^{b}	36.2±8.40^{a}
Phenylalanine*	38.5±7.500^{a}	9.7±1.40^{b}	31.1±5.60^{a}
Threonine*	21.7±7.600	18.4±8.50	24.5±3.20
Valine*	33.5±8.200^{ab}	29.1±7.70^{a}	42.9±9.50^{b}
NH_3	1.2±0.100	1.3±0.30	1.3±0.20
Total amino acids	812.4±177.6	807.0±48.1	845.2±77.6

$^{a-c}$Values with different letters within each row are significantly different ($p<0.05$); Amino acid with (*) is essential amino acids for piglets

no significant difference was observed between the control group and chlortetracycline group ($p>0.05$). Furthermore, both the lactoferrampin-lactoferricin group and chlortetracycline group had a significant increase in serum NO levels ($p<0.05$) compared with control group.

Serum amino acid levels: In all treatment groups, the serum total amino acid levels did not change ($p>0.05$) (Table 5). Dietary lactoferrampin-lactoferricin supplementation significantly increased ($p<0.05$) the serum cysteine, histidine and arginine levels and significantly decreased ($p<0.05$) the serum serine, alanine, methionine, leucine, tyrosine, phenylalanine and proline levels. Dietary chlortetracycline supplementation significantly increased ($p<0.05$) the serum histidine and arginine levels. Compared with the lactoferrampin-lactoferricin group, dietary chlortetracycline supplementation significantly increased ($p<0.05$) the serum serine, valine, methionine, leucine, tyrosine and phenylalanine levels and significantly decreased ($p<0.05$) the serum cysteine and histidine levels. Compared with the control group, the serum aspartic acid, threonine, glycine, isoleucine, lysine and ammonia (NH_3) levels had no significant difference ($p>0.05$) in the other treatment groups.

The use of antimicrobial peptides as feed additives for piglets is aimed primarily at developing an alternative to antibiotics. Many studies have indicated that antimicrobial peptides improve the growth performance of piglets and decrease their diarrhea rate (Liu *et al*., 2008; Bao *et al*., 2009; Tang *et al*., 2009). However, little is known about the side effects of lactoferrampin-lactoferricin supplementation on the development of the intestines and immunity of the weaning pigs. The fusion peptide lactoferrampin-lactoferricin, an antimicrobial peptide with possible practical application has a broader antimicrobial spectrum and stronger antimicrobial activity than its constituent peptides (lactoferrampin and lactoferricin) (Bolscher *et al*., 2009; Tang *et al*., 2010). In the current study, chlortetracycline was used as the control antibacterial agent to evaluate their side effects in piglets especially on immune status and intestinal barrier function of lactoferrampin-lactoferricin supplementation.

In the current study, supplementation with lactoferrampin-lactoferricin and chlortetracycline significantly increased the ADG and the final weight and decreased the feed conversion. Lactoferrampin-lactoferricin and chlortetracycline supplementation slightly increased ADFI; however, no significant difference was observed compare with the control group. Both lactoferrampin-lactoferricin and chlortetracycline supplementation decreased the diarrhea rate dramatically which is consistent with previous studies (Shan *et al*., 2007; Liu *et al*., 2009; Tang *et al*., 2009). The control group had a high feed to gain ratio and low ADG and final BW which may be attributed to its high diarrhea rate compared with the other groups. To a certain extent, lactoferrampin-lactoferricin and chlortetracycline have consistent effects: both lactoferrampin-lactoferricin and chlortetracycline effectively inhibit ETEC and decrease the diarrhea rate (Shan *et al*., 2007; Wang *et al*., 2007).

The current study evaluated the humoral and cell-mediated immunity of the piglets by determining some indices. Compared with the control group, the innate inflammation factors IL-1β, TNF-α and IL-6 did not change. Hence, lactoferrampin-lactoferricin and chlortetracycline did not cause inflammation in the intestines of the piglets. These cytokines (IL-1β, TNF-α, and IL-6) may be related to immune tolerance. Supplementation with lactoferrampin-lactoferricin and chlortetracycline decreased the IgG and IgM levels. Lactoferrampin-lactoferricin or chlortetracycline may have inhibited the humoral immunity of the piglets by decreasing the incidence of ETEC infections (Wang *et al*.,

2011). Interestingly, lactoferrampin-lactoferricin and chlortetracycline supplementation also significantly decreases the antibody secretions and diarrhea rate. Lactoferrampin-lactoferricin and chlortetracycline may have effectively inhibited some pathogens that cause diarrhea in piglets such as *E. coli*, Salmonella and Staphylococcus.

The serum DAO, ET-1 and d-lactate levels were determined using ELISA to evaluate intestinal function and injury (Song *et al*., 2009; Guo *et al*., 2010). D-Lactate is a product of intestinal bacterial metabolism. No endogenous enzymes are available to metabolize d-lactate in mammals. Increasing serum d-lactate concentrations indicate decreasing intestinal barrier function or increasing intestinal permeability (Smith *et al*., 1986; Chen *et al*., 1998). DAO is a secretion of intestinal epithelial cells and its increasing activity indicates injury to intestinal barrier function or increased intestinal permeability (Smith *et al*., 1986; Chen *et al*., 1998). In the current study, no change in DAO levels was observed among the treatment groups which indicate that lactoferrampin-lactoferricin and chlortetracycline supplementation does not cause injury to the intestines nor increase intestinal permeability. Compared with the control and chlortetracycline groups, lactoferrampin-lactoferricin supplementation produced lower d-lactate levels. Lactoferrampin-lactoferricin supplementation may have improved intestinal barrier function; however, its mechanism of action is unclear and requires further exploration. ET-1, produced by vascular endothelial cells, is the most potent vasoconstrictor known to date (Yanagisawa *et al*., 1988). It participates in the regulation of smooth muscle tone in both the physiologic and pathophysiologic settings (Boros *et al*., 1998). In animal models, serum and mucus ET-1 concentrations increase upon gastric mucosal injury (Wang *et al*., 2011). In the current study, the ET-1 levels exhibited no change in all treatment groups which further indicated that lactoferrampin-lactoferricin and chlortetracycline supplementation do not result in intestinal injury. NO, produced from arginine is a major vasodilator that regulates vascular tone and hemodynamics. This free radical also plays an important role in vascular endothelial growth factor secretion, modulation of platelet aggregation and adhesion, maintenance of endothelial function, stimulation of epithelial cell migration and inhibition of ET-1 release (Ziche *et al*., 1997; Lee *et al*., 1999; Wu and Meininger 2000; Rhoads *et al*., 2004). Interestingly although, lactoferrampin-lactoferricin and chlortetracycline supplementation significantly increased the NO levels compared with the control, no change in ET-1 levels was observed among the treatment groups which indicates that NO plays a significant role in maintaining the structure and function of the intestines (Han *et al*., 1995; Warner, 1999).

The serum amino acid levels were also analyzed. The concentrations of essential amino acids such as methionine, leucine and phenylalanine in serum decreased significantly in the lactoferrampin-lactoferricin group compared with the control group. The arginine and histidine concentrations increased significantly in the lactoferrampin-lactoferricin group, compared with the control group. High serum arginine concentrations may lead to high NO concentrations in piglets (Jobgen *et al*., 2009). These results are similar to that in the chlortetracycline group with high NO concentrations and slightly high arginine concentrations compared with the control group.

In addition, Wu *et al*. (1996) reported that arginine deficiency causes elevated NH_3 levels and reduces serum arginine levels which was further supported by Tan *et al*. (2009). The NH_3 levels reported in the current study represent free NH_3 concentration which was not significantly different among the treatment groups. In the current study, although lactoferrampin-lactoferricin supplementation changed the serum concentrations of 10 amino acids as discussed above, the total amino acid was not changed significantly compared with the control group. However, compared with the control group, the serum histidine concentration in the chlortetracycline group was significantly increased. Limited data was obtained on the influence of feed additive supplementation on serum amino acid concentration. Thus, further research is required to account for the observed phenomenon.

CONCLUSION

Dietary supplementation with lactoferrampin-lactoferricin or chlortetracycline improves the growth performance and feed conversion as well as decreases the diarrhea rate of weaned piglets challenged with ETEC. The results of the present study suggest that lactoferrampin-lactoferricin and chlortetracycline have similar effects on weaned piglets. The outcome of lactoferrampin-lactoferricin supplementation with its fewer side effects, provides an important reference for pig production. However, consumer safety and the possible side effects of lactoferrampin-lactoferricin as a genetically modified product, on both the target animals and humans remain to be evaluated and further tests are required prior to large-scale application. The use of lactoferrampin-lactoferricin combined with other additives will also be considered.

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