Polyphenol Extracts from Punica granatum and Terminalia chebula Are Anti-inflammatory and Increase the Survival Rate of Chickens Challenged with Escherichia coli

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Avian pathogenic Escherichia coli (APEC) causes inflammation in multiple organs of chickens called avian colibacillosis, and results in serious economic loss to the chicken industry. Polyphenolic compounds possess a wide range of physiological activities that may contribute to their beneficial effects against inflammation-related diseases. In this study, the curative effect and mechanism of action of the polyphenolic extracts from Punica granatum L. and Terminalia chebula Retz. in chickens challenged with APEC were studied. Specific-pathogen-free white Leghorn chickens (males, 21-d old) were challenged with APEC and then given oral administration of extracts of P. granatum and T. chebula. The extracts decreased the morbidity and inflammation induced by APEC. Data from quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay showed that the extracts of P. granatum and T. chebula polyphenols (GCP) reversed the over-expression genes of the Toll-like receptor (TLR) 2, 4, and 5, down-regulated the activation of nuclear factor-kappa B signal transduction pathways, and inhibited the production of pro-inflammatory cytokines. Naturally occurring GCP may be a potential alternative medicine for the prevention or treatment of avian colibacillosis.

Key words polyphenol; Escherichia coli; specific-pathogen-free chick; anti-inflammatory

Avian pathogenic Escherichia coli (APEC)1 are E. coli strains that can cause acute and predominant systemic colibacillosis in birds. Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perhepatitis and with peritonitis being most typical; and the additional diseases, septicaemia, chronic respiratory disease, vitelitus infection, salpingitis, peritonitis, chronic skin infections, and osteomyelitis,2) and swollen head syndrome3) are usually involved. APEC is found in the intestinal microflora of healthy birds and most of the diseases associated with them result from environmental and host predisposing factors. APEC strains cause invasive infection in chickens, and more specifically in broilers. Diseases resulting from APEC decrease growth rate, increase mortality and morbidity, and cause significant economic losses in the poultry industry. APEC also can be transmitted to other animals or even humans by certain vectors, and can be a human health risk.3,4)

At present, the approach for the prevention and control of APEC infections include the control of environmental contamination and environmental parameters such as humidity and ventilation. Vaccines containing killed or attenuated virulent bacteria protect against infection with the homologous strain, but are less efficient against heterologous strains. Therefore, vaccination for colibacillosis is not widely practiced due to the large variety of strains involved in field outbreaks.1) Chemical drugs (antibiotics) are widely used, but antimicrobial compounds often eradicate intestinal commensal bacteria as well as pathogenic bacteria. The increasing antibiotic resistance makes the use of antibiotic treatments less effective.5,6) As a result, the potential drug residues in livestock and poultry caused by the high use of antibiotics threaten the safety of our food supply.4) Thus, it is highly desirable to find alternative ways to control APEC.

Pomegranate (Punica granatum L.) and fruit of the Harītakī deciduous tree (Terminalia chebula Retz.) are used in traditional herbal Chinese medicine, and are used as an intestine astringent to relieve diarrhea and enteritis. P. granatum contains a wide range of phytochemicals which are primarily polyphenols.7) Studies have shown that activity of peel extracts of P. granatum is related to the polyphenol content. Polyphenols found in P. granatum have been shown to exert anti-inflammatory, antioxidant, and anticarcinogenic activity,9,10) and promote wound healing.11,12) Extracts of P. granatum were effective at protecting human skin fibroblast from cell death following UV exposure, which was related to reduced activation of the pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB).13) Extracts of P. granatum were also a potent inhibitor of a number of bacteria, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, and Salmonella enteritidis,14) and may be used to control various bacteria associated with oral infections in humans.15) The pharmacological effects of pomegranate have been known for a very long time and were mentioned in Greek and Egyptian documents.16,17) Terminalia chebula Retz. has been used for its healing properties throughout the ages in India and South-East Asia, and is anti-inflammatory, antioxidant, antimicrobial, and has demonstrated wound healing activity.18,19) The fruits of T. chebula are rich in tannins, phenolics, and polyphenols, which are related to the antigenotoxic and chemopreventive activities of T. chebula.19,20)
APEC invades Birds primarily by damaging the intestine mucosa, which induces regional inflammation. Activation of the Toll-like receptor (TLR) signaling pathway plays an important role in defense against invading pathogens.\textsuperscript{21} The TLR pathway strongly potentiates NF-κB activity and induces enhanced levels of the innate immune response.\textsuperscript{22} However, the over-activation of the TLR pathway may cause it to break out of immune equilibrium and lead to injury of the host. Therefore, the TLR pathway activation must be controlled within a certain range. In the present study, the polyphenols from \textit{P. granatum} and \textit{T. chebula} modulation of TLR/NF-κB signaling and pro-inflammatory mechanism was investigated in specific-pathogen-free (SPF) chickens following challenge with APEC.

**MATERIALS AND METHODS**

The experimental procedures involving animals in this study were approved by the Animal Care Center of the Beijing University of Agriculture, Beijing, China.

**Reagents and Materials** \textit{P. granatum} and \textit{T. chebula} were purchased from the Tongrentang Traditional Chinese Medicine Shop (Beijing, China). Folin-Ciocalteau reagent was procured from Sigma Chemical (Beijing, China). Other reagents were all analytical grade and from Beijing Chemical Reagents Co. (Beijing, China). Primers were synthesized by the Shanghai Shengong Bio-tech Co., Ltd. (Beijing, China). The GCP extract dissolved in distilled water at a dose of 0.9 g/kg BW; (3) 3 times of clinical dose of the GCP extract dissolved in distilled water at a dose of 0.9 g/kg BW; (4) 4 times of clinical dose of the GCP extract treatment (5×): birds were received a daily oral gavage of the GCP extract dissolved in distilled water at a dose of 1.5 g/kg BW. On the 8th day, all birds were sacrificed. The GCP toxicity was assessed by autopsy, histological examination and blood biochemical index.

**Effects of the GCP Extract on Survival Rate of Chickens Challenged with APEC** At 28-d of age, the 150 chickens were randomly divided into five groups according to treatment; all groups received treatment for 7d at 8:00 pm by controlled oral intake using syringe delivery of saline or the GCP extract. During the experimental period, the birds were provided water and feed \textit{ad libitum}. The chickens were divided into the following groups: (1) Negative control (NC): the birds received standard water and feed ration, and were daily given sterile saline by oral gavage; (2) Positive control (PC): birds were injected in the abdomen with 200 μL of an aequous APEC solution at a concentration of \textit{2×10}^6 colony forming unit (cfu)/mL at the beginning of the experiment. 12h after challenge, and each following day the birds received an oral gavage of sterile saline at a dose of 0.3 mL/kg BW; (3) PC plus a low dosage of the GCP extract treatment (PC+LT): birds were administrated APEC as was the PC, and 12h after challenge the birds received a daily oral gavage of the GCP extract dissolved in distilled water at a dose of 0.15 g/kg for seven consecutive day; (4) PC plus a medium dosage of the GCP extract treatment (PC+MT): birds were administrated APEC as was the PC, and 12h after challenge the birds re-
ceived a daily oral gavage of the GCP extract dissolved in distilled water at a dose of 0.3 g/kg BW for seven consecutive days; (5) PC plus a high dosage of the GCP extract treatment (PC+HT): birds were administered APEC as the PC, and 12 h after challenge the birds received a daily oral gavage of the GCP extract dissolved in distilled water at a dose of 0.6 g/kg BW for seven consecutive days. The mortality rate for each group was carried out at day 8.

The Anti-inflammatory Effect of APEC Infected Chickens Treated with GCP Fifty-four chickens at 28 d of age were divided equally into three groups and provided water and feed ad libitum. (1) Negative control (NC), the birds received daily oral gavage of sterile saline at a dose of 0.3 mL/kg BW. (2) Positive control (P), animals were injected in the abdomen with 200 kg BW. (5) PC plus a high dosage of the GCP extract treatment. Twelve hours after challenge, the birds received a daily oral gavage of the GCP extract dissolved in distilled water at a dose of 0.3 g/kg BW. (3) Positive control plus treatment (P+T): birds were administered APEC as was the P, and beginning 12 h after challenge, the birds received a daily oral gavage of the GCP extract dissolved in distilled water at dose of 0.3 g/kg BW.

At 24, 72, and 120 h post-challenge, six birds were randomly chosen from each group. The birds were anesthetized and sacrificed by jugular exsanguination. Approximately 2 cm of ileum intestinal sections were collected under sterile conditions. Samples were stored at –80°C until analyzed by polymerase chain reaction (PCR) and ELISA.

Total RNA Extraction, Reverse-Transcription and Quantitative Real-Time PCR The ileum intestinal tissue was homogenized, and the total RNA was isolated from each homogenized tissue sample using the TRIzol extraction method (Invitrogen, Beijing, China). The total RNA was quantitated using an ND-1000 spectrophotometer by determining the 260 nm/280 nm absorbance ratio. The total RNA was reverse transcribed to cDNA using the TRIzol extraction method (Promega Biotech Co., Ltd., Beijing, China) in 1× reverse transcriptase buffer. The reaction mixture was incubated at 37°C for 1 h.

All primers for genes encoding for TLR4, NF-κB(p50), IL-1β, IL-8, TNF-α, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM-1) were designed using Primer premier 5.0 and the DNAMAN software program using sequences acquired from GenBank. The specificity of the designed primers was initially examined by comparison of the primers with sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) through BLAST analysis, and then experimentally verified through PCR assays. The PCR conditions for each target gene were optimized, and the optimal conditions are listed in Table 1.

QRT-PCR assays were performed on an Applied Biosystems ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with Brilliant II SYBR Green QPCR Master Mix, 150 ng of purified RNA, 0.5 µL of each diluted cDNA sample was added to a 25 µL reaction mixture containing 12.5 µL of Brilliant SYBR Green QPCR master Mix, 150 nm (each) primers, and 0.375 µL ROX reference dye. Cycling parameters were as follows: an initial denaturation at 95°C for 10 min; 40 cycles of 30 s at 94°C, 1 min at the annealing temperature of 58°C, and extension for 0.5 min at 72°C. The conditions used for the dissociation curves were as follows: 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

Validation of the Expression for IL-1β and TNF-α Using ELISA Analysis To further validate the PCR data, an ELISA method was used to determine IL-1β and TNF-α.

Statistical Analysis The results are presented as the mean with the standard error (S.E.). The data were analyzed using GraphPad Prism 5 software to determine the significant dif-

<table>
<thead>
<tr>
<th>Table 1. PCR Primers</th>
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<tr>
<td>Gene</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NF-κB1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>TLR2</td>
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<tr>
<td></td>
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<td>TLR4</td>
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<td></td>
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<tr>
<td>TLR5</td>
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<tr>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
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<tr>
<td></td>
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<tr>
<td>TNF-α</td>
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<td></td>
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<tr>
<td>ICAM-1</td>
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<td></td>
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<td>VCAM-1</td>
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ference. The t-test values of less than 0.05 were considered statistically significant.

RESULTS

APEC infections were validated based on the clinical manifestation of signs, pathologic examination, and isolation of \textit{E. coli}. All \textit{E. coli} strains were isolated from the liver surface of debilitated or dead chickens. The colony characteristics of the isolated strains were observed on MacConkey agar and Eosin methylene blue agar plates, and biochemical tests and serotype confirmed that these were confirmed to be APEC strains.

**Extract Yield and Polyphenols Content**

Previous study showed that \textit{Punica granatum} L. and \textit{Terminalia chebula} RETZ contained relative higher polyphenolic substance. In this work, we extracted the polyphenols using water–ethanol solution, and obtained a 33% yield of extracted dried material. The total phenolic content was determined as gallic acid equivalents in dried extract material.

**The GCP Extract Showed No Toxicity on SPF Chickens**

To assess the toxicity, the mental status, food intake and other behavior for the chickens from four groups were investigated in the whole experiment. The GCP toxicity was further diagnosed at the 8th day by the means of autopsy, pathological examination, and biochemical test. After hematoxylin and eosin (HE) staining, the liver, kidney, lung histopathology were investigated under light microscopy. The results indicated that all the groups were normal; the representative histopathology of liver, kidney, and lung from the control and the highest dosage group (5\times) was shown in Fig. 1. The blood chemistry indexes were shown in Table 2, which were no significant difference between the control group and the three GCP treatment groups. The present results indicate that GCP, under the dosage, was non-toxicity under the described experimental conditions.

**The GCP Extract Increased the Survival Rate of Chickens Challenged with APEC Strains**

In this study, the GCP extract curative effect on APEC signs in SPF chickens was initiated using an APEC challenge dose equivalent to a 40% SPF chicken survival rate. The study demonstrated that the GCP extract alleviated the state of illness and prevented the mortality of chickens challenged with APEC. APEC caused acute systemic infection and inflammation. Chickens in each group began to die about 24 h after challenge, but the mortality of the APEC positive control group was higher than the other GCP extract treated groups. The spiritual status and ill signs showed a significant improvement in a dose-dependent relationship. The bird survival rates in the GCP treated

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>BUN (mmol/L)</th>
<th>UA (umol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.00±2.10</td>
<td>225.0±42.05</td>
<td>0.43±0.10</td>
<td>188.17±12.37</td>
</tr>
<tr>
<td>1\times</td>
<td>3.67±1.03</td>
<td>235.0±18.25</td>
<td>0.40±0.09</td>
<td>192.83±45.52</td>
</tr>
<tr>
<td>3\times</td>
<td>3.33±1.03</td>
<td>265.0±43.51</td>
<td>0.32±0.04</td>
<td>197.83±63.39</td>
</tr>
<tr>
<td>5\times</td>
<td>4.67±1.86</td>
<td>262.5±33.39</td>
<td>0.37±0.08</td>
<td>170.83±25.54</td>
</tr>
</tbody>
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Table 2. Blood Biochemical Index of the GCP Extract on SPF Chickens

<table>
<thead>
<tr>
<th>Daily weight gain (g)</th>
<th>NC</th>
<th>PC</th>
<th>PC+LT</th>
<th>PC+MT</th>
<th>PC+HT</th>
</tr>
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<tr>
<td></td>
<td>14.49±2.05</td>
<td>7.00±3.26</td>
<td>12.68±2.29</td>
<td>11.57±2.26</td>
<td>11.62±2.26</td>
</tr>
<tr>
<td>Feed conversion</td>
<td>2.13±0.13</td>
<td>3.02±0.48</td>
<td>2.63±0.36</td>
<td>2.24±0.27</td>
<td>2.07±0.20</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>100</td>
<td>36.67</td>
<td>43.33</td>
<td>53.33</td>
<td>60.00</td>
</tr>
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</table>

The difference was significant among different alphabets (p>0.05).
groups were shown in Table 3. When the GCP extract dosage increased from 0.15 g/kg to 0.6 g/kg BW, the survival rate increased from 43.3% to 60%. This effect was attributed to the GCP extract used to treat the chickens.

**The GCP Extract Inhibited TLR2, 4, and 5 Expression and the Activation of NF-κB in Chickens Challenged with APEC**

The TLR family is a highly conserved group of proteins that participate in pathogen recognition and in the initiation and regulation of the innate and adaptive immune responses. In this study, as shown in Fig. 2, the control group intestinal tissue expressed a baseline response for TLR2, 4, and 5, and NF-κB; whereas, the APEC challenge stimulated the expression of the TLRs and the activation of NF-κB ($p<0.05$). The GCP extract inhibited the over-expression of the TLRs and activation of NF-κB to APEC ($p<0.05$).

**GCP Extracts Decreased the Inflammatory Cytokine Concentration in Chicken Intestinal Tissue Following APEC Challenge**

The mRNA expression of genes encoding for the cytokines, TNF-α, IL-1β, IL-8, ICAM1, and VCAM1 in intestinal tissues were measured by QRT-PCR. The cytokines were expressed in the intestinal tissue of the negative chicken group, and were significantly enhanced in response to APEC challenge ($p<0.05$). The expression levels of the cytokines decreased at all three time points after receiving GCP treatment (Fig. 3).

The gene expression end-products, IL-1β and TNF-α, were further validated using an ELISA, the two different types of data were relatively consistent. As shown in Fig. 4, IL-1β and TNF-α in the positive control group both increased post-challenge, while treatment with GCP extracts inhibited the over-expression of the pro-inflammatory cytokines.

**DISCUSSION**

The extractable chemical constituents of *Punica granatum* L. and *Terminalia chebula* RETZ. belong mainly to the polyphenols. The polyphenol content is 25–30% in *P. granatum*, and 23–37% in *T. chebula* RETZ. Their extracts have shown multiple pharmacological activities, such as antimicrobial,8,15,25 cardiac action, antioxidant,11,26 antitumor,10,27,28 antiviral,29 and the promotion of wound healing.12,13

The intestinal tract contains the largest reservoir of bacteria and endotoxins in an animal’s body. The intestinal tissue is the first line of defense against invading microbial pathogens. Intestinal bacterial infection can lead to lower growth rate, disease and even death. APEC strains are the first pathogens associated with digestive tract diseases in poultry production. The infected tissues respond to APEC infection by producing pro-inflammatory cytokines to combat the invading APEC strains.

In this research, intraperitoneal injection of APEC was used following a high challenge dose of APEC ($2 \times 10^9$), the occurrence of death in infected chickens decreased following treatment by the GCP extract in a dose-dependent manner. For studying the pharmacological action mechanism of the GCP extract, the APEC challenge dose used was $8 \times 10^8$. This dosage caused chickens 100% morbidity without mortality. The advantage of this dosage ensured a good consistency between samples. The GCP toxicity of 1, 3, 5 times of clinical dose and consecutive administration of drug for 21 d on SPF chicken was previously tested, and the results showed that GCP had no affect on survival rate, blood biochemistry, histopathology. So we did not set the GCP control group in the study.

TLRs play an important role in the intestinal innate immune system initially in helping the intestinal tract recognize
bacteria. TLR2, 4, and 5 participate in the innate immune response to APEC and the development of enteritis. TLRs not only are immune recognition receptors on the cell surface, but also are transmembrane signal transduction molecules. TLR stimulation by pathogen-associated molecular patterns (PAMPs) with subsequent changes in the cytokine profile are major components of innate immunity, which are major interactions involved in the innate resistance to Gram-negative bacteria, but also in the pathogenesis of septic shock. TLR 2, 4, and 5 recognize APEC strains. TLR 2, 4, 5, respectively identify bacterial teichoic acid, LPS and flagellin. But inactive *Escherichia coli* induce TLR 2 and 4. In the process of *Escherichia coli* pathogen, LPS and other ingredients together help initiate the pro-inflammatory cytokines.

TLRs initiate a series of signal transduction intermediaries and activate NF-κB to induce the production of pro-inflammatory...
tory cytokines. NF-κB activation is essential for the expression of a variety of cytokines.\(^2\) In the study, \textit{Escherichia coli} induced expression of TLR 2, 4, 5 in various degrees. TLR4 was firstly induced, and reached peak at the third day, recovered close to normal at the fifth day. TLR2, TLR5 expressions were later induced, and kept higher at the fifth day. NF-κB1 (p50) was jointly induced by TLR2, 4, and 5, and partly recovered at the fifth day. It indicated TLRs-NF-κB pro-inflammation pathway was activated in the course of the disease. The GCP treatment significantly reversed the over-expression of TLR2, 4, 5, and NF-κB1 (p50).

Although TLR-mediated signaling is paramount in eradicating microbial infections and promoting tissue repair, the regulation must be tight. TLRs-NF-κB signal pathway induces the transcription and translation of pro-inflammatory cytokines, which leads to the massive release of inflammatory cytokines and adhesion molecules, including TNF-α, IL-1β, IL-6, IL-8, ICAM1, VCAM1, and promote the proliferation of epithelial cells and inhibit intestinal bacterial translocation.\(^3\)

However, over-production of cytokines can cause impairment to the host. Macrophages and monocytes secreted pro-inflammatory cytokines like TNF-α, IL-1β, and IL-8 following the bacteria infectious stimulation. These cytokines activated inflammatory cells like neutrophils, macrophages, and monocytes released large amounts of the toxic oxidizing radicals, the result caused organ injury via the peroxidation of membrane lipids and the oxidative damage of proteins and DNA, further led to augment of local tissue injury.\(^3\) TNF-α and IL-1β are thought to be relative pro-cytokines that can trigger the inflammatory cascade and induce other cells to produce other cellular factors. Blocking the production of these cytokines by receptor antagonists inhibits the previous mentioned diseases. In local regions, these molecules lead to apoptosis of intestinal mucosal epithelial cells and damage organs and tissues of the intestinal tract. Cytokines can also act on distant organs and amplify systemic inflammatory reactions.\(^3\) Compared to wild-type mice, intestinal epithelial damage caused by colitis was milder in TLR4-deficient mice.\(^3\) Expression of NF-κB might contribute to the ureter damage observed in obstructive uropathy, and inhibition of NF-κB could attenuate the tissue damage of obstructed ureters.\(^3\) VCAM-1 and ICAM-1 participate in firm adhesion of leukocytes to endothelial cells, which are secreted by endothelial cells activated by proinflammatory factors and are thought to play a central role in the development of tissue inflammation. Inflammation can induce VCAM-1 and ICAM-1, cause local thrombosis, and thrombosis can amplify inflammation.\(^3\)

In the study, TNF-α, IL-1β, IL-8, VCAM-1, and ICAM-1 were all induced by \textit{Escherichia coli} challenge, The overexpression of cytokines indicated that the chickens were in an inflammatory reaction status, while the down-regulation of these inflammatory by administration of the GCP extract may partly explain the beneficial anti-inflammatory and anti-thrombotic effects, which exhibited protection of intestinal tissue in APEC infected chickens.

In summary, APEC infection elevated levels of TLR2, 4, and 5, induced the activation of NF-κB and enhanced the production of TNF-α, IL-1β, and other cytokines in chickens. The GCP extract treatment inhibited the TLRs/NF-κB signaling pathway, and down-regulated the production of TNF-α, IL-1β, IL-8, ICAM1, and VCAM1. The treatment exerted an anti-inflammatory action, and resulted in the decreased mortality rate after challenge with APEC strains.

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REFERENCES

17) Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D. Antiox-


