THE PATHOGEN INHIBITION EFFECTS OF PROBIOTICS AND PREBIOTICS AGAINST

SALMONELLA SPP. IN CHICKEN

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Abstract
This current study investigated the effect of probiotics and prebiotics on the control of Salmonella spp. isolated from chicken. One hundred and eleven bacterial isolates were recovered from different chicken farms, and nine Salmonella isolates were detected with 8%. The serogroup analysis of nine Salmonella isolates showed three different groups identified as (4) S. enteritidis, (3) S. typhimurium, and (2) un-typed group. The positive-identified Salmonella was using PCR and genus-specific primer OMPCF (outer membrane protein reverse) with a target size of 204 bp. The results of Salmonella enteritidis with target size are also 304 bp and target 401 bp for Salmonella typhi-
murium. In addition, one hundred chicks were grouped into five groups (1, 2, 3, 4 and 5) containing 20 broiler chicks. The broilers of groups 1, 2, 3, 4 and 5 were orally inoculated with a dose of 1×10⁸ CFU nalidixic acid-resistant Salmonella enteritidis. Experimental groups were as follows: Group 1: negative control, Group 2: positive control (infected chickens), Group 3: infected chickens and treated with B. subtilis probiotic, Group 4: infected chickens and treated with levosyl prebiotic, Group 5: infected chickens and treated with panflor antibiotic. Results showed significantly increased survival percentage against those challenged with a virulent strain of nalidixic acid probiotics and prebiotics. The oral application significantly improved the survival percentage against with a virulent strain of nalidixic acid-resistant Salmonella spp. The best result of B. subtilis was 1×10⁸ which reduced the growth of the microorganism under study (S. typhimurium – S. enteritidis).

Key words: Salmonella spp., control, chicken, probiotics, prebiotics

Salmonella enterica represents the most pathogenic species. It can be transmitted to humans and the farm-to-
fork continuum, through contaminated foods of animal origin (Jajere, 2019). Additionally, the incredibly greater fork continuum, through contaminated foods of animal international importance. According to the preliminary report from FoodNet on the incidence of infection with food-borne pathogens, the ongoing efforts to reduce cases of salmonellosis associated with consuming contaminated meat, poultry product and other foods are showing success. This report indicates that fewer cultures of raw broiler chicken samples yielded Salmonella in 2009 (7.2%) than in 2006 (11.4%) (CDC FoodNet Report, 2009; Sanders, 2017).

Lilly and Stillwell (1965) described the growth-promoting factors produced by microorganisms and were the first to introduce the term “probiotic”. Crawford (1979) defined probiotics as a culture of specific living microorganisms, primarily Lactobacillus spp., which im-
plants in the organisms and ensures the rapid and practical establishment of beneficial intestinal populations. In addition, Zhang et al. (2021) investigated the effects of commercial probiotic supplementation to water on broil-
er chicks’ performance, carcass traits, immune function, and antioxidant capacity. Authors found that probiotic additives positively impact body weight, average daily feed intake (ADFI), and average daily weight gain for female chicks. Similar results were reported by Abd El-
Hack et al. (2017, 2018, 2019), Mahrose et al. (2019) and Othman et al. (2019). In contrast, these probiotics significantly reduced ADFI and the feed conversion ratio of male chicks. The present study aimed to investigate the efficacy of probiotics and prebiotics in controlling Salmonella spp. isolated from chicken.
Material and methods

Experimental chicks

Two hundred and thirty chicks, either freshly dead or moribund, aged 1–40 days and of different breeds (Balady, broilers and Saso) were collected from different localities in Sharkia Governorate, Egypt and subjected to clinical and postmortem examination. Specimens from the liver, lung, kidney, heart and yolk sac were aseptically collected and subjected to bacterial isolation and identification.

The commercial probiotic and prebiotic

Lypholac: The commercial probiotic produced by Microbiotech, USA containing Lactobacillus acidophilus, 1×10⁸ CFU/g.

Levoxyl: The commercial probiotic produced by New Feed Team (NFT), Italy, contains mannooligosaccharide and betaglucan.

Bacteriological media

The media contained nutrient agar medium (Oxoid, CMS), buffered peptone water (Difco), Rappaport-Vassiliadis Soy Peptone (RVS) Broth (Merck), MacConkey’s agar (Oxoid, CM7), Christensen’s urea agar base medium (Difco), Muller-Hinton broth (Oxoid), and Muller-Hinton agar (Oxoid code: CM0337).

Reagents of API 20 E: API NaCl 0.85% medium, 5 ml (Ref. 20 230) or API suspension medium, 5 ml (Ref. 20 150).

Materials used for PCR

Mini Kit – Catalog no. 51304

The contents of the kit: Buffer AL – Buffer ATL – Buffer AW1 (concentrate).

Buffer AW2 (concentrate) – Buffer AE – QIAGEN® protease.

Protease solvent – Proteinase K – Silica membrane (QIAamp Mini Spin Columns), PBS (phosphate buffer saline), Ethanol (96–100%), DNA molecular weight marker, enzyme (2500 bp), primers used in PCR are OMPCF (outer membrane protein c) forward, OMPCF (outer membrane protein c) reverse, ENTF, ENTR, TYPF, TYPHR.

Isolation and cultivation of Salmonella were carried out after (ISO 6579:2002), biotyping using API 20E (BioMerieux, 1992) for the identification of the isolates.

Isolation and identification of bacteria from commercial probiotic preparations were performed according to Collins et al. (1995). Probiotic bacterial strains were re-isolated from lypholac. The identification of growing colonies was carried out using colonial morphology and microscopic examination.

The evaluation of phagocytic activity was done according to Wilkinson (1977). The number of ingested cells represents the phagocytic percentage (p%).

In vitro growth inhibition of Salmonella was performed according to Piatek et al. (2020), Serological identification of Salmonella was carried out according to Kauffman–White scheme (Kauffman, 1972).

The extraction of DNA was done according to Oliveira et al. (2003). The yield of DNA was stored at –20°C till being used. The agarose gel electrophoresis was performed following Sambrook et al. (1989) method.

Preparation of Salmonella typhimurium and Salmonella enteritidis resistant strains was performed. The strains were sub-cultured in eight successive broth cultures containing increasing quantities of nalidixic acid, starting with 0.01 gm and ending by 1 g (nalidixic acid/litre).

Experimental design

This work aimed to study the efficacy of using some probiotics and prebiotics against Salmonella spp. isolates on experimental seven day-old broiler chicks. One hundred broiler chicks (one-day-old Avian-48) were grouped into five equal groups (1, 2, 3, 4 and 5) containing 20 broiler chicks. Chicks in groups 2, 3, 4 and 5 were orally inoculated with a dose of 1×10⁸ CFU of nalidixic acid-resistant S. enteritidis. Chicks in group one remained a negative control. Chicks in group 2 served as a positive control. Chicks in group 3 were orally treated with B. subtilis natto with a dose of 1×10⁸ CFU/bird for five days post-infection. Group 4 was orally treated with Levoxyl (prebiotic) 1 ml/liter in drinking water for five successive days post-infection. Group 5 was treated with panflor 1 ml/liter for five days post-infection. Panflor is a broad-spectrum antibiotic composed of fluorifincol. Broilers were reared separately on floor during the experimental period (6 weeks). Clinical observation of the infected chicks was carried out to record morbidity and gross lesion. Re-isolation trails of inoculated pathogens were performed. The total erythrocytes and total leukocytes were counted according to Schalm et al. (1975).

Blood sampling

The first sample was collected without adding an anticoagulant for serum separation. The second sample was collected with EDTA to estimate the total erythrocytic, leukocytic count and different leukocytic counts (DLC).

Statistical analysis

According to Tamhane and Dunlop (2000), the obtained data were statistically analyzed.

Results

Incidence of Salmonella spp. among chicken farms

Suspected Salmonella was detected with an incidence of 8.1% of farms. This was an incidence of farm infection of 20.5% (Table 1).
The control of poultry salmonellosis

Identification of Salmonella isolates from chicken

API 20 E kits

API 20 E protocol was carried out on nine randomly selected isolates. API 20 E results were parallel with the conventional biochemical identification results for these nine Salmonella isolates as both identified the isolates as Salmonella. API 20 E revealed different 7-digit profile numbers interpreted through API 20 E analytical profile index (Ref. 20190). Profile 6704752 was the most prevalent one, referring to 4 isolates (Figure 1).

Serotyping of tested Salmonella isolates from examined chickens

The serogroup analysis of the nine tested Salmonella showed that three groups were identified: S. enteritidis, S. typhimurium and un-typed. S. enteritidis and S. typhimurium were the most prevalent serogroups, with 44.4% and 33.3%, respectively. Another serogroup was un-typed with 22.2% (Table 2).

Molecular identification of tested Salmonella

Multiplex PCR was used for genotyping of nine biochemically and serologically identified Salmonella isolates. Primers were specific to Salmonella, OMPCF (outer membrane protein C forward) and OMPCR (outer membrane protein C reverse), with a target PCR amplicon size of 240 Pb.

The other pairs of primers were specific for the most common Salmonella serovars including S. typhimurium (target size 401 Pb) and S. enteritidis (target size 304 Pb). The positive identified Salmonella using PCR and genus-specific primers OMPCF and OMPCR with target PCR amplicon size of 204 bp are presented in Table 3 and Figure 2.

Table 1. Incidence of infection in tested farms and samples

<table>
<thead>
<tr>
<th>Examined farm</th>
<th>Total samples</th>
<th>+ ve sample</th>
<th>% of Salmonella spp.</th>
<th>Total farms</th>
<th>% of farm infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abo Hamad</td>
<td>11</td>
<td>2</td>
<td>18</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Abo Kaber</td>
<td>15</td>
<td>1</td>
<td>6.6</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>Zagazig</td>
<td>15</td>
<td>1</td>
<td>6.6</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Kanayat</td>
<td>15</td>
<td>1</td>
<td>76</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>DearbNegm</td>
<td>15</td>
<td>2</td>
<td>13.3</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>El-Salhia</td>
<td>14</td>
<td>1</td>
<td>7.14</td>
<td>6</td>
<td>16.6</td>
</tr>
<tr>
<td>Fakos</td>
<td>13</td>
<td>1</td>
<td>7.60</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Menca El-Kamh</td>
<td>15</td>
<td>–</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>9</td>
<td>8.1</td>
<td>34</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Figure 1. Biochemical identification of Salmonella isolates using API 20 E kits showing excellent Salmonella identification (seven-digit code number: 6704752 id%: 99.9 T index: 0.97)

Table 2. Frequency occurrence of different Salmonella serogroups isolated from infected chickens

<table>
<thead>
<tr>
<th>Identified strains</th>
<th>Antigen structure</th>
<th>No strain</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enteritidis</td>
<td>3, 4, 5, 9 c/h: 1.4</td>
<td>4</td>
<td>44.4</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>1, 6, 7 i:1.2</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>S. spp.</td>
<td>2, 8</td>
<td>2</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Table 3. Antibiotic sensitivity pattern of various Salmonella isolates

<table>
<thead>
<tr>
<th>Name of antibiotic</th>
<th>Sensitivity</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florphenicol</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>88.8%</td>
<td>11.2%</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>55.5%</td>
<td>43.5%</td>
</tr>
<tr>
<td>Amoxycelline</td>
<td>44.4%</td>
<td>55.6%</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>33.3%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Trimethoprime</td>
<td>33.3%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Neomycin</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>0%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Also, the results of Salmonella enterica were positive by using PCR and species-specific primers (ENTF and ENTR) with a target size of 304 bp for detecting S. enteritidis and primers (TYPHF and TYPHR) with a target size of 401 bp for detecting S. typhimurium are presented in Figure 2.

Lane M.: 2500 base-pair size marker ladder; Lane c: positive referring to Salmonella spp. (204 bp), S. enteritidis (304 bp), S. typhimurium (401 bp). Lane 1, 6, 7 S. typhimurium with amplicon size (304 bp). Lane 3, 4, 5, 9: refers to the species S. enteritidis. Lane 1, 6, 7: refers to S. typhimurium. Lane 2, 8: to the un-typed Salmonella, Lane 3, 4, 5, 9 refers to S. enteritidis with amplicon size (304 bp). All Lanes 1–9 showed +ve amplicon and molecular size 204 bp for Salmonella spp.

PCR for detection of the most common virulence genes in different Salmonella isolates

Using five sets of primers, PCR was used to detect virulence genes that proved to play an important role in the virulence of Salmonella isolates (AvrA, Stn, Mgtc, SopB, Bcfc). It was applied to five different Salmonella isolates. The PCR products of the obtained virulence genes gave characteristic bands at 600, 422, 677, 517 and 467 bp for Stn, AvrA, Mgtc, SopB and Bcfc virulence genes, respectively (Figures 3–7).
**Antibiotic sensitivity**

The nine isolates of *Salmonella* showed that isolates were highly sensitive to Florophenicol (100%), Ciprofloxacin (88.8%), Gentamycin (55.5%), Amoxicillin (44.4%), Kanamycin (33.3%), Trimethoprim (33.3%), Neomycin (0%) and Spectinomycin (0%), (Table 3).

**Bio-control of *Salmonella* (typhimurium and enteritidis) by *B. subtilis***

Results of the *in vitro* growth showed that the best result of *Bacillus subtilis* was $1 \times 10^8$, which reduced the growth of microorganisms under study (*S. typhimurium* and *S. enteritidis*). Probiotics were used to determine the antimicrobial activity and produced microbial inhibition zone in *Salmonella* spp. (Table 4).

<table>
<thead>
<tr>
<th>Concentration of B. subtilis</th>
<th>Zone inhibition of S. typhimurium</th>
<th>Zone inhibition of S. enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{10}$</td>
<td>2.3 cm</td>
<td>2.1 cm</td>
</tr>
<tr>
<td>$1 \times 10^9$</td>
<td>2.3 cm</td>
<td>2.1 cm</td>
</tr>
<tr>
<td>$1 \times 10^8$</td>
<td>2.0 cm</td>
<td>1.95 cm</td>
</tr>
</tbody>
</table>

– Performance parameters (body weight and weight gain) at 28 days post-infection in group 2 broilers showed a significantly lower body weight than the control (Table 5).

– Broilers treated with probiotics and prebiotics showed a significant increase ($P<0.05$) in total erythrocytic and total leukocytic counts all over the experimental period compared with the infected broilers only (Table 6 and Figure 8).

**Table 5. The effect of probiotic and prebiotic on mortality and performance parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of broilers</th>
<th>Dose/ Broilers</th>
<th>Route of administration</th>
<th>Mortality</th>
<th>Reisolation</th>
<th>7 days PI</th>
<th>14 days PI</th>
<th>28 days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (healthy chickens)</td>
<td>20</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>380±40 a</td>
<td>840±112 a</td>
<td>1956±140 a</td>
</tr>
<tr>
<td>Infected chicken group</td>
<td>20</td>
<td>$1 \times 10^8$</td>
<td>Orally</td>
<td>8/20</td>
<td>7/20</td>
<td>142±33 c</td>
<td>360±85 d</td>
<td>1540±160</td>
</tr>
<tr>
<td>Infected group and treated with <em>B. subtilis</em> (probiotic)</td>
<td>20</td>
<td>$1 \times 10^8$</td>
<td>Orally</td>
<td>1/20</td>
<td>0/20</td>
<td>220±52 b</td>
<td>640±69 b</td>
<td>1970±80 a</td>
</tr>
<tr>
<td>Infected group and treated with levovyl (prebiotic)</td>
<td>20</td>
<td>$1 \times 10^8$</td>
<td>Orally</td>
<td>2/20</td>
<td>1/20</td>
<td>283±39 c</td>
<td>674±110 b</td>
<td>1940±70 a</td>
</tr>
<tr>
<td>Infected group and treated with panflor (antibiotic)</td>
<td>20</td>
<td>$1 \times 10^8$</td>
<td>Orally</td>
<td>2/20</td>
<td>2/20</td>
<td>253±60 d</td>
<td>549±120 c</td>
<td>1820±90 b</td>
</tr>
</tbody>
</table>

PI: post infection.

a, b, c, d, e – means in the same column with different letters are significantly different ($P<0.05$).

**Table 6. Effect of probiotic and prebiotic given in drinking water for five successive days on total erythrocyte and total leukocyte count**

<table>
<thead>
<tr>
<th>Parameter Groups</th>
<th>Total RBC counts</th>
<th>Total WBC count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 day PI</td>
<td>14 day PI</td>
</tr>
<tr>
<td>Control group (healthy chickens)</td>
<td>4.46±0.09 a</td>
<td>4.54±0.07 a</td>
</tr>
<tr>
<td>Infected chicken group</td>
<td>3.05±0.02 c</td>
<td>3.22±0.03 d</td>
</tr>
<tr>
<td>Infected group and treated with <em>B. subtilis</em> (probiotic)</td>
<td>3.05±0.02 b</td>
<td>4.04±0.05 b</td>
</tr>
<tr>
<td>Infected group and treated with levovyl (prebiotic)</td>
<td>3.48±0.01 b</td>
<td>3.57±0.08 c</td>
</tr>
<tr>
<td>Infected group and treated with panflor (antibiotic)</td>
<td>3.12±0.07 c</td>
<td>3.42±0.01 c</td>
</tr>
</tbody>
</table>

RBC: Red blood cells, WBC: White blood cells, PI: Post infection.
a, b, c, d, e – means in the same column with different letters are significantly different ($P<0.05$).

Figure 8. Impacts on total erythrocytic and total leukocytic count are all over the experimental period compared with infected broilers only.
– Broilers treated with probiotics and prebiotics showed a significant increase (P≤0.05) in hemoglobin ratio compared to the untreated broilers and those infected with Salmonella spp. (Table 7).

Table 7. Effect of probiotic and prebiotic given in drinking water for five successive days on hemoglobin ratio (Hb) in healthy and experimentally infected broilers with Salmonella organism

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hemoglobin ratio (Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days PI</td>
</tr>
<tr>
<td>Control group (healthy chickens)</td>
<td>14.15±0.04 a</td>
</tr>
<tr>
<td>Infected chicken group</td>
<td>9.4±0.07 c</td>
</tr>
<tr>
<td>Infected group and treated with</td>
<td>10.8±0.17 b</td>
</tr>
<tr>
<td>B. subtilis (probiotic)</td>
<td>9.55±0.08 c</td>
</tr>
<tr>
<td>Infected group and treated with</td>
<td>9.55±0.08 c</td>
</tr>
<tr>
<td>levoxyl (prebiotic)</td>
<td></td>
</tr>
</tbody>
</table>

PI: Post infection.

(a, b, c – means in the same column with different letters are significantly different (P<0.05).

Discussion

In this study, nine pooled samples from poultry aged 1 to 40 days were suspected to be Salmonella-positive out of 111 collected pooled samples with an incidence of 8.1% in seven positive farms from a total of 34 farms with a farm infection rate of 20.5%. Microbiological, biochemical and serological examinations confirmed that all suspected isolates belong to the genus Salmonella. Also, PCR examination confirmed that all suspected isolates belong to the genus Salmonella by using specific primers (Alvarez et al., 2004).

Furthermore, multiplex PCR analysis identified four isolates as S. enterica serovar enteritidis, three as S. enterica serovar Typhimurium and two Salmonellae un-typed (Alvarez et al., 2004). For the effect of B. subtilis on microbial activity, it is noticeable that the use of B. subtilis reduced the growth of selected microorganisms (Salmonella spp.). This result agrees with those of Khodary and El-Sayed (1997).

In the present study, PCR was used to detect five virulence genes that proved to play a role in the virulence of Salmonella genes (Stn, AvrA, Mgtc, SopB, and Bcfc). Two isolates had five virulence genes, while three isolates had four virulence genes (Stn, Mgtc, SopB and Bcfc). The PCR products of the obtained virulence genes gave characteristic bands at 422, 600, 677, 517, and 467 pb for AvrA, Stn, Mgtc, SopB and Bcfc virulence genes, respectively. These results agree with those obtained by Zou et al. (2012), who found that all the 425 isolates tested by PCR were positive for the presence of virulence genes Mg2+ transportation system (Mgtc), production enterotoxin (Stn) and Salmonella outer protein production (SopB). Salmonella on all clinical isolates gave us highlights on the role of this gene in the production of enterotoxin.

Also, MurugKar et al. (2003) confirmed the presence of the Stn gene in all Salmonella serotypes and reported that the Stn gene obtained a unique sequence to Salmonella strain field samples. The experimental infection of broiler chicks with Salmonella spp. showed clinical signs after the incubation period of 36 hours. Similar results were obtained by Reda et al. (2019).

The clinical signs were the information of depression, weakness, off food, ocular discharge, closed eye and greenish diarrhea in the two infected groups. The mortality rates of chicks of groups 2, 3, 4 and 5 were 40%, 5%, 10% and 10%, respectively. The inoculated nalidixic acid-resistant Salmonella percentage was high from the caeca, intestine and liver of orally infected chicks (0%, 35%, 0%, 5% and 10%, respectively). Also, Hui and Dus (2001) assured that the highest percentage of Salmonella may exist in the intestine and liver. Performance parameters (body weight and weight gain) at 28 days post-infection in group 2 broilers showed the lowest mean body weight, 1540 g, compared to the control group, 1950 g. While in groups 4 and 5, body weights were 1940 g and 1830 g, in group 3. These results are similar to those of Khodary and El-Sayed (1997).

The effect of probiotics and prebiotics after 35 days of treatment showed an increase in feed consumption compared to the control. This result agrees with Andreeva and Dimitrov (2002), who reported increased feed consumption for probiotics and prebiotics-treated groups concerning body weight gain. Also, Jin et al. (1997), Alagawany et al. (2016, 2018) and Soomro et al. (2019) observed good growth elevation with increased feed intake in birds treated with probiotics and prebiotics.

Conclusions

Probiotics and prebiotics are potential tools for reducing intestinal contamination caused by food-borne bacteria. They could be successfully used as nutritional tools in poultry feed to promote growth, modulate intestinal microflora and inhibit pathogens. Lactic acid-producing bacteria and Bacillus subtilis species have been recently used as commercial probiotics. So, probiotics and prebiotics could be used as antibiotic alternatives to improve poultry health and production. Based on our findings, the best result of B. subtilis was 1×108 which reduced the growth of the microorganism under study (S. typhimurium – S. enteritidis).

Author Contributions


Ethical approval

Animal maintenance and care adhered to Zagazig University’s criteria for the care and use of laboratory animals and those of the Egyptian Research Ethics Committee.
Data Availability Statement

The datasets generated for this study are available on request from the principal or corresponding authors.

Conflict of interest

The author declares no conflicts of interest.

Acknowledgment

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