Effects of dietary supplementation of *Ecklonia cava* with or without probiotics on the growth performance, nutrient digestibility, immunity and intestinal health in weanling pigs

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**ABSTRACT**

The present study investigated the effect of dietary supplementation of *Ecklonia cava*, probiotics or their combination on the growth performance and gut health in weanling pigs. A total of 240 weaned pigs (Landrace × Yorkshire × Duroc, 7.04 ± 0.23 kg) were allotted to four treatments. The dietary treatments were basal diet (control) or diet supplemented with 1.5 g/kg *Ecklonia cava* (EC), 3.0 g/kg fermented probiotic product (P) or combination of both 1.5 g/kg *Ecklonia cava* and 3.0 g/kg fermented probiotic product (ECP) fed in meal form for 2 phases. Average daily gain (ADG) was improved consistently in phases I, II and overall result of the experiment for supplemented groups, and feed efficiency was improved (*p* < 0.05) in the PR and EC throughout the experiment. The digestibility of dry matter (DM), gross energy (GE) and crude protein (CP) was improved (*p* < 0.05) in both phases for PR and EC. The cecal *Lactobacillus* spp. was increased (*p* < 0.01) for PR and EC while *E. coli* was decreased (*p* < 0.05) in both supplements. Serum IgG, IgM and IgA were increased (*p* < 0.05) in the EC groups. The interaction between PR and EC was significant for IgG at the second phase as well. The villus height of duodenum, jejunum and ileum was increased (*p* < 0.05) in EC and PR groups, while crypt depth was not affected by the treatments. This suggested that both *Ecklonia cava* and probiotics are beneficial for weanling pigs; however, there were no interactions.

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**Introduction**

Pigs are subjected to different kinds of nutritional, environmental and social stress at the time of weaning that are associated with changes in the gut morphology, reduced growth rates and array of weaning related disorders (Pluske et al. 1996). Previously, these issues were addressed by exploring different in-feed additives such as antibiotics (Williams et al. 2001; O’Doherty et al. 2005). Excessive use of these additives created other issues such as resistance to antibiotics, gut microbial imbalance and environmental concerns (Pierce et al. 2006). Considering the above facts, search for different in-feed additives is ongoing.

Recently, few types of seaweed have been explored as an alternative to the above discussed in-feed additives due to their rich content of non-digestible polysaccharides and soluble dietary fibres (Gahan et al. 2009). *Ecklonia cava*, a brown marine alga, is one of the seaweeds highly persistent in clear waters and found mostly in South Korea, Japan and China. It contains about 50% carbohydrate, 28% protein, 1.2% lipid and almost 20% polyphenols in dry matter (Ahn et al. 2011). Previous studies conducted on different species suggested in-feed supplementation of *Ecklonia cava* can improve growth in fishes and immunity in murine (Ahn et al. 2008; Kim et al. 2014). However, as per our knowledge, role of *Ecklonia cava* as feed additive in pigs is still unexplored.

Probiotics are friendly bacteria that positively regulate the gut microflora and maintain the host body balance (Czerucka & Rampal 2002). Previous studies suggested that, intestinal bacteria play an integral role in animal health and nutrition with over 400 species and total numbers around 10^{14} (Holzapfel et al. 1998). The addition of lactic acid bacteria and bacillus-based probiotic products has been reported to increase the growth and nutrients digestibility in pigs (Giang et al. 2010). However, there are few studies that reported the negative results in practical feeding trials of growing-finishing pigs (Vitini et al. 2000).
Therefore, the aim of the present experiment was to evaluate the effect of *Ecklonia cava* with or without fermented probiotic product on the growth performance, coefficient of total tract apparent digestibility (CTTAD) of nutrients, serum immunoglobulins, cecal microflora and intestinal morphology of weaning pigs. It was hypothesised that the inclusion of fermented probiotic product in combination with *Ecklonia cava* would improve the performance of weanling pigs by modifying the gastrointestinal tract microflora, digestibility and immunity.

**Materials and methods**

The present experiment was conducted at the facility of Kangwon National University farm. The protocol for this experiment was approved by the Institute Animal Care and Use Committee of Kangwon National University, Chuncheon, South Korea.

**Ecklonia cava collection and processing**

The powder form of *Ecklonia cava* was used in this study. Leaves of seaweed *Ecklonia cava* were directly collected from the sea near Jeju Island, washed three times in water to remove the impurities and then dried in oven at 36°C. These leaves were then ground to the particle size of about 100 μm and were directly used in the feed.

**Preparation of probiotic product**

To prepare the fermented probiotic product, *Lactobacillus acidophilus*, *Bacillus subtilis* and *Saccharomyces cerevisiae* were isolated from the faeces of weaned pigs, natto (fermented soybeans) and koji (malted wheat) respectively, and were maintained as stock culture in the laboratory (MRS Broth for *lactobacillus acidophilus*, TSB culture for *Bacillus subtilis* and *Saccharomyces cerevisiae*, Difco Laboratories, Detroit, MI, USA). A corn and molasses (CM) medium containing 60.0 mL corn steep liquor, 40.0 mL molasses, 3.0 g/L yeast extract, 5.0 g/L KH₂PO₄ and 2.5 g/L K₂HPO₄ in distilled water was prepared and autoclaved before use. Two liters of autoclaved CM were inoculated with 20 mL of culture of each microbe separately and subjected to fermentation for 48 h. The *L. acidophilus* and *B. subtilis* were incubated at 37°C at pH 7.0 (with addition of 3.5 gr NaOH to CM), whereas *S. cerevisiae* was incubated at 32°C and pH 4.0 (with addition of HCl to adjust the pH to 4). The microbes grown on CM were directly sprayed on corn:soybean meal (1:1 for corn:soybean ratio) followed by drying at 40°C for 72 h. These microbes were used as starter and the pasteurised corn:soybean meal (1:1) mixture was used as the substrate for fermentation as previously described by Shim et al. (2010). The substrates (13.0 kg) were then inoculated with 2.0 L of starter and fermented for 7 days at 32°C and at pH 7.0. After 7 days fermentation, the whole mixture was dried at 40°C for 72 h and mixed to obtain fermented probiotic product. The counts of *L. acidophilus*, *B. subtilis* and *S. cerevisiae* in fermented probiotic product were $4.0 \times 10^8$, $4.8 \times 10^9$ and $1.0 \times 10^8$ cfu/g respectively.

**Animals and experimental design**

A total of 240 weaned pigs (Landrace × Yorkshire × Duroc, initially 7.04 ± 0.23 kg body weight and 28 ± 2 day of age) were randomly allotted to 4 treatments on the basis of body weight. There were 5 replicate pens in each treatment with 12 pigs per pen. All the pigs were housed in partially slatted concrete floor pens (1.90 × 2.54 m) equipped with self-feeder and nipple drinker to allow ad libitum access to feed and water. The dietary treatments included a basal diet (control) or basal diet supplemented with 1.5 g/kg *Ecklonia cava* (EC), 3.0 g/kg fermented probiotic product (P) or combination of both 1.5 g/kg *Ecklonia cava* and 3.0 g/kg fermented probiotic product respectively. The experimental diets (Table 1) were fed in a meal form in 2 phases (d 0–14, phase I and d 15–28, phase II). Diets were formulated to contain 14.22 MJ/kg ME and 15.3 g/kg lysine (phase I; Table 1) and 14.02 MJ/kg ME and 14.0 g/kg lysine (phase II; Table 1). All diets met or exceeded the nutrient requirements as recommended by National Research Council (2012).

**Sample preparation and measurements**

The pigs were weighed individually at the beginning and at the end of each phase. Consumption of feed was recorded at the end of each phase. On the bases of these records, average daily gain (ADG), average daily feed intake (ADFI) and feed to gain ratio (F/G) were calculated.

To evaluate the digestibility, 2.5 g/kg of chromium was added in the diets as an indigestible marker during the last seven days of each phase, and then faecal grab samples were randomly collected from four pigs of each pen during the last three days of each phase and pooled per pen. Faeces were pooled and dried in an air forced drying oven at 60°C for 72 h and ground in a Wiley laboratory mill (Thomas Model 4 Wiley® Mill, Thomas scientific, Swedesboro, NJ) using a 1-mm screen for chemical analysis.
Table 1. Ingredient and chemical composition of basal diets (as-fed basis).

| Ingredient, g/kg | Phase I (d 0–14) | Phase II (d 15–28) |
|-----------------|------------------|---------------------|
| Ingredient, g/kg |                  |                     |
| Corn             | 338.3            | 497.8               |
| Whey powder      | 200.0            | 153.8               |
| Fish meal        | 50.0             | 30.0                |
| Soybean meal dehulled | 232.8   | 229.2               |
| Soy Protein Concentrate | 50.0    | 30.0                |
| Soy oil          | 36.4             | 30.5                |
| Monocalcium Phosphate | 3.1      | 7.0                 |
| Limestone        | 6.4              | 8.6                 |
| Salt             | 2.0              | 2.0                 |
| DL-Methionine (980 g/kg) | 1.4   | 1.1                 |
| L-Lysine (780 g/kg) | 1.6        | 2.0                 |
| Vitamin premix* | 2.0              | 2.0                 |
| Mineral premix*  | 2.5              | 2.5                 |
| Choline chloride (500 g/kg) | 0.5  | 0.5                 |
| Zinc Oxide       | 3.0              | 3.0                 |
| Lactose          | 70.0             | -                   |
| Chemical composition |          |                     |
| Metabolic Energy, MJ/kg | 14.22 | 14.02               |
| Crude Protein, g/kg   | 230.0           | 210.0               |
| Calcium, g/kg       | 8.0             | 8.0                 |
| Available Phosphorus, g/kg | 4.6 | 4.6                 |
| Lysine, g/kg        | 15.3            | 14.0                |
| Methionine + Cystine, g/kg | 8.7 | 7.9                 |
| Lactose, g/kg       | 200.0           | 100.0               |

The dietary treatments were: Control (basal diet) and basal diet supplemented with 1.5 g/kg Ecklonia cava, 3.0 g/kg probiotics or combination of both Ecklonia cava (1.5 g/kg) and probiotics (3.0 g/kg) respectively.

*Supplied per kilogram of diet: 20 000 U vitamin A, 4200 U vitamin D3, 4.2 mg vitamin B6, 0.042 mg vitamin B12, 14 mg pantothenic acid, 42 mg niacin, 0.105 mg biotin, 1.05 mg folic acid.

To analyse the concentrations of immunoglobulins (IgG, IgA and IgM), 10 mL blood sample were collected from 2 randomly selected pigs from each pen at d 14 and 28 of the experiment. The sampling was done by jugular vein puncture using a disposable vacutainer tube without anticoagulants (Becton Dickinson, Franklin, NJ). The blood samples were then centrifuged (3000 x g for 15 min 4 °C) for the separation of serum which were then stored at −20 °C until analysis.

To study the effect of diets on the small intestinal morphology and cecal microflora, pigs from each treatment (2 per replicate) based on average body weight were selected and sacrificed by electrocution at the end of the experiment (d 28). The cecum contents were collected in sterilised plastic bottle and stored at 7 °C for bacterial analysis. The intestinal samples from the region of duodenum, jejunum and ileum were also collected after removing its contents and flushing it with physiological saline. The samples were submerged in a fixative solution (0.1 M collidine buffer, pH 7.3) containing 30 g/L glutaraldehyde, 20 g/L paraformaldehyde and 15 g/L acrolein and then brought to the laboratory for studying the morphological changes.

Chemical and microbial analyses

Experimental diets and excreta samples were analysed in triplicate for dry matter (DM, method 930.15; AOAC 2007), crude protein (CP, method 990.03; AOAC 2007), ash (method 942.05; AOAC 2007), calcium, and phosphorus (method 985.01; AOAC 2007). The gross energy (GE) of diets and faeces were measured by a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL, USA), and chromium concentration was determined with an automated spectrophotometer (Jasco V-650, Jasco Corp., Tokyo, Japan). Amino acid composition of feed samples was determined by HPLC (Waters 486, Waters Corp., Milford, MA) after acid hydrolysis. The methionine and cystine were determined following oxidation with performic acid (Moore 1963). The concentrations of serum IgG, IgA and IgM were analysed using radial immune-diffusion kits (Tripple J Farms, Bellingham, WA).

The microbiological assay of cecal samples was carried out by the procedure published previously (Torrallardona et al. 2003). One gram of mixed content was diluted with 9 mL of Butterfields phosphate buffer solution, followed by further serial dilutions in the phosphate buffer solution. Duplicate plates were then inoculated with 0.1 mL sample and incubated. The microbial groups enumerated were total anaerobic bacteria (TAB, plate count agar, Difco Laboratories, Detroit, MI, USA), E. coli (MacConkey agar-incubated for 24 h at 37 °C), Lactobacillus spp. (MRS agar, Oxoid, Hampshire, UK). The anaerobic conditions during the assay of total anaerobic bacteria and Clostridium spp. were created by using gas-pak anaerobic system (BBL, No. 260678, Difco, Detroit, MI). The microbial populations were log transformed before statistical analysis.

Small intestinal morphology

Three cross-sections for each intestinal sample were prepared after staining with azure A and eosin using standard paraffin embedding procedures. A total of 10 intact and well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height was measured from the tip to the villus crypt junction. Crypt depth was measured as the depth between adjacent villi. All morphological measurements (villus height or crypt depth) were made in 10 μm increments by using an image processing and analysis system (Optimus software version 6.5, Media Cybergenetics, North Reading, MA).
Statistical analysis

Data generated in the present experiment was analysed as a 2×2 factorial arrangement in a completely randomised design. Pens were considered the experimental unit for growth performance and digestibility, whereas piglets were experimental units for intestinal sampling and blood samples. The main effects of fermented probiotic product and Ecklonia cava, and their interaction were determined by mixed procedure of SAS statistical program (SAS Inst., Inc., Cary, NC). p Values ≤0.05 were considered statistically significant.

Results

Growth performance

During the all periods, no significant interaction was found between Ecklonia cava and fermented probiotic product dosage in terms of ADG, ADFI and F/G (Table 2). Inclusion of EC and PR significantly improved the average BW gain and decreased the feed-to-gain ratio in phase 1. A similar pattern was observed for growth performance in the second phase and in the overall period with a greater ADG and feed-to-gain ratio for both EC and P. The ADFI did not show any significant change.

Digestibility

At phase I the CTTAD of DM was increased (p<0.05) in both PR and EC groups while the digestibility of CP and GE was increased (p<0.05) only in the probiotics supplemented diet (Table 3). At phase II DM, GE and CP digestibility was enhanced (p<0.05) in both PR and EC. No significant difference was observed in the interaction between the dietary treatments.

Cecal microbial populations

The population of cecal Lactobacillus spp. was increased in the diet added with PR and EC and the population of E. coli decreased (p<0.05) in both supplements (Table 4). The population of TAB and Clostridium spp. was similar and not affected (p>0.05) by dietary treatments. No significant interaction was found between supplemented groups.

Serum immunoglobulins

Dietary fermented probiotics had similar levels of immunoglobulins in both phases. However, the IgM levels improved (p<0.05) in the Ecklonia cava in phase I, and also significant differences on IgG, IgA and IgM were observed in EC supplemented diet. No interaction was observed between dietary PR and EC for IgA and IgM, however, the interaction between PR and EC was significant for IgG at the second phase (Table 5).

Intestinal morphology

The villus height and crypt depth of duodenum, jejunum and ileum was increased (p<0.05) in the PR (Table 6). EC supplemented diets also showed...
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Table 5. Effects of supplementation of probiotics (PR) and Ecklonia cava (EC) on serum immunoglobulins (mg/mL) of weanling pigs.

| Item     | EC 0 | 1.5 g/kg | PR 0 | 3 g/kg | SEM* | p value | EC 0 | PR 0 | EC*PR |
|----------|------|----------|------|--------|------|---------|------|------|-------|
| d14 IgG  | 6.23 | 6.37     | 6.28 | 6.32   | 0.11 | 0.246   | 0.736 | 0.403 |
| IgA      | 0.34 | 0.35     | 0.34 | 0.35   | 0.01 | 0.129   | 0.804 | 0.225 |
| IgM      | 0.83 | 0.86     | 0.84 | 0.84   | 0.01 | 0.017   | 0.771 | 0.5  |
| d28 IgG  | 6.36 | 6.63     | 6.48 | 6.51   | 0.06 | <0.001  | 0.603 | 0.035 |
| IgA      | 0.35 | 0.39     | 0.37 | 0.37   | 0.01 | 0.001   | 0.806 | 0.625 |
| IgM      | 0.85 | 0.88     | 0.87 | 0.87   | 0.01 | 0.004   | 1     | 0.073 |

*Standard error of means.

Table 6. Effects of supplementation of probiotics (PR) and Ecklonia cava (EC) on small intestinal morphology in weanling pigs (d 28).

| Item       | EC 0 | 1.5 g/kg | PR 0 | 3 g/kg | SEM* | p value | EC 0 | PR 0 | EC*PR |
|------------|------|----------|------|--------|------|---------|------|------|-------|
| Villus length, μm |      |          |      |        |      |         |      |      |       |
| Duodenum   | 430  | 454      | 428  | 456    | 9.23 | 0.017   | 0.006 | 0.344 |
| Jejunum    | 425  | 441      | 423  | 443    | 6.39 | 0.024   | 0.005 | 0.101 |
| Ileum      | 320  | 332      | 317  | 335    | 4.34 | 0.012   | 0.001 | 0.635 |
| Crypt depth, μm |      |          |      |        |      |         |      |      |       |
| Duodenum   | 248  | 256      | 247  | 257    | 3.44 | 0.024   | 0.009 | 0.461 |
| Jejunum    | 243  | 250      | 243  | 251    | 2.47 | 0.011   | 0.006 | 0.029 |
| Ileum      | 193  | 198      | 192  | 199    | 2.34 | 0.077   | 0.012 | 0.282 |
| VH/CD*     |      |          |      |        |      |         |      |      |       |
| Duodenum   | 1.74 | 1.78     | 1.73 | 1.78   | 0.04 | 0.402   | 0.333 | 0.689 |
| Jejunum    | 1.75 | 1.76     | 1.74 | 1.77   | 0.03 | 0.657   | 0.414 | 0.849 |
| Ileum      | 1.65 | 1.68     | 1.65 | 1.68   | 0.02 | 0.342   | 0.185 | 0.749 |

*Standard error of means.

Discussion

In order to improve the performance of pigs, different alternatives have been explored. Seaweeds and probiotic products are among few of them (Grinstead et al. 2000; Davis et al. 2008; Giang et al. 2010). Ecklonia cava is rich in polyphenolic contents such as Eckol, Dieckol, Phloroglucinol, Phlorofucofuroeckol, Phlorotannins, Fucoidanetc (Kwon et al. 2013). It is also well known for antitumor, anticoagulant, antioxidant and antiviral activities to inhibit the viral replication (Kwon et al. 2013). While probiotics products are known to improve gut heath by regulating the gut microflora (Czerucka & Rampal 2002).

In the present study, supplementation of Ecklonia cava, fermented probiotic product improved ADG in pigs. This is in line with the previous studies of Grinstead et al. (2000) who reported improvement in ADG of piglets when the diets were provided in meal form and supplemented with seaweed. Similarly, different studies on the supplementation of different microbes such as Saccharomyces cerevisiae, lactobacillus acidophilus or Bacillus-based DFM suggested improvement of ADG and feed conversion ratio in weaning pigs (Baum et al. 2002; Davis et al. 2008; Giang et al. 2010). The gut is a rich source of nutrients for bacteria, and the ability of pathogenic bacteria to rapidly utilise the energy source may reduce the lag phase of bacterial growth (Wilson & Perini 1988). Increase in ADG might be due to the competing nature of probiotic products against the pathogenic bacteria, improvement in the gut health, and intestinal morphology (Malago & Koninkx 2011).

Probiotics are known to possess high fermentative capacity and stimulate digestion (Hong et al. 2002). In the present study, the digestibility of DM, GE and CP were considerably improved in both PR and EC supplemented diets. This agreed with the previous studies where supplementation of Bacillus subtilis or Lactobacillus acidophilus in pig’s diet increased the digestibility of GE and CP in weaning pigs (Giang et al. 2010; Meng et al. 2010). Higher concentration of organic acids was reported in the gut contents of pigs supplemented with probiotic complexes (Hogberg & Lindberg 2006), which may decrease the pH in the gut and may have resulted in better nutrient digestibility (Lyberg et al. 2006).

In the present studies, there was a considerable increase in the population of lactobacillus spp. and decrease in E. coli in both PR and EC supplemented diets. This correlates the previous studies where inclusion of the LAB complex together with the mixture of Bacillus and Saccharomyces increased LAB counts and decreased E. coli counts in the pigs (Giang et al. 2011). The deficiency of even one essential nutrient can inhibit the microbial growth and decrease in the E. coli population as the presence of probiotics may compete for nutrients and absorption sites with pathogenic bacteria (Malago & Koninkx 2011). Lactobacillus spp. are acid resistance. Lorca and de Valdez (2001) suggested that acid resistance in Lactobacillus acidophilus appeared to be mediated by membrane ATPases. Moreover, Czerucka and Rampal (2002) reported that Saccharomyces can produce antimicrobial substances that may decrease the levels of potential pathogens in the gut lumen. Similarly, Bacillus spp. which are not the principal members of the normal intestinal flora, but it consumes oxygen rapidly and thus reduces the pH, which may favour Lactobacilli and inhibits E. coli (Wu et al. 2011). There was no variation in the microbial population in Ecklonia cava
treated pigs. This is in line with the previous reports of Walsh et al. (2013), where they reported no variation on the *Lactobacillus* spp. population in the pigs containing seaweed extract in diets.

The first few weeks after birth are critical for the piglets as their immune system is poorly developed and are more susceptible to diseases until 4–5 week of age (Wang et al. 2004). Immunoglobulins act as an important part of the immune response to bind with specific antigens. Various immunoglobulin isotypes can offer a conception about the complex humoral immune response (Lefranc & Lefranc 2001).

In the present study, levels of serum immunoglobulins (IgG, IgA and IgM) were higher in *Ecklonia cava* supplemented diets. The tendency for an interaction between PR and EC for serum immunoglobulins was observed. This might be due to the presence of polyphenolic compounds in the *Ecklonia cava* as earlier studies reported that compounds isolated from *E. cava* have shown strong antiviral activity against porcine epidemic diarrhea virus, inhibiting viral entry and/or viral replication (Kwon et al. 2013). Some probiotic strains are capable to act as an immunomodulators by enhancing the serum immunoglobulin levels (Vitini et al. 2000). However in the present study no change was observed in any of the immunoglobulin types in pigs fed the probiotic supplemented diets in comparison to control. This difference of immune response from the previous studies could be due to preparation of probiotics product, low survival rate of the strains, probiotic doses, administration frequency, age, stress, health, nutritional status and animal type (Vitini et al. 2000; Bomba et al. 2002).

Intestinal morphology is the indicator of gut health. In the present study, the villi height of duodenum, jejunum and ileum were increased in the *Ecklonia cava* treated pigs. This is in line with the previous reports where pigs offered seaweed extract had higher villous height compared with pigs offered the basal diet (Walsh et al. 2013). Similarly, the villi height of the whole intestine (duodenum, jejunum and ileum) was considerably increased in pigs fed probiotic. This is in line with the previous studies where supplementation of *Lactobacillus* spp. as probiotics increased the villus height of piglets (Willing & Van Kessel 2007). The crypt depth of duodenum and jejunum was affected in the *Ecklonia cava* or probiotics group. In the previous studies, Scharek et al. (2005) reported no change in the crypt depth in proximal jejunum of pigs suggesting inconsistent effects of probiotics on crypt depth; however Willing and Van Kessel (2007) reported increase in crypt depth of piglets inoculated with *Lactobacillus* spp. This may probably due to the variations of the strains, dosage or the application of used probiotics product and can be an important observation as negative alterations normally occur in the intestinal morphology of pigs during the initial post-weaning and are directly related with the nutrient digestion and absorption capacity of the small intestine (Pluske et al. 1996).

**Conclusions**

From the present study it can be concluded that the supplementation of *Ecklonia cava* or fermented probiotic product as feed additive has shown beneficial effects on performance, digestibility and gut health. Serum immunoglobulins as an effective factor on immune system were improved by *Ecklonia cava*. However, supplementation of fermented probiotic product with *Ecklonia cava* in combination did not show an obvious interaction.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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