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SHORT COMMUNICATION

In vitro assay for evaluating the effects of three anti-nutritional factors on the primary-cultured intestinal epithelial cells isolated from Japanese flounder, *Paralichthys olivaceus*

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Aquaculture is one of the most rapidly growing food-producing sectors. Its average annual growth rate between 1970 and 2009 was 8.3% (FAO 2012). However, the shortage of fish meal supply is limiting the development of aquaculture. Thus, alternative protein sources are being widely studied and used in aquaculture. Soybean meal is the most promising one, which is regarded as an economical and nutritious alternative due to its relatively low cost, high crude protein content and reasonably balanced amino acid profile (Lim, Kim, Ko, Song, Oh, Kim, Kim & Lee 2011; Lin & Luo 2011; Silva-Carrillo, Hernández, Hardy, González-Rodríguez & Castillo-Vargasmachuca 2012). However, high inclusion of soybean protein has been reported to inhibit the growth performance of fish which was usually accompanied with the histological damages of intestine (Refstie, Landsverk, Bakke-McKellep, Ringø, Sundby, Shearer & Krogdahl 2006; Ringø, Sperstad, Myklebust, Refstie & Krogdahl 2006; Bakke-McKellep, Penn, Salas, Refstie, Sperstad, Landsverk, Ringø & Krogdahl 2007). Anti-nutritional factors in soybean meal have been demonstrated to be the main factor causing these damages to the growth and intestinal health of fish (Francis, Makkar & Becker 2001; NRC 2011).

In vivo studies with effects of anti-nutritional factors on intestinal health of fish have been extensively studied. Soyasaponins could cause significant intestinal damage in rainbow trout, chinook salmon, Atlantic salmon and Japanese flounder respectively (Krogdahl, Roem & Baeverfjord 1995; Bureau, Harris & Cho 1998; Iwashita, Yamamoto, Furuita, Sugita & Suzuki 2008; Chen, Ai, Mai, Xu, Liufu, Zhang & Cai 2011). Dietary phytic acid could induce histological lesions in digestive tract of chinook salmon (Richardson, Higgs, Beames & McBride 1985) and carp (Hossain & Jauncey 1993). For raffinose, both positive (Van den Ingh, Krogdahl, Olli, Hendriks & Koninkx 1991; Krogdahl *et al.* 1995; Sørensen, Penn, El-Mowafi, Storebakken, Cai, Øverland & Krogdahl 2011) and negative (Olli & Krogdahl 1995a,b; Refstie, Svihus, Shearer & Storebakken 1999 & Grisdale-Helland, Helland & Gatlin 2008) effects were reported on fish. These three anti-nutritional factors are heat stable and more information is warranted regarding the effects of these anti-nutritional factors on the intestinal epithelial cells of fish.

The efficacy of *in vivo* studies with influences of anti-nutritional factors on intestine was usually limited by the interferences such as complex cell

interactions, stresses from environments and feeding conditions, as well as the high cost considering the diversity of both anti-nutritional factors and fish species. Thus, *in vitro* approaches were worthwhile, which simplified the procedures and could be undertaken under highly controlled conditions (Castillo, Teles, Mackenzie & Tort 2009; Seierstad, Haugland, Larsen, Waagbø & Evensen 2009; Ryckaert, Bossier, D'Herde, Diez-Fraile, Sorgeloos, Haesebrouck & Pasmans 2010; Li, Ai, Mai, Xu & Zheng 2012). Fish feeds of the future will likely be much more complex than those currently used and development will require research from a wide range of disciplines, including *in vitro* approaches with cell.

Intestinal epithelial cells function as site for digestion and nutrition intake, as well as a component of the structural and physiological barriers covering the surface of intestinal mucosa. To date, a rainbow trout intestinal epithelial cell line has been established by Kawano, Haiduk, Schirmer, Hanner, Lee, Dixon and Bols (2011), which was pointed out that it should be a useful *in vitro* tool for studying problems of nutrition and gastrointestinal health. However, it has been found that there are functional differences between primary-cultured and immortalized cells. Primary-cultured cells are expected to retain most of the physiological features of the original cells(Kaiserlian, Vidal & Revillard 1989; Vidal, Grosjean, Revillard, Gespach & Kaiserlian 1993). In mouse, it has been investigated that the primary-cultured intestinal epithelial cells can be used in the function studies, such as mucosal immunity (Yamada, Sato, Morishita, Kaminogawa & Totsuka 2009). Most studies on the primary culture of intestinal epithelial cells mainly focus on mouse (Booth, Patel, Bennion & Potten 1995; Whitehead, Demmler, Rockman & Watson 1999; Macartney, Baumgart, Carding, Brubaker & Offit 2000; Yamada *et al.* 2009), rat (Evans, Flint, Somers, Eyden & Potten 1992; Booth, Evans & Potten 1995; Kaeffer & Briollais 1998) and human (Perreault & Jean-Francois 1996; Perreault & Beaulieu 1998; Whitehead *et al.* 1999; Panja 2000). Few attempts with primary-cultured intestinal epithelial cells have been made in fish, not to mention the study of the effects of anti-nutritional factors on the primary-cultured cells. In the present study, an *in vitro* model of primary culture of intestinal epithelial cells was developed and was used to detect the effects of three heat stable anti-nutritional factors, soyasaponins, phytic acid and raffinose, on viability, morphology and functions of primary-cultured intestinal epithelial cells. Intestinal epithelial cells were isolated from Japanese flounder (*Paralichthys olivaceus*), which is an economically important carnivorous species for aquaculture in North China and sensitive to the replacement of fishmeal by soybean meal (Kikuchi 1999). High inclusion of fish meal in commercial feed of Japanese flounder is limiting the development of its culture industry.

Materials and methods

Experimental fish

Japanese flounder (around 80 g) was obtained from Maoyu Aquaculture Co. Ltd. (Qingdao, China). Prior to the start of the experiment, fish were stocked in a flow-through system in Ocean University of China. Fish were hand-fed commercial diets (Nisshin Flour Milling Co., Ltd., Tokyo, Japan) to apparent satiation once daily. The rearing tanks were provided with continuous aeration and maintained under natural photoperiod. The temperature ranged from 20.0 to 22.0°C, the salinity 30 to 33 mg L^{-1}, and pH 7.7–7.9.

Preparation of anti-nutritional factors solution

Solutions of 2 g L^{-1} soyasaponins (North China Pharmaceutical Group Corporation, China), 4 g L^{-1} sodium phytate (Sigma, Saint Louis, MO, USA) and 8 g L^{-1} raffinose (Amresco, Solon, OH, USA) were made up with complete Dulbecco's Modified Eagle's Medium (cDMEM, Gibco, Carlsbad, CA, USA), which contains high glucose, 5% foetal bovine serum (FBS), 2 mM glutamine, 2 μg mL^{-1} insulin, 20 ng mL^{-1} epidermal growth factor (EGF), 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2.5 μg mL^{-1} amphotericin. All solutions used in the present study were sterilized by pushing them through 0.22 μm filter. Solutions were stored at 4°C before use.

Isolation and primary culture of epithelial cells

Isolation of intestinal epithelial cells was conducted according to the method described by Upreti, Kannan and Pant (2007) with some modifications. Briefly, (i) Japanese flounder (fasted for 48 h) was force fed with 1 ml DMEM with 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (P/S, Sigma); Then the fish was taken into a tank with

sterilized seawater for 2 h, which contained 200 IU mL^{-1} penicillin and 200 μg mL^{-1} streptomycin; After that, the fish was washed three times by ultrapure water and sterilized by 75% ethanol; (ii) the sterilized fish was blooded after anaesthetized with eugenol (1:10 000) (Shanghai Reagent, China). Then, fish was dissected and the intestine was pulled out, the intestine was put into the sterilized D-Hanks solution (4°C, Gibco) and washed; (iii) the enteric cavity was washed carefully with digestive solution A (1 mM dithiothreitol in PBS, pH 7.2). The caecal end of the intestine was ligated with artery forceps after washed. The other end was tied after digestive solution B (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4 and 5.6 mM Na_2HPO_4; pH 7.3) was filled in the intestine. The intestine was incubated by gently shaking for 15 min at 25°C; (iv) digestive solution B was discarded after incubation. Digestive solution C (1.5 mM EDTA and 0.5 mM DTT in PBS, pH 7.2) was poured into the intestine and both sides of intestine were tied again. The intestine was gently shaken for 2 min at 25°C and the incubated solution was collected. The last step (from digestive solution C poured into the intestine) was repeated three times at different duration of incubation (2 min, 3 min and 4 min respectively) and the incubated solutions were pooled. The intestinal epithelial cells were isolated from the pooled solutions by centrifugation (300 *g*, 10 min, 25°C). Cells were washed twice with DMEM and resuspended in cDMEM to 1×10^6 cells mL^{-1}; (v) cells were seeded in 12-well cell culture plates (Hyclone, Logan, UT, USA) precoated with collagen protein and grown at 25°C in a humidified atmosphere of 5%-CO_2 incubator (Thermo, Fisher Scientific, Waltham, MA, USA). The culture medium was changed every 48 h.

Morphology of primary-cultured intestinal epithelial cells

A quantity of 2 mL intestinal epithelial cell suspension (1×10^6 mL^{-1}) was seeded in each well of cell culture plates (12-well). After attachment for 48 h, cells were washed with D-Hanks solution for twice to remove non-adherent cells. Three solutions of anti-nutritional factors with final concentration of 2 mg mL^{-1} soyasaponins, 4 mg mL^{-1} sodium phytate and 8 mg mL^{-1} raffinose, respectively, were added into the cell plates, and DMEM was added as the control. All treatments were designed in triplicate wells. Cells were incubated in 5%-CO_2 incubator at 25°C. Morphology of epithelial cells was observed with phase contrast inverted microscope (Nikon, Tokyo, Japan) at 24 h and 48 h respectively.

The survival of primary-cultured intestinal epithelial cells

After stimulation to the cells with anti-nutritional factors (see details above) for 24 h, Trypan Blue method (Freshney 1987) was used to detect the survival of epithelial cells. Briefly, 0.1 mL of 0.4% Trypan Blue was added into 0.5 mL cell suspension of each treatment; allow the mixture to stand 5 min at room temperature; fill a haemocytometer with the mixture as for cell counting; observe if non-viable are stained and viable cells excluded the stain under a microscope.

The viability of primary-cultured intestinal epithelial cells

Cell viability was assessed with quantified spectro-photometrical 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Saint Louis, MO, USA) assay (Greenman, Rutten, Fowler, Scheffler, Shortridge, Brown, Sheppard, Deveney, Deveney & Trunkey 1998). After two washes to remove non-adherent cells, 180 μL anti-nutritional factors solution (2 mg mL^{-1} soyasaponins, 4 mg mL^{-1} sodium phytate and 8 mg mL^{-1} raffinose respectively) was added into the 96-well cell plates and 180 μL DMEM was added as the control. All treatments were designed in six repeats. Cells were incubated in 5%-CO_2 incubator at 25°C. After 12 h incubation, MTT working solutions were added to cell plate wells and incubated for 4 h followed by fully dissolving formazan crystals in DMSO, and absorbance was measured in 96 microplate readers (Thermo) at 570 nm. The decrease in absorbance was considered as signs of cell viability loss.

Lactate dehydrogenase (LDH) activity of primary-cultured intestinal epithelial cells

After attachment for 48 h, cells in each well (1×10^6 mL^{-1}, 2 mL) were washed twice with D-Hanks solution to remove non-adherent cells. Three solutions of anti-nutritional factors (2 mg mL^{-1} soyasaponins, 4 mg mL^{-1} sodium phytate

and 8 mg mL^{-1} raffinose respectively) were added into the cell plates(12-well, Hyclone), and DMEM was added as the control. All treatments were designed in triplicate wells. Cells were incubated for 24 h in 5%-CO_2 incubator at 25°C. After incubation, the activity of LDH was assayed by a LDH assay kit (NJJCBIO assay kits, Nanjing, China). Briefly, LDH in the medium catalysed the supplemented lactic acid into pyruvic acid, which reacted with 2, 4-dinitrophenylhydrazine into pyruvic acid (2, 4-dinitrophenyl) hydrazone. Pyruvic acid (2, 4-dinitrophenyl) hydrazone presented in red in alkaline solutions and can be measured spectrophotochemically. The absorbance was measured at 440 nm.

Alkaline phosphatase (AKP) activity of primary-cultured intestinal epithelial cells

After incubation with anti-nutritional factors for 24 h (see details above), the AKP activity was detected by using AKP kit (NJJCBIO assay kits, Nanjing, China). Briefly, AKP in the homogenate catalysed disodium phenyl phosphate into free phenol and phosphoric acid. Free phenol in alkaline solutions (with potassium ferricyanide) reacted with 4-aminoantipyrine into red quinone derivatives. AKP activity was determined by measuring quinone derivatives spectrophotochemically. The absorbance was measured at 520 nm.

Statistical analysis

All data were subjected to one-way analysis of variance in SPSS 15.0 (IBM Corporation, Armomk, NY, USA) for Windows. Differences between the means were tested by Tukey's multiple range test, and were considered significant when probability (P) values < 0.05 were obtained. Results were presented as means ± SEM (standard error of the mean).

Results

Primary culture of intestinal epithelial cells

In primary culture of Japanese flounder intestinal epithelial cells, adhesion of the cultured cells to the cell plate and expansion of epithelial-like cells were observed. The epithelial-like cells had the typical features of normal epithelial cells, such as an adherent monolayer and flagstone-like appearance. Each cell had polygonal, flattened shape with a large and oval nucleus. After incubation for 72 h, the epithelial-like cells cultured *in vitro* still had the characteristic of the epithelial cells (Fig. 1).

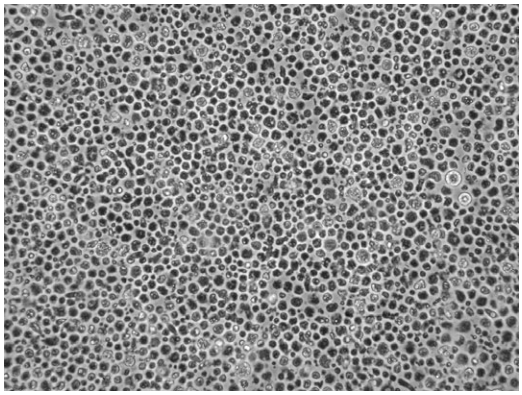

Figure 1 Representative microphotographs of intestinal epithelial cells in primary culture incubated for 72 h (Original magnification is 100×).

Effects of anti-nutritional factors on cells morphology

After incubation with different anti-nutritional factors for 24 h, the cultured cell was showed as Fig. 2. Compared with the control group, no obviously floated or damaged cells were observed in raffinose treatment group (Fig. 2a and d). However, obviously more detached and deformed cells in soyasaponins treatment group were observed compared with the control group (Fig. 2b). Detachment of cells observed in phytic acid group was more than those of the raffinose and control group but lower than those in the soyasaponins group. Less cell morphology alteration was observed in the phytic acid group compared with the soyasaponins group (Fig. 2b and c).

After incubation with different anti-nutritional factors for 48 h, cells in control and raffinose group remained adhered to the surface of the cell plate and had the normal epithelial morphology, which showed very high density and shaped in a tightly packed monolayer (Fig. 3a and d). However, more cells treated with both soyasaponins and phytic acid were detached from the surface of cell plates and the morphology of cells deformed obviously compared with the control group and the 24 h incubation. The cells treated with soyasaponins had the worse situation (Fig. 3b and c).

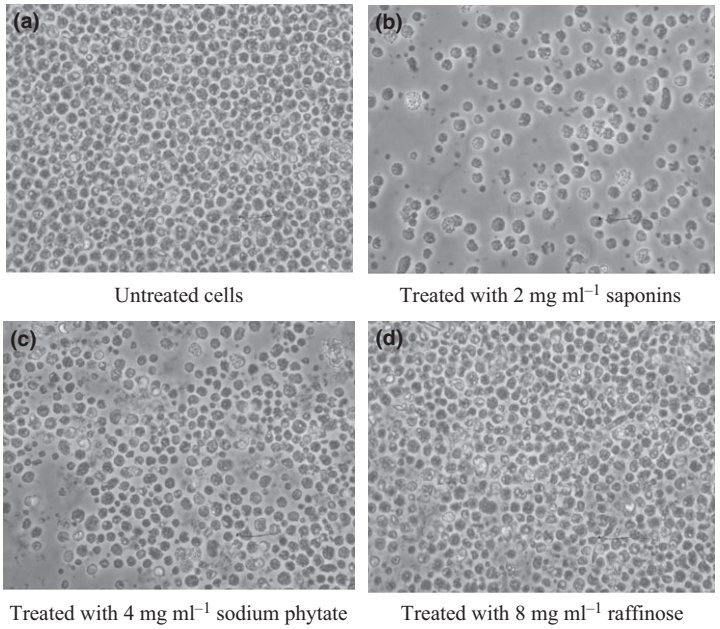

Figure 2 Representative microphotographs of intestinal epithelial cells demonstrating the morphological alterations and growth pattern 24 h after exposure to anti-nutritional factors (Original magnification is 200×).

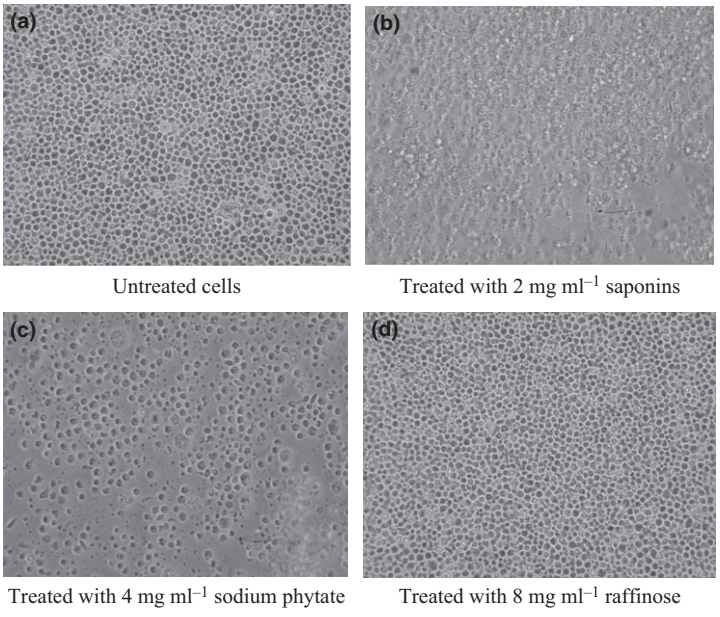

Figure 3 Representative microphotographs of intestinal epithelial cells demonstrating the morphological alterations and growth pattern 48 h after exposure to anti-nutritional factors (Original magnification is 100×).

Effects of anti-nutritional factors on survival rate of cells

After incubation for 24 h, no significant difference was observed in survival rate of cells between the control group and the raffinose group ($P > 0.05$). However, the survival rate of cells in phytic acid and soyasaponins groups were significantly lower compared with the other two groups, and the lowest value was observed in the soyasaponins group ($P < 0.05$) (Table 1).

Effects of anti-nutritional factors on cell viability

Cell viability was not significantly affected by raffinose supplementation, however, both soyasaponins and phytic acid supplementation significantly decreased the cell viability ($P < 0.05$). The lowest

Table 1 Effect of anti-nutritional factors on survival, viability, lactate dehydrogenase (LDH) and alkaline phosphatase (AKP) activities of primary-cultured intestinal epithelial cells of Japanese flounder*

Medium	Survial (%)	Viability (MTT OD)	LDH (U g^{-1} protein)	AKP (U mg^{-1} protein)
Control (0.0)	92.33 ± 1.20[a]	0.65 ± 0.01[a]	17.90 ± 1.36[c]	4.62 ± 0.06[a]
Saponins (2 g L^{-1})	66.00 ± 2.08[c]	0.28 ± 0.01[c]	113.20 ± 5.35[a]	3.32 ± 0.05[b]
Phytic acid (4 g L^{-1})	82.00 ± 1.15[b]	0.49 ± 0.02[b]	42.30 ± 1.84[b]	3.14 ± 0.09[b]
Raffinose (8 g L^{-1})	93.33 ± 0.88[a]	0.68 ± 0.02[a]	21.93 ± 1.30[c]	4.82 ± 0.11[a]
ANOVA†				
F value	81.67	123.60	219.92	113.26
P value	<0.001	<0.001	<0.001	<0.001

*Values are presented as means of triples. Means in the same column with different superscripts are significantly different from each other determined by Tukey's test ($P < 0.05$).
†ANOVA: one-way analysis of variance.

cell viability was observed in the soysaponins group ($P < 0.05$) (Table 1).

Effects of anti-nutritional factors on AKP activity cells

No significant difference in AKP activity was observed between the control and raffinose group ($P > 0.05$). However, AKP activity of cells treated with soyasaponins and phytic acid was significantly lower compared with the control and raffinose group ($P < 0.05$). There were no significant differences in AKP activity between the soyasaponin group and phytic acid group ($P > 0.05$) (Table 1).

Effects of anti-nutritional factors on LDH activity of cells

No significant difference in LDH activity was observed between the control and raffinose group. However, soyasaponin and phytic acid supplementation significantly increased the activity of LDH compared with the control and raffinose group ($P < 0.05$). Particularly, the LDH activity in the soyasaponins group was significantly higher than that in the phytic acid group ($P < 0.05$) (Table 1).

Discussion

In the present study, with the methods stated above, we obtained the primary-cultured intestinal epithelial cells isolated from Japanese flounder which showed the typical features of intestinal epithelial cells. The survival, viability, membrane integrity and morphology of primary-cultured intestinal epithelial cells maintained well and the adhesion of cells was observed after 72 h incubation. In the present method, ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) was used as chelant to react with Ca^{2+} and Mg^{2+}, so as to break the cell junctions to isolate the intestinal crypts, which kept the morphology of epithelial cells in crypts. In addition, DTT could break the disulphide bonds among molecules after its uptake into cells and remove the mucus on the cell surface. Via this method, mechanical damage of epithelial cells was avoided because the cell isolation was conducted in the intestinal canal without cutting the tissue into pieces. After the isolation procedure, the out intestinal wall and mesentery was intact and separated well from the epithelial cells, which avoided the pollution of epithelial cells by epidermal cells and fibroblasts. The intestinal epithelial cells of Japanese flounder based on primary cell culture maintained biological activities of normal intestinal cells, like viability and AKP activity. They could be efficiently simulated by anti-nutritional factors and this indicated that they responded well as normal intestinal cells.

It is observed in this study that soyasaponins of 2 g L^{-1} and phytic acid of 4 g L^{-1} significantly decreased the survival and viability of primary-cultured intestinal epithelial cells, damaged the membrane integrity and altered the cell morphology, indicating that the anti-nutritional factors, soyasaponins and phytic acid, inhibited the survival and viability of fish epithelial cells *in vitro.* Lactate dehydrogenase (LDH) is a glycolytic enzyme observed in all tissues. Its release is often used to indicate the damage of the integrity of cell membrane (Konjevic, Jurišic & Spuzic 1997), as it cannot transit through the cell membrane. AKP is an enzyme on brush border membrane of intestinal

epithelial cells (Mozes, Lenhardt & Martinkova 1998), which takes part in intracellular digestion and extracellular digestion after epithelial cells desquamate to the enteric cavity. The activity of AKP in epithelial cells indicates the metabolism of cells indirectly. The results of the present study showed that both soyasaponins of 2 g L^{-1} and phytic acid of 4 g L^{-1} significantly decreased the AKP activity and increased the LDH activity of primary-cultured intestinal epithelial cells, indicating that the cultured cells were damaged by the supplementation of soyasaponins and phytic acid.

To date, no *in vitro* study has been reported on effects of soyasaponins and phytic acid on intestinal epithelial cells in fish. However, the results of the present study were in accordance with our previous *in vivo* studies on Japanese flounder, which showed that dietary saponins (3.2 g kg^{-1} or 6.4 g kg^{-1}) and dietary phytic acid (4 g kg^{-1} or 8 g kg^{-1}), respectively, significantly depressed the growth performance and impaired the integrity of intestinal histological structure of Japanese flounder (Cai 2006; Chen *et al.* 2011). In another study on Atlantic salmon, it was showed that dietary soyasaponins compromise gut integrity in the distal intestine by increasing the intestinal epithelial permeability (Knudsen, Jutfelt, Sundh, Sundell, Koppe & Frøkiær 2008). It has been identified that saponins had the ability to disrupt biological membranes (Francis, Makkar & Becker 2002; Sparg, Light & van Staden 2004). This might be related to that saponin had special structures which are surface-active to the membrane. Sung, Kendall and Rao (1995) examined the effects of soyasaponins on cancer cell lines (HCT-15) of epithelial cells of rectum and found that more than 600 ppm (0.6 g kg^{-1}) soyasaponins significantly influenced the histology of cancer cells, e.g. increased the vesicles, decreased the cytoplasm density and changed the cell morphology. That indicated that the interaction between saponin and cell membrane may be responsible for the alteration of the cell morphology.

Few studies were conducted to study the effect of dietary phytic acid on the intestinal histology in aquatic animals and the mechanisms involved in the damage of intestinal epithelial cells caused by phytic acid were poorly understood. Studies on chinook salmon (Richardson *et al.* 1985) and carp (Hossain & Jauncey 1993) showed that dietary phytic acid induced histological lesions in digestive tract of fish, such as enlargement of pyloric caecum or intestinal epithelial cells. It was identified that phytic acid had the ability to chelate the divalent or trivalent metal irons and protein molecules, and then inhibited the utilization of minerals (Storebakken, Kvien, Shearer, Grisdale-Helland, Helland & Berge 1998; Sugiura, Raboy, Young, Dong & Hardy 1999; Vielma, Maekinen, Ekholm & Koskela 2000; Apines, Satoh, Kiron, Watanabe & Aoki 2003) and protein (Francis *et al.* 2001) by animals. Ca^{2+} and Mg^{2+} of the cultured medium could be chelated by phytic acid. It subsequently reduced the Ca^{2+} and Mg^{2+} in membrane of cultured cell which may lead to the stability of the cell membrane structure.

In this study, raffinose (8 g L^{-1}) did not influence the morphology, survival, viability and membrane integrity of intestinal epithelial cells *in vitro*. This was consistent with our previous *in vivo* study with Japanese flounder, which showed that dietary raffinose (from 5 to 15 g kg^{-1}) had no affection on growth performance and intestinal histology of Japanese flounder (Cai 2006). The study with Atlantic salmon also showed that no significant morphological changes were observed in the intestines of fish fed raffinose (Van den Ingh *et al.* 1991; Krogdahl *et al.* 1995; Sørensen *et al.* 2011). However, soy oligosaccharides could cause the inflammation of intestine in pig which may be attributed to the bacterial populations in the ileum and colon of pigs (Krause, Easter & Mackie 1994; Liying, Li, Qiao, Johnson, Li, Thacker & Han 2003; Karr-Lilienthal, Kadzere, Grieshop & Fahey Jr.G.C. 2005). The bacterial populations of fish usually are different from the warm-blooded animals (Ringø, Strbm & Tabachek 1995). This may be related to that fish lack the microbacteria in intestine to avoid the flatulent effects of fermentation observed in terrestrial animals.

In this study, we found that primary-cultured cells displayed the functional properties of intestinal epithelial cells isolated from intestine of Japanese flounder. The results of this study *in vitro* showed the high consistency with the studies *in vivo*. It was indicated that the primary-cultured intestinal epithelial cells may have the possibility to study the anti-nutritional factors *in vitro*. Furthermore, the primary-culture model of fish intestinal epithelial cells might facilitate studies of the functions of particular genes in intestinal epithelial cells in response to the plant protein. The primary-culture model may be used to analyse the relationship between the intestinal epithelial cells and the

intestinal microbiota, because the intestinal epithelial cells can be prepared and cultured in germ-free environment under a simple and controllable condition.

Conclusion

The present *in vitro* study with primary-cultured epithelial cells isolated from Japanese flounder suggested that supplementation of soyasaponin (2 g L^{-1}) or phytic acid (4 g L^{-1}) in the culture medium could significantly inhibit the survival and viability of cells, reduce the alkaline phosphatase (AKP) activity, damage the integrity of cell membrane and alter the cell morphology. However, supplementation of raffinose (8 g L^{-1}) showed no significant influence on morphology, survival, viability, membrane integrity and AKP activity of primary-cultured cells. Primary-cultured intestinal epithelial cells might be a good model to analyse intestinal epithelial cells of fish responses to stimulations, especially in the study on replacement of fish meal with plant protein sources. Furthermore, paralleled studies *in vivo* need to be conducted to verify the results *in vitro*, although the results of this study *in vitro* showed some consistency with some previous studies *in vivo*.

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