



Effect of bile salts on intestinal epithelial function in gilthead seabream (*Sparus aurata*)

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Abstract In the context of modern aquaculture, the effort to reduce the reliance on fishmeal/marine ingredients in fish diets has led to the exploration of plant-based protein sources as potential substitutes, a dietary shift that disrupts the bile acid profile in fish. Therefore, bile salts are being sought as additives. However, artificially increased intestinal levels of bile acids may significantly impact mucosal function. Therefore, here, we explored the regulatory role in the intestine of gilthead sea bream (*Sparus aurata*) of (i) chenodeoxycholic acid (CDC), (ii) a mixture formed by two bile acids, 3% cholic acid and

97% deoxycholic acid (MIX), and (iii) a conjugated bile salt sodium taurocholate (TC) in Ussing chambers with the epithelial voltage clamp technique. We tested the bile salts in a 50–500 µg/ml concentration range, and all of them promoted ion absorption. Yet, clear concentration-dependent and more pronounced effects on the ion transport were observed in the posterior intestine. On the other hand, bile salts had no or minor effects on tissue resistance. However, there are indications that the MIX could have adverse effects at high concentrations (500 µg/ml), promoting a threefold increase in tissue permeability measured using FITC-dextran (4 kD) regardless of the intestinal region, thus suggesting an alteration in intestinal permeability at high bile salt concentrations. The

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findings from our study emphasize the importance of considering intestinal function when contemplating the possible use of a particular bile salt as a dietary supplement. It appears that bile salts, whether acting individually or in combination, play a pivotal role in orchestrating nutrient absorption by influencing the function of epithelial ion transport. However further research is needed to fully grasp the region-dependent nuances of bile salt effects on ion transport and the ultimate consequences for nutrient absorption in the context of fish aquaculture.

Keywords Sea bream · Intestine · Ussing chamber · Bile salts · Intestine

Introduction

Bile acids are classically known for their roles in facilitating the digestion of fats, although many other functions have been acknowledged in the last few years. They act as regulators of epithelial homeostasis, transport, and barrier function at the intestinal level in vertebrates (Hegyí et al. 2018) and also support antimicrobial activities that can influence the gut microbiome (Hagey et al. 2010). Moreover, bile acids can act as pro-inflammatory agents that activate nuclear receptors and cell signaling pathways to regulate lipid, glucose, and energy metabolism (Chiang 2009). Additionally, in the case of fish, there is some evidence that bile acids act as pheromones (Giaquinto and Hara 2008), affect taste stimuli (Rolen and Caprio 2008), and likely function in inter- and intraspecific communication (Zhang et al. 2001).

In the current aquaculture, efforts to reduce the reliance on fishmeal in fish diets have led to the exploration of plant-based protein sources as potential substitutes. This dietary shift can have repercussions on digestion, an often-overlooked facet. Plant protein-based diets typically have lower taurine levels, which is highly present in fishmeal (Aragão et al. 2014). Taurine plays a critical role in the conjugation of bile acids in fish (Hofmann et al. 2010). Therefore, feeding fish with diets deficient in taurine results in diminished bile salt concentrations due to a reduction in its availability, as shown in Korean rockfish *Sebastes schlegelii* (Kim et al. 2015). Also, low dietary levels of taurine may impair fat digestion indirectly considering that taurocholate is the primary salt

present in fish bile (Goto et al. 1996; Kortner et al. 2014) and that taurine-conjugated bile acids are more efficient fat emulsifiers than glycine-conjugated bile acids (Goto et al. 1993; Chesney et al. 1998). From a strictly practical point of view, there is evidence that using plant protein meals in diets for some aquacultured fish is often associated with decreased lipid digestibility, reduced bile acid levels, and hypocholesterolemia (Krogdahl et al. 2010; Tocher et al. 2008; Francis et al. 2001). Using such ingredients disrupts the bile acid status, resulting in increased excretion/decreased intestinal reabsorption and modified bile acid synthesis (Staessen et al. 2020; Kortner et al. 2014). As detailed in a recent review by Romano et al. (2020), there is an association between impaired bile acid transport and intestinal inflammation induced by several anti-nutritional factors like saponins, oligosaccharides, high molecular mass proteins, fibers, and specific amino acid residues that may bind bile salts preventing their reabsorption. Furthermore, low contents of cholesterol and the absence of taurine in plant protein sources are likely to reduce bile acid synthesis.

Bile salts are shown to be capable of reducing or mitigating the effects of plant-based diets. In Sparids, bile acid supplementation showed growth-promoting effects in the sea bream (*Sparus aurata*) at levels of 0.06–0.12% (Ruiz et al. 2023a), but not in the black sea bream (*Acanthopagrus schlegelii*) at levels of 200 mg/kg of feed (Jin et al. 2019), with benefits to integrity and immune responses in both species. In rainbow trout (*Oncorhynchus mykiss*), dietary supplementation with bovine bile acids (1.5%) or synthetic taurocholate (1%) prevents decreased growth and distal intestine and liver abnormalities caused by soybean meal or soy antinutrients (Iwashita et al. 2008, 2007; Yamamoto et al. 2007). In contrast, neither purified taurocholate nor bovine bile salt (at 1.8% supplementation) could revert the poor growth performance, lipid digestibility, and intestinal inflammation caused by dietary plant protein in Atlantic salmon (Kortner et al. 2016). Dietary supplementation with taurocholate attenuated but did not completely counteract soybean meal-induced enteritis in *Scophthalmus maximus* (Gu et al., 2016). These observations point to a possible negative interaction of the supplementary bile salts with some plant compounds like lectins or flavonoids that neutralize their potential beneficial effect.

On the other hand, artificially increased intestinal levels of bile acids may significantly impact mucosal function. Studies in humans demonstrate that low concentrations of bile acids stimulate enterocyte proliferation, while higher concentrations cause cell death. This effect is closely related to the typology of the bile acid, i.e., conjugated, hydrophilic, deconjugated or dehydroxylated (Yui et al. 2009; Katona et al. 2009). Also, it is well established that exposure to increasing concentrations of bile acids leads to rapid loss of epithelial barrier function and a consequent increase in mucosal permeability to macromolecules in the Caco-2 cell line (Raimondi et al. 2008). Sustained loss of epithelial barrier function in this way can have severe pathologic implications because it increases the ability of luminal bacteria to penetrate the mucosa, thereby causing inflammation in the human colon (Münch et al. 2007).

The molecular mechanisms by which bile acids alter intestinal epithelial transport still need to be fully clarified. Therefore, bile acids are receiving much attention as significant regulators of mammalian epithelial function in health and disease (Kelly et al., 2013; Hegyi et al. 2018). Crucial differences exist in intestinal tissue structure and function between mammals and fish, and the potential effects of different concentrations of bile salts on intestinal function in fish have received limited attention.

In a recent study, Fuentes et al. (2018) evaluated the epithelial function of physiological (0.4–4 mM) levels of taurocholic and tauro lithocholic acids in the intestine of Senegalese sole (*Solea senegalensis*) using the short-circuit current technique. They demonstrated that bile salts alter ionic transport in the intestine, intensifying the absorptive pathway in a dose-dependent and reversible manner without compromising the integrity of the epithelium, at least in vitro. The present study was performed bearing in mind the above-detailed effects of bile salts already determined in the sole intestine. We aimed at evaluating the effect of variable concentrations of different bile salts on the epithelial function of the most important aquacultured species in the Mediterranean Basin, the gilthead seabream (*Sparus aurata*), which has been shown to respond positively to bile salt supplementation in growth trials (Ruiz et al. 2023a, 2023b). Considering the growing trend to include bile acid supplements in high-plant diets for fish to compensate for some of their anti-nutritional effects, the study

was considered an important preliminary step in decision-making for bile salt type and level of inclusion in feeds for this species.

Materials and methods

Chemicals

Bile acids or salts were used in the experiments: a primary bile acid, chenodeoxycholic acid (CDC, Nutriad, Spain); a mixture formed by two primary bile acids, 2.8% cholic acid and 96.7% deoxycholic acid (MIX, Nutriad, Spain); and a conjugated bile salt, sodium taurocholate (TC, Sigma, Madrid). Concentrated stocks (50 mg/ml) were prepared in distilled water to provide final concentrations of 50, 250, and 500 µg/ml in the Ussing chamber during the experiments. Fluorescein isothiocyanate-dextran (FITC mol wt 4000, Sigma) was used as a marker for permeability assays.

Fish

Sea bream (*Sparus aurata*) juveniles 70–80 g were obtained from the in-house stock of Ramalhete Marine Station (University of Algarve, Faro, Portugal) and maintained in 500-l tanks with running seawater at a density < 5 kg/m³ and hand fed three times/week at 1.5% body weight (or the closest they would ingest, considering the low temperature), with a commercial sea bream diet (Aquagold, Sorgal SA, Portugal: 44% crude protein, 14% crude fat, 8% ash, 2.5% crude fibers, 1% phosphorus). Fish were acclimated for 1 month in flowing seawater (salinity 35 p.p.t.; water temperature 16–17 °C) under a natural photoperiod for January in the Algarve, Portugal.

Tissue removal and definition

In total, tissues from 43 fish were isolated. In most cases, more than one portion of the anterior or the posterior intestine was obtained from each donor fish. In these cases, tissues were allocated to different in vitro experiments, and no tissues from the same intestinal region of an individual fish were included in the same experimental group. The anterior intestine was defined as a portion of about 3 cm in length extending caudally from the point of insertion of the

last pyloric caeca and perfectly distinguishable from the mid intestine by the transition of musculature. The posterior intestine was defined as the segment anterior to the rectal sphincter and did not include the rectum, a wider, more flexible region perfectly delimited by the rectal and the anal sphincters. Based on our previous experience of tissue viability (Fuentes et al. 2006), the maximum expected duration of the *in vitro* experiment was 3.5 h, considering that only a single bile salt type was analyzed in each set of tissues from the same fish in the Ussing chamber.

Voltage clamp in Ussing chambers

On the day of the experiments, 24-h starved fish were sacrificed, and the whole intestine from stomach to rectum was isolated and transferred to ice-chilled pre-gassed (0.3% CO₂+99.7% O₂) saline. The intestinal regions defined above were isolated, the luminal content was flushed with saline, the tissue fat was removed, and no attempt was made to remove the serosa muscle layer as previously described (Carvalho et al. 2012). On the day of the experiments, tissues were used in batches of 12 (the maximum number we could run in parallel experiments), mounted on tissue holders of 0.5 cm², and positioned between half-chambers of a P2400 Ussing type chamber (Physiologic Instruments, San Diego, USA) containing 2 ml of physiological saline (160 mmol l⁻¹ NaCl, 1 mmol l⁻¹ MgSO₄, 2 mmol l⁻¹ NaH₂PO₄, 1.5 mmol l⁻¹ CaCl₂, 5 mmol l⁻¹ NaHCO₃, 3 mmol l⁻¹ KCl, 5.5 mmol l⁻¹ glucose, and 5 mmol l⁻¹ HEPES, pH 7.800). During the experiments, the tissue was bilaterally gassed with 0.3% CO₂+99.7% O₂ and maintained at 17 °C. All experiments were carried out under the short-circuit mode by application of a direct current to set the potential difference (PD) to 0 mV. Under these conditions, changes in short-circuit current, I_{sc} (ΔI_{sc}), represent changes in the transepithelial net ion transfer. Voltage clamping and current injections were performed using VCC MC6 or VCC MC8 voltage clamp amplifiers (Physiologic Instruments, San Diego, USA). Bioelectrical parameters for each tissue were recorded continuously onto LabScribe3 running in a McIntosh computer using an iWorx118 data acquisition system from the time of mounting until the end of the experimental period. I_{sc} was continuously recorded, and epithelial resistance (R_t , Ω cm²) was manually calculated (Ohm's law,

$R_t = \Delta V / \Delta I$) using the current deflections induced by a bilateral ± 1 mV pulse of 3 s every minute. The apical side of the preparation was considered as the ground. Once R_t and I_{sc} achieved a steady state, usually within 25–35 min, control values were recorded, and the preparations received incremental levels of individual bile acids prepared from concentrated stocks at 30-min intervals to provide final concentrations of 50, 250, and 500 μ g/ml. Experiments were terminated 30 min after adding the highest concentration of bile salt. Positive values for ΔI_{sc} (μ Amp/cm²) represent stimulation of absorptive currents or inhibition of secretory currents, with the same result regarding net ion movement.

Permeability assays

In separate experiments, the anterior intestine and the posterior intestine were collected and processed as described above, but no attempt was made to monitor electrophysiological variables. After 30 min of tissue stabilization, the saline was replaced with fresh pre-gassed solution at 17 °C to a final volume of 2 ml per half-chamber. FITC-dextran (average mol wt 4000, Sigma, Madrid) from a concentrated stock (100 mg/ml) was added to achieve a final concentration of 0.5 mg/ml in the apical chamber, and a sample (0.1 ml) was collected from the apical and basolateral compartments after 5 min of mixing to establish time zero. After exactly 1 h, samples from the donor (apical) and receiver (basolateral) compartments were collected into fresh vials and established control periods for apparent permeability (Papp). Individual bile acids from concentrated stocks were sequentially added to the apical chamber to give final concentrations of 250 μ g/ml and 500 μ g/ml, and permeability was tested in the same tissues for 2 additional hour periods. In these experiments, the 50 μ g/ml experimental group was not tested due to putative issues with the preparations' viability due to the experiments' length (Fuentes et al. 2006).

Fluorescence measurements were performed using a Multi-Mode Microplate Reader BioTek SynergyTM 4 (BioTek Instruments, Winooski, VT, USA) set for excitation wavelength at 492 nm and emission wavelength at 520 nm. The apparent permeability (Papp) was estimated using the following equation: $Papp = (V \cdot dC) / (A \cdot C_0 \cdot dT)$, where Papp is the permeability in centimeters per second, V is the volume of

the receiver chamber in μl , A is the surface area of the tissue in square centimeters, C_0 is the starting concentration in the donor compartment (apical) in $\text{ng}/\mu\text{l}$, and dC/dT is the rate of concentration change (ng/s) of FITC in the receiving chamber (basolateral).

Statistical analysis

Results are presented as means \pm SEM of ten individual tissues per group for the electrophysiology experiments and eight for the permeability measurements. All statistics were performed after assessing the homogeneity of variance (Brown-Forsythe homogeneity test), normality (Kolmogorov–Smirnov normality test), and Rout’s test to identify potential outliers. Comparison of basal parameters between intestinal regions was carried out by the Student t -test. Effects of bile salts were evaluated with a two-way analysis of variance, considering the intestinal region and bile salt dose as main factors, complemented with the Sidak test to identify significant effects. All statistical analyses were performed with Prism 8.0 (GraphPad Prism 8.0 for McIntosh, GraphPad Software, San Diego, California, USA), and groups were considered significantly different at $p < 0.05$.

Multivariate analysis was performed using the software Primer v7.0.21 from PRIMER-e (Massey University, Albany, New Zealand; Clarke and Gorley 2015). For each parameter under study (delta current, resistance, and permeability), the corresponding resemblance matrix (Euclidean distance) was obtained using all samples or samples from the same biliary salt experiment. The following routines were then used in sequence: (i) CLUSTER to obtain hierarchical clustering into sample groups and the respective cophenetic correlation, together with SIMPROF to test for structure in the data and identify sample groups with 95% confidence, and (ii) permutation-based hypothesis testing (ANOSIM), an analog of univariate ANOVA, run on the resemblance matrix to test for differences between groups of samples from different experimental treatments (different biliary salts, same intestinal region) and between groups of samples from the same treatment (same biliary salt, two intestinal regions). Finally, a principal coordinate (PCO) analysis was used to produce a configuration plot to visualize the level of similarity of individual samples of each dataset.

Results

Basal measurements in sea bream intestine

Basal epithelial parameters of intestinal preparations in the sea bream anterior and posterior intestine are shown in Table 1. In symmetric conditions and under voltage clamp, the intestine of sea bream shows significantly different short-circuit currents (I_{sc}) between the anterior and the posterior intestine. In addition, tissue resistance (Rt) was significantly higher in the anterior than in the posterior intestine, showing 166.6 ± 15.9 vs. $78.1 \pm 3.9 \Omega \text{ cm}^2$, respectively. Apparent permeability measured using 4 kD FITC-dextran was between 2 and $3 \times 10^{-7} \text{ cm/s}$ in both regions, without significant differences.

Effects of bile acids on short-circuit current

Increasing CDC concentrations, 50, 250, and 500 $\mu\text{g}/\text{ml}$ applied in Ussing chambers, did not produce an effect in the anterior intestine ΔI_{sc} ($\mu\text{Amp}/\text{cm}^2$). However, the posterior intestine responded with graded increases of ΔI_{sc} in a dose-dependent manner. The two-way ANOVA analysis (Fig. 1) evidenced significant effects of the dosage ($p < 0.001$) and the intestinal region ($p = 0.008$), as well as a significant interaction between both factors ($p = 0.0227$). Similar results were obtained when testing increasing concentrations of TC and the bile acid MIX (Figs. 2 and 3).

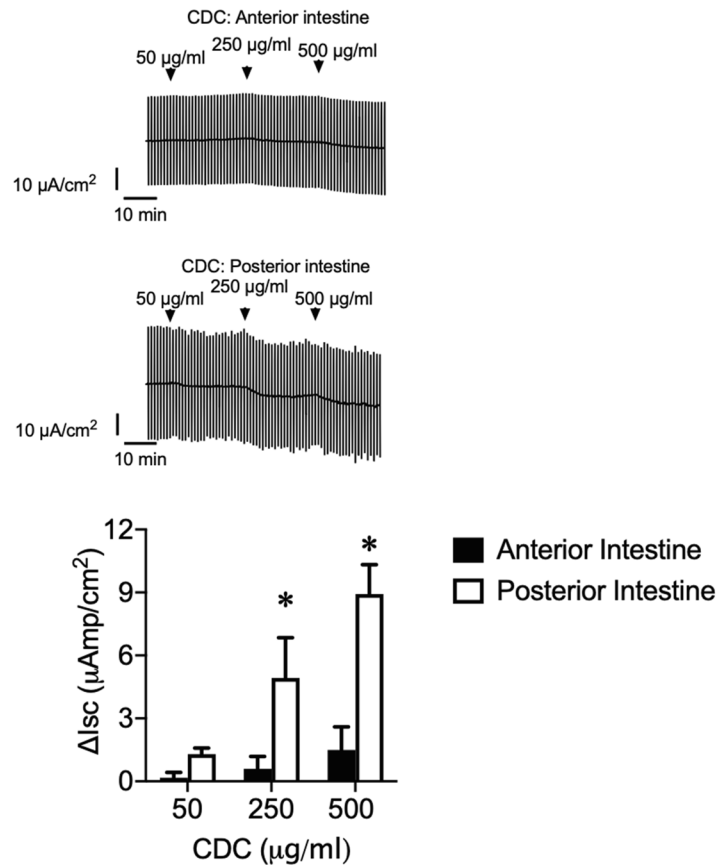
Effects of bile acids on tissue resistance

Increasing concentrations of CDC and MIX (50, 250, and 500 $\mu\text{g}/\text{ml}$) applied to in vitro preparations in Ussing chambers did not affect tissue resistance.

Table 1 Basal epithelial parameters of short-circuit current (I_{sc} , $\mu\text{Amp}/\text{cm}^2$), tissue resistance (Rt, $\Omega \text{ cm}^2$), and permeability (Papp, $\times 10^{-7} \text{ cm/s}$) in the anterior and posterior intestine of the sea bream (*Sparus aurata*) used in this experiment. The number of tissues for each parameter is given in parentheses. Asterisks represent differences between the anterior and posterior intestine (Student t -test, $p < 0.001$)

	Anterior Intestine	Posterior Intestine
I_{sc} ($\mu\text{Amp}/\text{cm}^2$)	1.13 ± 0.28 (30)	3.94 ± 0.49 (30)*
Rt ($\Omega \text{ cm}^2$)	166.6 ± 15.9 (30)	78.1 ± 3.9 (30)*
Papp ($\times 10^{-7} \text{ cm/s}$)	2.83 ± 0.49 (24)	2.03 ± 0.42 (24)

Fig. 1 Original traces of short-circuit current (I_{sc} , $\mu\text{A}/\text{cm}^2$) in the anterior intestine and the posterior intestine of sea bream juveniles were recorded after consecutive apical application of CDC 50, 250, and 500 $\mu\text{g}/\text{ml}$. Vertical current deflections are generated by 1 mV pulses to calculate Rt. Lower panel: changes in short-circuit current (ΔI_{sc} , $\mu\text{A}/\text{cm}^2$) in sea bream juveniles' anterior and posterior intestines after consecutive apical application of CDC 50, 250, and 500 $\mu\text{g}/\text{ml}$. Each column represents the average + SEM ($n=10$). Asterisks represent significant differences between regions for a given dose (two-way ANOVA, followed by Sidak multi-comparison test)



Source of Variation	% of total variation	P value
Interaction	8.039	0.0227
Region	16.22	0.0008
Dose	22.31	<0.0001

However, applying TC significantly increased tissue resistance in the anterior but not in the posterior intestine. Results from the two-way ANOVA (Fig. 4) showed a significant effect of the dosage ($p=0.0343$) and the intestinal region (0.0026), as well as a significant interaction ($p=0.035$) between both factors.

Effects of bile acids on tissue permeability

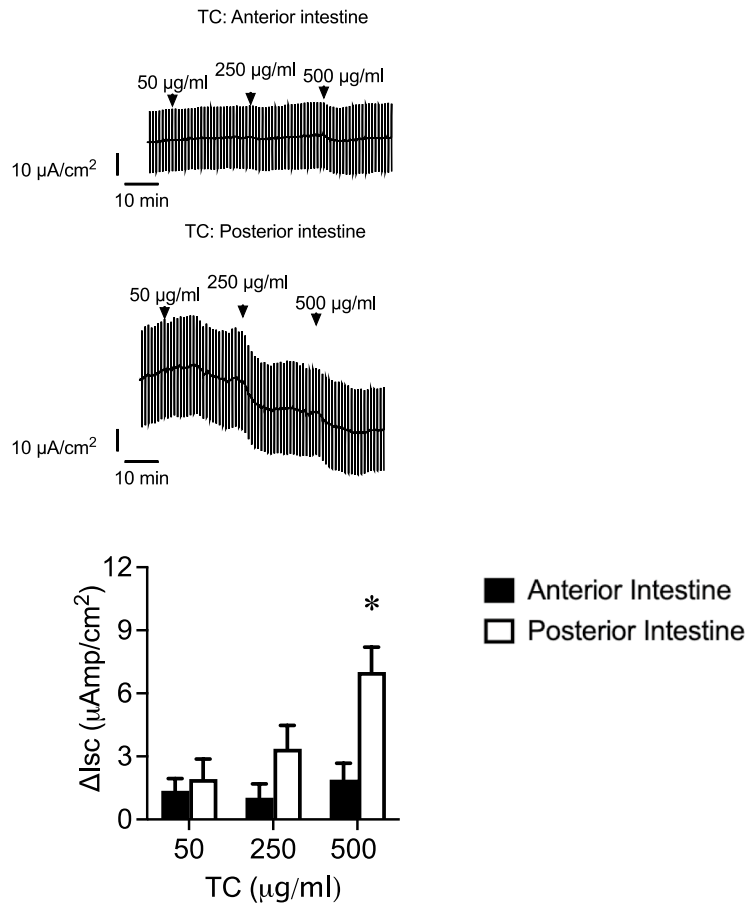
Figure 5 shows the effects of CDC, MIX, and TC on tissue permeability in the anterior and posterior sea bream intestine. The application of increasing concentrations of CDC and TC (250 and 500 $\mu\text{g}/\text{ml}$) produced no effect either in the anterior or posterior intestine ($p>0.05$, two-way ANOVA). However, increasing doses of bile acid MIX determined a

significant permeability increase ($p=0.0015$), without effects at levels of 250 $\mu\text{g}/\text{ml}$ (two-way ANOVA followed by Sidak multi-comparison test), regardless of the intestinal region (Fig. 5).

Multivariate analysis

The multivariate analysis performed considering either of the three parameters under study showed neither significant differences between the overall effect of CDC, MIX, or TC nor between the respective effect of each biliary salt in the anterior or the posterior intestinal regions. However, significant differences were detected between the two intestinal areas for the effect of CDC in the delta current (Supplementary Fig. 1; ANOSIM (R)=0.849; $p=0.001$)

Fig. 2 Original traces of short-circuit current (I_{sc} , $\mu\text{Amp}/\text{cm}^2$) in sea bream juveniles' anterior intestine and posterior intestine were recorded after consecutive apical application of TC 50, 250, and 500 $\mu\text{g}/\text{ml}$. Vertical current deflections are generated by 1 mV pulses to calculate Rt. Lower panel: changes in short-circuit current (ΔI_{sc} , $\mu\text{Amp}/\text{cm}^2$) in the anterior and posterior intestine of sea bream juveniles after consecutive apical application of TC 50, 250, and 500 $\mu\text{g}/\text{ml}$. Each column represents the average \pm SEM ($n = 10$). Asterisks represent significant differences between regions for a given dose (two-way ANOVA, followed by Sidak multi-comparison test)



Source of Variation	% of total variation	P value
Interaction	7.646	0.0478
Region	12.81	0.0073
Dose	15.40	0.0007

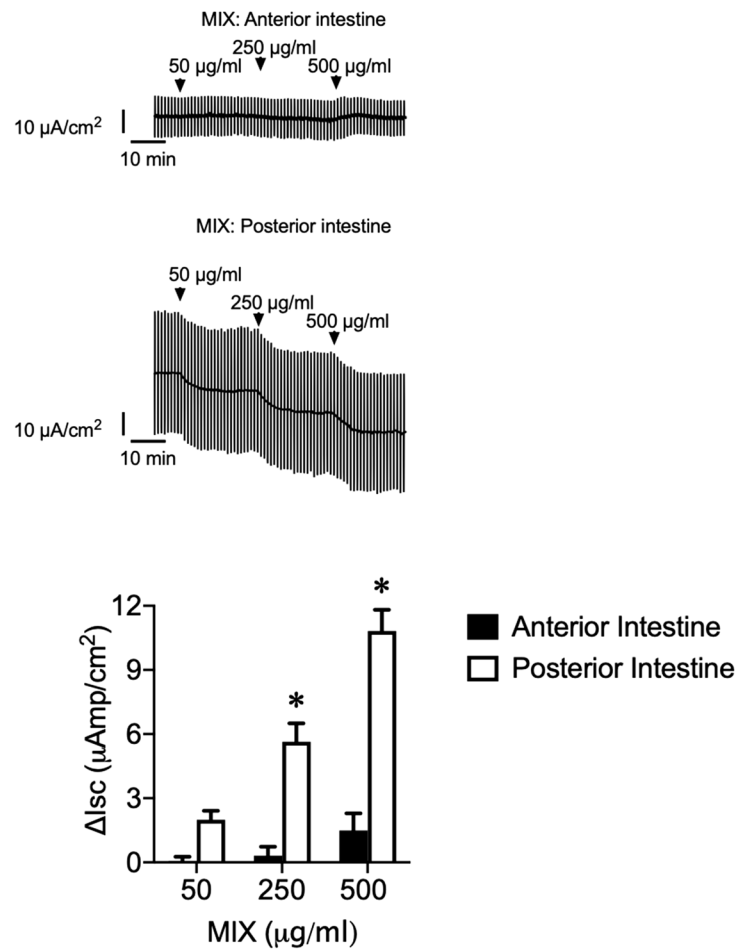
and in the permeability (Supplementary Fig. 2; ANOSIM (R)=0.835; p =0.003) and for the effect of MIX in delta current (Supplementary Fig. 2; ANOSIM (R)=0.987; p =0.001). A borderline difference was detected (ANOSIM (R)=0.51; p =0.002) between the effect of TC on the epithelial resistance of each intestinal region.

Discussion

The primary objective of this study was to unveil the physiological basis that sustains the potential impact of bile salts on the epithelial functionality of the

sea bream's intestine. We approached the work taking into consideration specific bile salts, the relative concentration, and the specific intestinal region being investigated. To accomplish this, we utilized the Ussing chamber, a specific methodology that allows for the measurement of three different parameters: the short-circuit current, tissue resistance, and permeability. The short-circuit current is an indicator of the net active transcellular ion transport across the intestinal epithelium. The process of active ion transport results in the establishment of a potential difference across the epithelium, referred to as TEP (transepithelial potential). We measured this voltage difference using two electrodes positioned as closely as

Fig. 3 Original traces of short-circuit current (I_{sc} , $\mu\text{A}/\text{cm}^2$) in the anterior intestine and the posterior intestine of sea bream juveniles were recorded after consecutive apical application of MIX 50, 250, and 500 $\mu\text{g}/\text{ml}$. Vertical current deflections are generated by 1 mV pulses to calculate Rt. Lower panel: changes in short-circuit current (ΔI_{sc} , $\mu\text{A}/\text{cm}^2$) in the anterior and posterior intestine of sea bream juveniles after consecutive apical application of MIX 50, 250, and 500 $\mu\text{g}/\text{ml}$. Each column represents the average \pm SEM ($n = 10$). Asterisks represent significant differences between regions for a given dose (two-way ANOVA, followed by Sidak multi-comparison test)

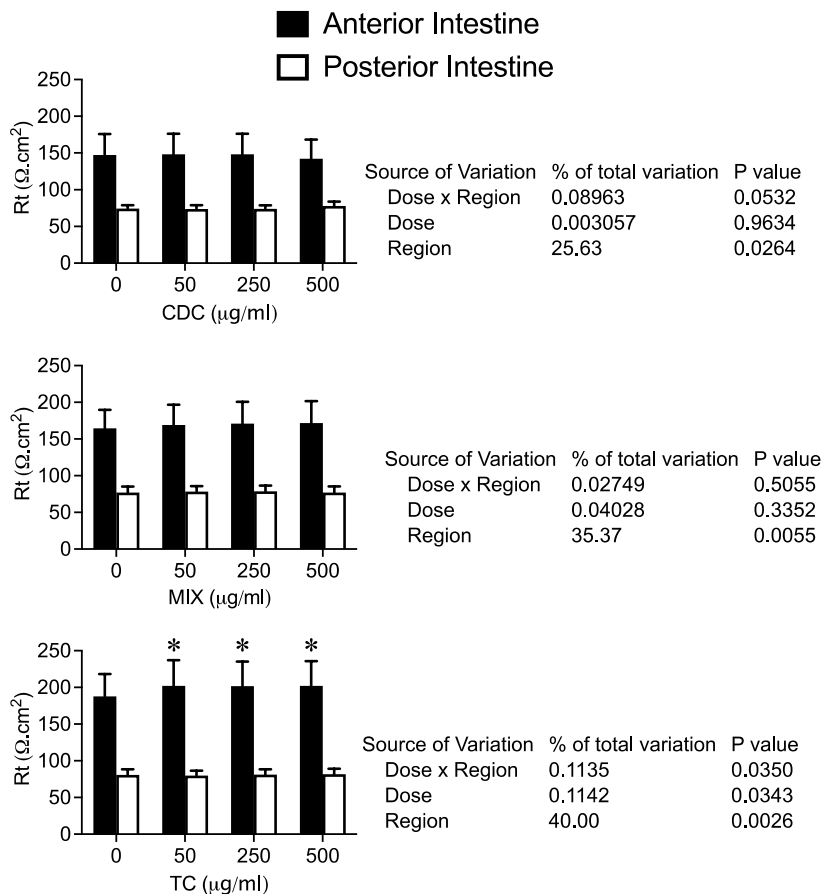


Source of Variation	% of total variation	P value
Interaction	11.94	<0.0001
Region	23.33	<0.0001
Dose	40.14	<0.0001

possible to the tissue or epithelium. To eliminate any spontaneous voltage, we introduced a counter-current using another set of two current electrodes at a distance from the epithelium. This externally applied current is known as the short-circuit current (I_{sc}) and provides an exact measurement of net ion transport across the epithelium when the tissue is short-circuited; I_{sc} serves as a precise reflection of the tissue's absorptive or secretory capacity (Clarke 2009). On the other hand, barrier functionality is primarily governed by epithelial cells and tight junctions, and in the context of epithelial systems, tissue resistance is regarded as the electrical manifestation of barrier

function (Clarke 2009) and the bioelectrical evaluation of resistance/integrity of the tissue. Preserving the integrity of the barrier function within the intestinal epithelium is of utmost importance as it segregates the internal and external compartments of the body (Clarke 2009). Numerous factors can potentially influence intestinal permeability, including alterations in gut microbiota, mucus layer changes, and epithelial lining damage, that can lead to translocation and infiltration of luminal contents to the intestinal tissue and plasma (Gieryńska et al. 2022; Stolfi et al. 2022). In the context of the fish intestine, this approach has been employed to assess insults to the epithelia

Fig. 4 Tissue resistance (R_t , $\Omega \text{ cm}^2$) of juvenile sea bream anterior and posterior intestine in basal conditions and in response to consecutive apical application of 50, 250, and 500 $\mu\text{g/ml}$ of CDC, MIX, and T009 (upper, central, and lower panel, respectively). Each column represents the average \pm SEM ($n = 10$). Asterisks represent significant differences from corresponding basal values (two-way ANOVA, followed by Sidak multi-comparison test)



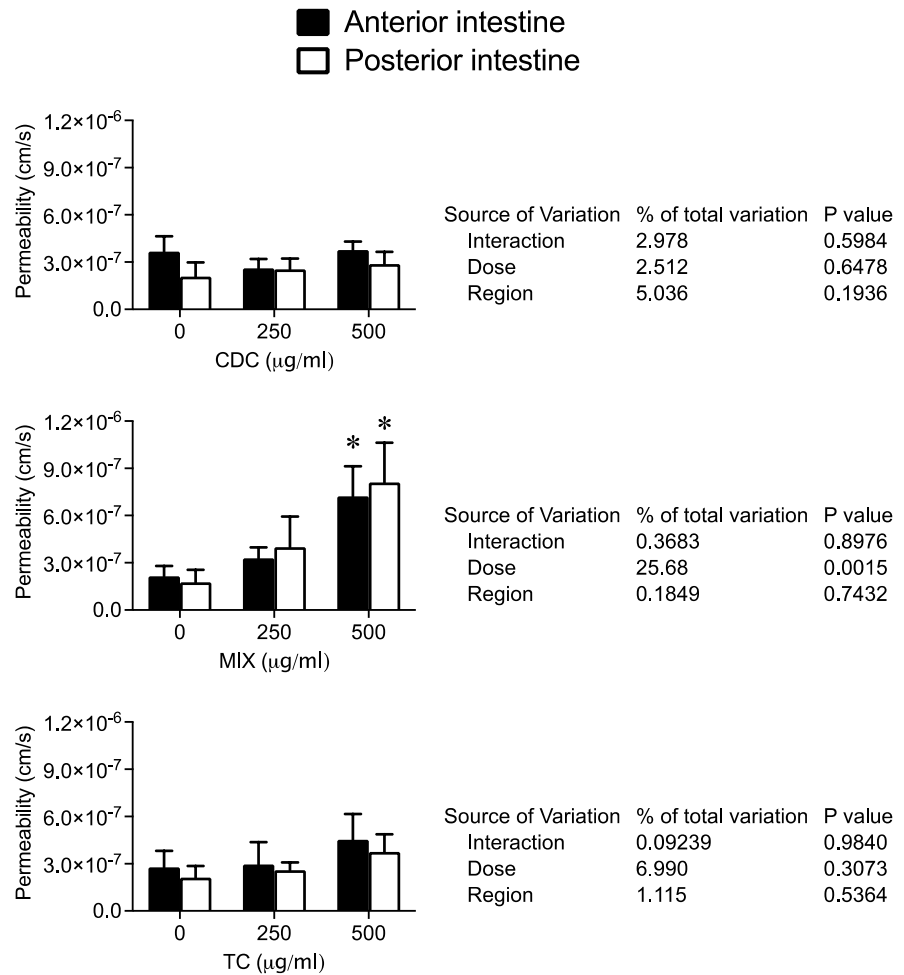
resulting from the introduction of plant protein or alternative protein sources in sea bream (Estensoro et al. 2016; Aragão et al. 2020; Molina-Roque et al. 2022), sea bass (Fonseca et al. 2023) or meagre (Sáenz de Rodríguez et al. 2013).

In the present study, we tested the putative effects of selected bile salts chenodeoxycholic acid (CDC), a mixture formed by two bile acids, 3% cholic acid and 97% deoxycholic acid (MIX), and a conjugated bile salt sodium taurocholate (TC) on the ion transport in the intestine of sea bream. The selection of the CDC and the mixture used in this study was based on previous research concerning the bile composition in fish, detailed in the “Introduction” section. For instance, chenodeoxycholic acid (CDC) was identified in the grass carp *Ctenopharyngodon idella*, as reported by Zhang et al. (2017). In contrast, aside from the Cypriniformes, primarily characterized by C27 bile alcohols (Hagey et al. 2010), most cultivated fish species are characterized by the prevalence of

C24 bile acids, and, in the case of Sparids like the red seabream (*Pagrosomus major*), CDC may account by 40% of total bile acids (Goto et al. 1996).

Notably, these bile acids are primarily conjugated with taurine, with taurine being the sole amino acid involved in the conjugation of bile acids in these species (Kortner et al. 2016; Kim et al. 2015; Yamamoto et al. 2007). The levels tested in vitro in this study were selected with the assumption of feeding at approximately 1.5% of the body weight per day, enriched with 50–500 mg bile acids per kg of feed. These values correspond to intermediate supplementation ranges identified in the literature for fish (Kim et al. 2015; Hagey et al. 2010; Goto et al. 1996). For a fish weighing around 100 g and considering a feeding rate of 1.5%, the ingestion rate of bile acids at 50–500 mg/kg of feed would amount to an estimated intake of 75–750 μg per fish, a range aligning with the most commonly published values (see above). These bile acids are anticipated to be retained in the

Fig. 5 Permeability (Papp, cm/s) of juvenile sea bream anterior and posterior intestine in basal conditions and in response to consecutive apical application of 250 and 500 $\mu\text{g/ml}$ of CDC, MIX, and T009 (upper, central, and lower panel, respectively). Each column represents the average \pm SEM ($n=8$). Asterisks represent significant differences from corresponding basal values (two-way ANOVA, followed by Sidak multi-comparison test)



intestinal lumen, resulting in concentrations ranging from 50 to 500 $\mu\text{g/ml}$ in the intestinal fluid, the range we used in our *in vitro* experiments. While some intestinal fluid may be lost through absorption into the bloodstream, there is also the potential for the uptake of bile salts in the anterior intestine, which could decrease luminal levels. However, if water absorption occurs throughout the intestine as it does along the intestine of the sea bream (Carvalho et al. 2012), both regions might maintain comparable concentrations.

We applied bile salts always on the apical/luminal compartment of our *in vitro* assays to simulate the putative effects of feed-bound bile salts in the intestine of sea bream. Results evidenced that the addition of either CDC or MIX at increasing concentrations did not affect tissue resistance either in the anterior or posterior intestine. In contrast,

sodium taurocholate induced a slight yet significant increase in tissue resistance in the anterior intestine of the sea bream. This observation was surprising since in our previous study, performed on the intestine of another fish species, the Senegalese sole, neither taurocholic nor tauro lithocholic acids affected tissue resistance (Fuentes et al. 2018). However, sodium taurocholate decreases tissue resistance in other epithelial models, such as the rabbit gastric mucosa (Birkett and Silen 1974), although at much higher concentrations (2.5 to 20 mM). These observations suggest that the effects on barrier function are both bile salt and species-specific, possibly exhibiting heterogeneity, as different sections of the intestine may respond differently. This is a crucial aspect to take into account when considering the potential utilization of a specific bile salt as a dietary supplement.

Electrophysiological measurements represent the gold standard for assessing tissue integrity. Nevertheless, we further characterized the barrier function concerning the influence of bile salts using the fluorescent small-size permeability marker FITC-dextran, which has a molecular weight of 4 kD. Our observations revealed no significant impact on permeability in tissues stimulated with either CDC or taurocholate. However, when tissues were stimulated with the MIX, we observed increased permeability at the highest concentration employed (500 $\mu\text{g/ml}$), which roughly equates to a range of 1–1.5 mM in the luminal compartment. This finding suggests that while the MIX may display favorable effects on the transcellular absorption pathway, as evidenced by the stimulation of I_{sc} , it could have adverse effects at higher concentrations, potentially leading to a disruption in intestinal barrier function. Similar effects have been observed in other models, such as the Caco-2 cell intestinal model (Zeng et al. 2022). However, whether the effect described here is temporary and reversible or results in a lasting disruption of the intestinal barrier function is a subject that requires further investigation. In addition, we need to consider how our results *in vitro* translate to an *in vivo* situation. Bile salt metabolism is complex and can be regulated by various factors, for example, bile acid synthesis autoregulation through the activation of the farnesoid X receptor (FXR) in the liver and intestine, which causes hepatic *cyp7a1* not to be activated and a reduced bile acid synthesis (Romano et al. 2020).

When examining together the present findings made on seabream in combination with those previously obtained with the Senegalese sole *in vitro* (Fuentes et al. 2018), a clear pattern emerges, pointing to more pronounced effects of bile salts on the ion transport in the posterior regions of the fish intestine. While our analysis focused on the impact of bile salts in distinct segments of the seabream's intestinal tract, specifically the most anterior and posterior sections, results reveal a progressively increasing influence on the absorption process from the anterior to the posterior regions of the intestine. Multivariate analysis revealed distinct responses between intestinal regions to each bile salt, albeit with variations in the affected parameters. Specifically, with CDC, disparities were evident in both delta current and permeability responses. In contrast, with MIX, discrepancies were solely detected in delta current, and with TC,

alterations were observed exclusively in epithelial resistance. However, the extent of significance attributed to the region-specific effects of bile salts warrants further investigation, as molecular mechanisms influenced by the chemical conformation of the bile salts utilized cannot be discounted.

In conclusion, the present study explored the effects of different bile salts on the ion transport in the intestine of sea bream. All bile salts had some stimulatory effects in the absorptive pathway, apparent region-dependent effects, and concentration-dependent stimulation. In addition, the mix of cholic and deoxycholic acids suggested a potential disruption of the intestinal barrier function. Although the degree of relevance associated with the region-dependent function of bile salts on ion transport remains a subject for further exploration, the effects described here are probably complexly linked to nutrient absorption. The uptake of protein components, encompassing amino acids and small peptides, demands a coordinated interplay between nutrient and ion transport mechanisms. This interplay connects nutrient sensing with the absorption of nutrients through the regulation of ion transport, a process mediated by enteroendocrine cells (McCauley et al., 2020). Given that a substantial portion of dietary amino acid and peptide transport relies on sodium (Na^+)- and hydrogen (H^+)-linked transporters (Broer and Fairweather 2018; Broer 2008), precise adjustments of ion concentrations within the intestinal lumen become paramount for maintaining and potentially enhancing the absorptive processes crucial for proper nutrition. Bile salts, whether acting individually or as a collective pool, are likely pivotal in coordinating nutrient absorption by influencing the function of epithelial ion transport. Considering this, using bile salts as feed additives could be contemplated mostly in fish diets, including ingredients whose composition may impair the normal intestinal absorption process.

Author contribution JF: study conception, experimental design, data evaluation, manuscript writing. SFG, FF, and RR: animal care, experiment execution, sampling, and data collection. JAMS: experimental design, data evaluation. FJM: study conception, experimental design, manuscript writing. All authors reviewed the manuscript.

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Data availability Data is provided within the manuscript. Derived data supporting the findings of this study are available from the corresponding author L. Fuentes on request.

Declarations

Competing interests The authors declare no competing interests.

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References

- Aragão C, Colen R, Ferreira S, Pinto W, Conceição LEC, Dias J (2014) Microencapsulation of taurine in Senegalese sole diets improves its metabolic availability. *Aquaculture* 431:53–58
- Aragão C, Cabano M, Colen R, Fuentes J, Dias J (2020) Alternative formulations for gilthead seabream diets: towards a more sustainable production. *Aquac Nutr* 26:444–455. <https://doi.org/10.1111/anu.13007>
- Birkett D, Silen W (1974) Alteration of the physical pathways through the gastric mucosa by sodium taurocholate. *Gastroenterology* 67(6):1131–1138
- Broer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* 88:249–286. <https://doi.org/10.1152/physrev.00018.2006>
- Broer S, Fairweather SJ (2018) Amino acid transport across the mammalian intestine. *Comp Physiol* 9:343–373. <https://doi.org/10.1002/cphy.c170041>
- Carvalho ES, Gregorio SF, Power DM, Canario AVM, Fuentes J (2012) Water absorption and bicarbonate secretion in the intestine of the sea bream: heterogeneous response to transmembrane and soluble adenylyl cyclase stimulation. *J Comp Physiol B* 182(8):1069–1080
- Chesney RW, Helms RA, Christensen M, Budreau AM, Han X, Sturman JA (1998) The role of taurine in infant nutrition. *Adv Exp Med Biol* 442:463–476
- Chiang JY (2009) Bile acids: regulation of synthesis. *J Lipid Res* 50(10):1955–1966. <https://doi.org/10.1194/jlr.R900010-JLR200>
- Clarke LL (2009) A guide to Ussing chamber studies of mouse intestine. *Am J Physiol Gastrointest Liver Physiol* 296:G1151–G1166. <https://doi.org/10.1152/ajpgi.90649.2008>
- Clarke KR, Gorley RN (2015) PRIMER v7: user manual/tutorial, 3rd edn. Plymouth, United Kingdom: Primer-E Ltd
- Estensoro I, Ballester-Lozano G, Benedito-Palos L, Grammes F, Martos-Sitcha JA, Mydland L-T, Caldach-Giner JA, Fuentes J, Karalazos V, Ortiz A, Øverland M, Sitjà-Bobadilla A, Pérez-Sánchez J (2016) Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. *PlosONE* 11(11):e0166564. <https://doi.org/10.1371/journal.pone.0166564>
- Fonseca F, Fuentes J, Vizcaíno AJ, Alarcón FJ, Mancera JM, Martínez-Rodríguez G, Martos-Sitcha JS (2023) From invasion to fish fodder: inclusion of the brown algae *Rugulopteryx okamuræ* in aquafeeds for European sea bass *Dicentrarchus labrax* (L., 1758). *Aquaculture* 568:739318
- Francis G, Makkar HPS, Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199:197–227
- Fuentes J, Figueiredo J, Power DM, Canario AVM (2006) Parathyroid hormone-related protein regulates intestinal calcium transport in the sea bream (*Sparus auratus*). *Am J Physiol Regul Integr Comp Physiol* 291:R1499–R150
- Fuentes J, Ribeiro L, Araújo C (2018) Bile salts regulate ion transport in the intestine of Senegalese sole. *Aquaculture* 495:842–848. <https://doi.org/10.1016/j.aquaculture.2018.06.050>
- Giaquinto PC, Hara TJ (2008) Discrimination of bile acids by the rainbow trout olfactory system: evidence as potential pheromone. *Biol Res* 41:33–42. <https://doi.org/10.4067/S0716-97602008000100005>
- Gieryńska M, Szulc-Dąbrowska L, Struzik J, Mielcarska MB, Gregorczyk-Zboroch KP (2022Jan 8) Integrity of the intestinal barrier: the involvement of epithelial cells and microbiota—a mutual relationship. *Animals (basel)* 12(2):145. <https://doi.org/10.3390/ani12020145>
- Goto T, Une T, Kihira K, Kuramoto T, Hoshita T (1993) Enzymatic formation of D-cysteinolic acid conjugated chanoxycolic acid in liver preparation from red seabream. *Pagrosomus Major Biol Pharm Bull* 16(12):1216–1219
- Goto T, Ui T, Une T, Kuramoto T, Kirira T, Hoshita T (1996) Bile salt composition and distribution of the D-cysteinolic acid conjugated bile salts in fish. *Fish Sci* 62:606–609
- Gu M, Bai N, Zhang YQ, Krogdahl Å (2016) Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high

- supplementation levels. *Aquaculture* 464:286–95. <https://doi.org/10.1016/j.aquaculture.2016.06.035>
- Hagey LR, Møller PR, Hofmann AF, Krasowski MD (2010) Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. *Physiol Biochem Zool* 83(2):308–321. <https://doi.org/10.1086/649966>
- Hegyí P, Maléth J, Walters JR, Hofmann AF, Keely SJ (2018) Guts and gall: bile acids in regulation of intestinal epithelial function in health and disease. *Physiol Rev* 98(4):1983–2023. <https://doi.org/10.1152/physrev.00054.2017>
- Hofmann AF, Hagey LR, Krasowski MD (2010) Bile salts of vertebrates: structural variation and possible evolutionary significance. *J Lipid Res* 51(2):226–246. <https://doi.org/10.1194/jlr.R000042>
- Iwashita Y, Suzuki N, Yamamoto T, Shibata J, Isokawa K, Soon AH, Ikehata Y, Furuita H, Sugita T, Goto T (2007) Supplemental effect of cholytaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout *Oncorhynchus mykiss*. *Fish Sci* 74:1083–1095. <https://doi.org/10.1111/j.1444-2906.2008.01628.x>
- Iwashita Y, Suzuki N, Matsunari H, Sugita T, Yamamoto T (2008) Influence of soya saponin, soya lectin, and cholytaurine supplemented to a casein-based semi-purified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. *Fish Sci* 75:1307–1315. <https://doi.org/10.1007/s12562-009-0158-1>
- Jin M, Pan T, Cheng X, Zhu TT, Sun P, Zhou F, Ding X, Zhou Q (2019) Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. *Aquaculture* 504:199–209. <https://doi.org/10.1016/j.aquaculture.2019.01.063>
- Katona BW, Anant S, Covey DF, Stenson WF (2009) Characterization of enantiomeric bile acid-induced apoptosis in colon cancer cell lines. *J Biol Chem* 284(5):3354–3364. <https://doi.org/10.1074/jbc.M805804200>
- Kelly OB, Mroz MS, Ward JB, Colliva C, Scharl M, Pellicciari R, Gilmer JF, Fallon PG, Hofmann AF, Roda A, Murray FE, Keely SJ (2013) Ursodeoxycholic acid attenuates colonic epithelial secretory function. *J Physiol* 591(2307–2318):2013. <https://doi.org/10.1113/jphysiol.2013.252544>
- Kim S-K, Kim K-G, Kim K-D, Kim K-W, Son M-H, Rust M, Johnson R (2015) Effect of dietary taurine levels on the conjugated bile acid composition and growth of juvenile Korean rockfish *Sebastes schlegelii* (Hilgendorf). *Aquac Res* 46:2768–2775
- Kortner TM, Björkhem I, Krasnov A, Timmerhaus G, Krogdahl A (2014) Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* 111:2089–103. <https://doi.org/10.1017/S0007114514000373>
- Kortner TM, Penn MH, Björkhem I, Måsøval K, Krogdahl Å (2016) Bile components and lecithin supplemented to plant based diets do not diminish diet related intestinal inflammation in Atlantic salmon. *BMC Vet Res* 12(1):190. <https://doi.org/10.1186/s12917-016-0819-0>
- Krogdahl Å, Penn M, Thorsen J, Refstie S, Bakke AM (2010) Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquacult Res* 41:333–344
- McCaughey HA, Matthis AL, Enriquez JR, Nichol JT, Sanchez JG, Stone WJ, Sundaram N, Helmrath MA, Montrose MH, Aihara E, Wells JM (2020) Enteroendocrine cells couple nutrient sensing to nutrient absorption by regulating ion transport. *Nature Communications* 11(1). <https://doi.org/10.1038/s41467-020-18536-z>
- Molina-Roque L, Bárány A, Sáez MI, Alarcón FJ, Tapia ST, Fuentes J, Mancera JM, Perera E, Martos-Sitcha JA (2022) Biotechnological treatment of microalgae enhances growth performance, hepatic carbohydrate metabolism and intestinal physiology in gilthead seabream (*Sparus aurata*) juveniles close to commercial size. *Aquaculture Reports* 25:101248
- Münch A, Ström M, Söderholm JD (2007) Dihydroxy bile acids increase mucosal permeability and bacterial uptake in human colon biopsies. *Scand J Gastroenterol* 42(10):1167–1174. <https://doi.org/10.1080/00365520701320463>
- Raimondi F, Santoro P, Barone MV, Pappacoda S, Barretta ML, Nanayakkara M, Apicella C, Capasso L, Paludetto R (2008) Bile acids modulate tight junction structure and barrier function of Caco-2 monolayers via EGFR activation. *Am J Physiol Gastrointest Liver Physiol* 29:G906–G913. <https://doi.org/10.1152/ajpgi.00043.2007>
- Rolen SH, Caprio J (2008) Bile salts are effective taste stimuli in channel catfish. *J Exp Biol* 21:2786–2791. <https://doi.org/10.1242/jeb.018648>
- Romano N, Kumar V, Yang G, Kaibaf K, Rubio M, Overturf KE, Brezas A, Hardy R (2020) Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. *Aquaculture* 12(3):1792–1817. <https://doi.org/10.1111/raq.12410>
- Ruiz A, Andree KB, Sanahuja I, Holhorea PG, Caldach-Gine JA, Morais S, Pastor JJ, Pérez-Sánchez J, Gisbert E (2023a) Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*). *Aquaculture* 566:739203. <https://doi.org/10.1016/j.aquaculture.2022.739203>
- Ruiz A, Andree KB, Furones D, Holhorea PG, Caldach-Giner JÀ, Viñas M, Pérez-Sánchez J, Gisbert E (2023) Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. *Front Microbiol* 14:1123716. <https://doi.org/10.3389/fmicb.2023.1123716>
- Sáenz de Rodríguez MA, Fuentes J, Moyano FJ, Ribeiro L (2013) *In vitro* evaluation of the effect of a high plant protein diet and nucleotide supplementation on intestinal integrity in meagre (*Argyrosomus regius*). *Fish Physiol Biochem* 39:1365–1370
- Staessen TWO, Verdegem MCJ, Koletsi P, Schrama JW (2020) The effect of dietary protein source (fishmeal vs. plant protein) and non-starch polysaccharide level on fat digestibility and faecal bile acid loss in rainbow trout (*Oncorhynchus mykiss*). *Aquac Res* 51(3):1170–1181. <https://doi.org/10.1111/are.14467>

- Stolfi C, Maresca C, Monteleone G, Laudisi F (2022) Implication of intestinal barrier dysfunction in gut dysbiosis and diseases. *Biomedicines* 10(2):289. <https://doi.org/10.3390/biomedicines10020289>
- Tocher DR, Bendiksen EA, Campbell PJ, Bell JG (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* 280:21–34
- Yamamoto T, Suzuki N, Furuita H, Sugita T, Tanaka N, Goto T (2007) Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout *Oncorhynchus mykiss*. *Fish Sci* 73:123–131. <https://doi.org/10.1111/j.1444-2906.2007.01310.x>
- Yui S, Kanamoto R, Saeki T (2009) Biphasic regulation of cell death and survival by hydrophobic bile acids in HCT116 cells. *Nutr Cancer* 61(3):374–380. <https://doi.org/10.1080/01635580802582744>
- Zeng Huawei, Safratowich Bryan D, Cheng Wen-Hsing, Larson Kate J, Briske-Anderson Mary (2022) Deoxycholic acid modulates cell-junction gene expression and increases intestinal barrier dysfunction. *Molecules* 27(3):723. <https://doi.org/10.3390/molecules27030723>
- Zhang C, Brown SB, Hara TJ (2001) Biochemical and physiological evidence that bile acids produced and released by lake char (*Salvelinus namaycush*) function as chemical signals. *J Comp Physiol B* 171:161–171
- Zhang J, Xiong F, Wang GT, Li WX, Li M, Zou H, Wu SG (2017) The influence of diet on the grass carp intestinal microbiota and bile acids. *Aquaculture Research* 48(9):4934–4944. <https://doi.org/10.1111/are.13312>

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