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EFFECTS OF SODIUM BUTYRATE TREATMENT ON HISTONE MODIFICATIONS AND THE EXPRESSION OF GENES RELATED TO EPIGENETIC REGULATORY MECHANISMS AND IMMUNE RESPONSE IN EUROPEAN SEA BASS (DICENTRARCHUS LABRAX) FED A PLANT-BASED DIET

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Short Title:	EFFECTS OF SODIUM BUTYRATE ON HISTONE MODIFICATIONS AND EPIGENETIC-RELATED GENES IN MARINE FISH
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Keywords:	Aquaculture; epigenetics; butyrate; Gene expression; histone acetylation; marine fish; immune system
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	dicer1. Statistical analysis by 2-way ANOVA for these genes showed not only significant differences due to the butyrate treatment, but also due to the interaction between tissue and treatment.
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Thank you for handling this paper.

Sincerely yours,
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Keywords: Aquaculture; epigenetics; butyrate; gene expression; histone acetylation; marine fish;

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Abstract

Bacteria that inhabit the epithelium of the animals' digestive tract play a critical role in the establishment and maintenance of their hosts' health. The gut microbiota provide the essential biochemical pathways for fermenting otherwise indigestible dietary fibers, leading to the production of short-chain fatty acids (SCFAs). Of the major SCFAs, butyrate has received particular attention due to its numerous positive effects on the health of the intestinal tract and peripheral tissues. Several lines of evidence suggest that this four-carbon chain organic acid molecule has potential immunomodulatory and anti-inflammatory properties, too. The mechanisms of action of butyrate are different; many of these are related to its potent regulatory effect on gene expression since butyrate is a type of histone deacetylase inhibitors that play a predominant role in the epigenetic regulation of gene expression and cell function. The inclusion of butyrate into the livestock diets can promote growth and have multiple beneficial effects on the intestinal tract and liver metabolism. Although such effects have been demonstrated in several terrestrial species, very few studies have assessed them in fish.

In the present work, we investigated in the European sea bass (*Dicentrarchus labrax*) the effects of butyrate used as a feed additive on fish epigenetics as well as its regulatory role in mucosal protection and immune homeostasis through impact on gene expression. Seven target genes related to inflammatory response and reinforcement of the epithelial defense barrier [*tnfα* (tumor necrosis factor alpha) *il1β,* (interleukin 1beta), *il-6, il-8, il-10,* and *muc2* (mucin 2)] and five target genes related to epigenetic modifications [*dicer1*(double-stranded RNA-specific endoribonuclease), *ehmt2* (euchromatic histone-lysine-N-methyltransferase 2), *pcgf2* (polycomb group ring finger 2), *hdac11* (histone deacetylase-11), and *jarid2a* (jumonji)] were analyzed in fish intestine and liver. We also investigated the effect of dietary butyrate supplementation on histone acetylation, by performing an immunoblotting analysis on liver

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Results of the 8-week-long feeding trial showed no significant differences in weight gain or SGR (specific growth rate) of sea bass that received 2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate. Dietary butyrate led to a threefold increase in the acetylation level of histone H4 at lysine 8, but showed no effects on the histone H3 at Lys9. Moreover, two different isoforms of histone H3 that might correspond to the H3.1 and H3.2 isoforms previously found in terrestrial animals were separated on the immunoblots. The expression of four (*il1β*, *il8*, *irf1,* and *tnfa*) out of seven analyzed genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only *il10* showed differences in expression due to the butyrate treatment. In addition, butyrate caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1*. Statistical analysis by 2-way ANOVA for these genes showed not only significant differences due to the butyrate treatment, but also due to the interaction between tissue and treatment.

Introduction

Bacteria associated with the epithelium of an animal's digestive tract play a critical role in establishing and maintaining their host's health. The intestinal microbiota is involved in the anaerobic fermentation of complex dietary carbohydrates such as cellulose, hemicellulose, pectin, and oligosaccharides that are otherwise indigestible as well as of digestible simple carbohydrates such as starch and glucose that escape digestion and absorption in the small intestine [1]. Intestinal mucus, sloughed cells from the epithelia, lysed microbial cells, and endogenous secretions provide other sources of fermentable substrates, especially proteins

and polysaccharides [1]. Nearly 75% of the energy content of the fermented carbohydrates is used to produce metabolic end products such as short chain fatty acids (SCFAs), which are then readily absorbed by the host; the remaining 25% is used for microbial growth and maintenance or lost as hydrogen, carbon dioxide, and methane [2]. Microbial fermentation mainly takes place in the forestomach (a fermentation chamber cranial to the acid-secreting part of the stomach) of foregut fermenters such as ruminants (cattle, sheep, goats, etc.) and in the cecum and large intestine of hindgut fermenters (the food is fermented after it has been digested by the stomach), such as rodents, elephants, and most carnivores and omnivores, including humans [1,3]. The produced SCFAs are waste products to the microbes but represent the main source of metabolic energy for colonocytes in hindgut fermenters or serve as a principal source of energy for the entire animal in the case of foregut fermenters. Indeed, ruminants depend on SCFAs for 80% of their maintenance energy [1,4,5].

SCFAs, also known as volatile fatty acids, are carboxylic acids with aliphatic tails of 1 to 6 carbon atoms that exist in straight- and branched-chain conformations. Common SCFAs include acetic (C2), propionic (C3), butyric (C4), valeric (C5), and caproic (C6) acid [4]. Being weak acids with modest pKas of approximately 3.6 to 4.7, SCFAs do not completely dissociate or dissolve in water. Owing to this and because the pH of that part of the gastrointestinal tract in which the fermentation occurs is nearly neutral (the colonic pH is about 6.0-7.5), more than 90% of SCFAs are present as anions rather than as free acids [1]. The predominant anions in either the rumen or large intestine are the short, straight-chain FAs such as acetate, propionate, and butyrate, whereas the short branched-chain FAs, isobutyrate and isovalerate, which are produced by fermentation of the amino acids valine and leucine, respectively, are found in much smaller amounts [1,6].

Among the SCFAs, butyrate has received particular attention due to its numerous positive effects on the health of intestinal tract and peripheral tissues [7]. In addition to being

97 the main respiratory fuel source of the colonic bacteria, and preferred to glucose or glutamine,
98 butyrate plays a major role in enhancing epithelial cell proliferation and differentiation and in
99 improving the intestinal absorptive function [8,9,4]. Furthermore, there are several lines of
100 evidence suggesting that butyrate has potential immunomodulatory and anti-inflammatory
101 properties in the intestine and may prevent colorectal cancer in humans [10,11,12].

102 Although the exact underlying mechanisms of action have not yet been elucidated, the
103 influence of butyrate on cell proliferation may be explained, at least in part, by its potent
104 regulatory effect on gene expression. This effect is often attributed to the ability of butyrate to
105 inhibit the activity of many histone deacetylases, leading to hyperacetylation of histones [12].
106 Histone acetylation modifies chromatin structure, allowing the binding of transcription factors
107 and polymerases and hence, the beginning of transcription. The modulation of gene expression
108 through core histone acetylation is one of the most relevant means by which cell function and
109 DNA methylation are epigenetically regulated [12,13,14]. A positive effect of butyrate on
110 transcriptomic activity of some pivotal genes at the intestinal level has also been suggested in
111 fish in two recent studies carried out on European sea bass (*Dicentrarchus labrax*) [15] and
112 gilthead sea bream (*Sparus aurata*) [16].

113 Much of the research on butyrate has focused on its role in the gut, while less is known
114 about whole-body metabolism of butyrate and, in particular, on how it might influence the
115 metabolic potential of the liver *in vivo* [17,18]. Although butyrate is largely taken up by the
116 intestinal epithelium, a small fraction can also reach the liver through the blood stream via the
117 portal vein [18,19]. In liver, butyrate is readily converted in mitochondria to butyryl CoA by
118 the enzyme butyryl-CoA synthetase to produce ketone bodies (rather unlikely in fed animals)
119 and acetyl CoA, which then enters into the Krebs cycle [19]. Butyrate is also a potent effector
120 of hepatic metabolism. It can reduce the mitochondrial oxidative phosphorylation yield and the
121 ATP content of the liver [20,21,7] and can influence mitochondrial ATP turnover, which is

linked to glycogen metabolism [22]. Hepatic metabolism and clearance of butyrate are substantial since evidence shows that close to 100% was removed in the liver of rodents adapted to a high-fiber diet [22], whereas in the human gut *in vivo* [18] butyrate release into the circulatory system was counterbalanced by hepatic butyrate uptake, indicating that the liver is highly involved in butyrate metabolism [22]. However, in contrast to single-stomached animals, *in vivo* studies in ruminants have shown that butyrate is taken up in the rumen but that the capacity to metabolize the four-carbon butyrate molecule is limited in the ruminal epithelium and liver [3]. Indeed, in ruminants, the proportion of butyrate that passes into the blood is low in relation to the amount produced in the rumen, but, of the butyrate that is absorbed, approximately 80 % is transported via the portal vein to the liver for hepatic gluconeogenesis. The fraction not absorbed is distributed to peripheral tissues and mammary glands for lipogenesis and milk fat synthesis [1,23,24].

For butyrate to exert its physiologic, cellular, and molecular effects, circulating concentrations would need to be maintained at a consistently high level. This is difficult to attain because plasma clearance of butyrate is very rapid, with a half-life on the order of 6 min when given intravenously in humans [25]. A possible solution to circumvent problems associated with rapid metabolism of butyrate would be to administer it orally by giving multiple daily doses of stable derivatives of butyrate, which are being developed for use in both humans and animals. Indeed, when stable derivatives of butyrate were given orally as opposed to intravenously in humans, the half-life was increased to 40 min., and circulating butyrate concentrations reached high enough values to be efficacious [25]. Furthermore, in farmed animals such as pigs and chickens, butyrate included in the diet has had a positive influence on body weight gain, feed utilization, and composition of intestinal microflora, as well as trophic effects on the intestinal epithelium through an increase in the villi length and crypt depth [26,27,28]. Applied as a nutritional supplement in poultry, butyrate caused *in vivo*

147 hyperacetylation of the hepatic core histones and modified the epigenetic regulation of
148 hepatocyte's function [7]. In addition, some authors have suggested significant improvements
149 in growth and food conversion rates of fish when butyrate is included in diets of some species
150 such as catfish (Owen et al. 2006), tilapia, carp [29], and sea bream [16], but not in others such
151 as salmon [30,31]. However, except for these studies, literature is scarce concerning the use of
152 butyrate or its derivatives as an additive in fish feed.

153 Accordingly, the present study aimed to evaluate in European sea bass (*Dicentrarchus*
154 *labrax*) the potential effects of butyrate as a feed additive on fish growth, as well as butyrate's
155 regulatory role on the mucosal protection and immune homeostasis through its effects on gene
156 expression. The target genes related to mucosal inflammatory response and reinforcement of
157 the mucous defense barrier included tumor necrosis factor alpha (*tnfα*), which is a cell-
158 signaling protein (cytokine) that makes up the inflammatory acute phase reaction and possesses
159 a wide range of proinflammatory actions [32]; interleukins such as *il1β, il-6, il-8,* and *il-10*,
160 which are well-known cytokines that regulate immune responses, inflammatory reactions, and
161 hematopoiesis; interferon regulatory factor 1 (*irf1*), which is a transcription factor that
162 stimulates both innate and acquired immune responses by activating specific target genes
163 expressed during inflammation, immune responses, and hematopoiesis [33]; and mucin 2
164 (*muc2*), which is a major component of intestinal mucus gel secretions that serve as a barrier
165 to protect the intestinal epithelium [34].

166 The second goal of the present study was to evaluate the epigenetic effects of dietary
167 butyrate in sea bass by monitoring both the acetylation state of hepatic core histones and the
168 hepatic and intestinal expression of a suite of genes related to epigenetic modifications [35].
169 These genes included: *dicer 1,* which encodes an active, small RNA component that represses
170 other gene expression [36]; *ehmt2* (euchromatic histone-lysine-N-methyltransferase 2), which
171 demethylates Lys9 in histone 3 in euchromatin, creating a tag for epigenetic transcription

repression [37,38]; *pcgf2* (polycomb group ring finger 2), which acts via chromatin remodeling and histone modification [39]; *hdac11* (histone deacetylase-11), which modifies core histone octamer packing chromatin in dense structures [40] or controls various histone methyltransferase complexes; and *jarid2a* (jumonji), which is a nuclear factor that functions as a powerful transcriptional repressor [41].

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Insubria, Varese, Italy. All of the protocols performed were approved by the Committee on the Ethics of Animal Experiments of the same University. Fish handling was performed under tricaine methanesulfonate (MS222) anesthesia, and all efforts were made to minimize discomfort, stress, and pain to the fish.

Fish and experimental set up

Juvenile European sea bass (*Dicentrarchus labrax*) were purchased from a commercial hatchery (Civitavechia, Italy). Upon arrival to the laboratory, fish were stocked for 40 days in two indoor tanks of 2.5 cubic meters to acclimate.

After removing fish deviating from the average weight of approximately 15 g, we distributed fish into six experimental tanks of 600 L each at a density of 35 fish per tank (3 replicates) and let them to acclimate over a period of 1 week. There were no significant differences in fish weight between the experimental tanks at the onset of the experiment ($P>0.05$).

Rearing facility and maintenance

All rearing tanks were located in an indoor facility. The tanks were equipped with re-circulating systems and photoperiod, temperature, and salinity could be strictly controlled with this equipment. The experimental layout consisted of six cylindrical fiberglass tanks of 600 liters and four rectangular tanks of 2500 liters, connected to a central main biofilter of 350 liters. The light source was the natural photoperiod enhanced with florescent light, providing a light intensity of 1200 lx during the day. The water was heated and maintained at 21 ± 1°C by using submersible aquarium heaters. The salinity was 22 ± 0.5 g/l.

Twice a week the following parameters were measured: dissolved oxygen, pH, and ammonia and nitrite levels. The levels of all parameters remained within the range considered optimal for sea bass growth throughout the experiment.

Diet formulation, and feeding

As a control diet we used a formulation of 40% crude protein and 16% fat which was based on plant protein and fishmeal. The control diet was similar to feed commercially available for growing European seabass. In addition to being used as is, this diet was supplemented with 2 g/kg (2%) of sodium butyrate to produce the experimental butyrate diet. Information about each diet composition is presented in Table 1. Diets were prepared using small-scale machinery for mixing ingredients and preparing pellets of 3.5 mm in diameter. Na-butyrate substituted an equivalent amount of filler in the butyrate diet.

Table 1. **Composition of the diets in g/100 g on a dry weight basis.**

Ingredients (g/100g)	**Control**	**Butyrate**
Fish meal	10.00	10.00
Soybean meal	30.00	30.00
Pea concentrate	16.00	16.00
Corn gluten	14.20	14.20
Wheat gluten	5.00	5.00
Fish oil	14.00	14.00
Stay-C 35d	0.03	0.03
Vitamin Mix	0.45	0.45
Mineral Mix	1.00	1.00
DL-Methionine	0.25	0.25
Lysine (98%)	0.05	0.05
Fish Hydrolysate	2.00	2.00
Dextrin	1.56	1.56
Sodium alginate	0.79	0.79
Dicalcium phosphate	0.72	0.72
Filler (gelatin)	4.00	2.00
Na-butyrate	-	2.00
Total	100.00	100.00

Each diet was provided to fish in duplicate (2 tanks/diet). Fish were fed twice a day and feeding rates were restricted to 3.0% of biomass during the feeding experiment based on four-weekly fish weight measurements to adjust the feed ration to a similar percentage of fish biomass in

222 both treatments. The feeding trial lasted 8 weeks. Fish specific growth rate (SGR) was
223 calculated using the following formula: (ln Wf - ln Wi)/t x 100, where Wf is the final weight
224 (g), Wi is the initial weight (g), and t is growth time (days).

225 Fish sampling

226 At the end of the 8-week-long feeding trial, fish in each tank were batch-weighed after
227 overnight food deprivation. Six fish from each treatment (3 fish/tank) were then randomly
228 selected, and sacrificed. Intestine and liver were excised from each sampled fish using sterile
229 instruments, snap-frozen in dry ice, and then kept at -80°C until nucleic acid extraction and
230 histone protein acetylation analysis.

231 Preparation of liver nuclear protein fraction

232 Liver nuclear protein extracts were prepared from six fish per group using 3 ml/g of tissue of
233 an extraction buffer containing: 10 mM Tris/HCl, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5
234 mM Pefabloc® (SIGMA-ALDRICH®), 0.5 mM DTT, 1 mM Na_3VO_4, and 1X protease inhibitor
235 cocktail (SIGMA-ALDRICH®). Tissue lysis and homogenization were carried out in a closed
236 system using the gentleMACS™ Dissociator and single-use gentleMACS™ M tubes (Miltenyi
237 Biotec). Liver lysates were then centrifuged at 1500 *g* for 20 min at 4°C. The supernatants
238 containing the cytosolic protein fraction were discarded while the nuclear pellets were stored
239 at −80°C until further histone isolation procedure.

240 Histone isolation

241 Purified histone extracts were isolated from nuclear fractions using the Histone Purification

Mini Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Active Motif's Histone Purification Kit preserves phosphoryl, acetyl, and methyl post-translational modifications on histones. Briefly, an equal volume of ice-cold extraction buffer was added to the nuclear suspension. After homogenization, samples were left overnight in the extraction buffer on a rotating platform at 4°C. Next day, tubes were centrifuged at maximum speed for 5 min in a microfuge at 4°C and the supernatants, which contained the crude histone extracts, were neutralized with one-fourth volume of 5x neutralization buffer (pH 8.0). Neutralized extracts were loaded to previously equilibrated histone isolation spin columns. After three washes with histone wash buffer, histones were eluted in 100 µl of histone elution buffer and precipitated overnight by adding 4% perchloric acid. On the following day, samples were centrifuged at maximum speed for 1 hour; histone pellets were washed first with 4% perchloric acid, later with acetone containing 0.2% HCl, and finally with pure acetone, after which they were air dried. Histones were suspended in sterile distilled water and the yield of total core histone proteins was quantified by measuring the absorbance at 230 nm.

Histone acetylation western blots

Western blotting analyses were performed according to the instructions of the Acetyl Histone Antibody Sampler Kit (Cell Signaling) and the protocol applied by Mátis *et al*. [7]. Histone proteins were diluted by 2x SDS and β-mercaptoethanol containing loading buffer (supplemented with 50 mM DTT), sonicated for 15 sec, and heat denatured at 95°C for 5 min. Histones were separated by SDS-PAGE on polyacrylamide (4-20%) precast gradient gels (Bio-Rad); 3 µg protein per lane were loaded for the detection of histones H2A, H2B, and H3, whereas 6 µg per lane were loaded for histone H4. After electrophoresis, proteins were blotted

onto PVDF membranes (0.22-µm pore size, Bio-Rad). Before proceeding to the immunodetection process, a reversible Ponceau staining was applied to membranes to check equal loading of gels and protein transfer. Histones were identified using antibodies furnished by the Acetyl Histone Antibody Sampler Kit. After blocking with 5% fat-free milk containing PBST for 3 h, the immunoblots were incubated overnight at 4°C with primary antibodies against histone H2A (1:1000), H2B (1:500), H3 (1:1000), H4 (1:500), and their acetylated forms. Each acetyl histone antibody was specific for the target histone modified at the lysine residue of the most frequent acetylation site (AcH2A and AcH2B: Lys 5, AcH3: Lys 9, AcH4: Lys 8). The primary antibody was detected using an anti-rabbit secondary antibody (1:2000) or an anti-mouse secondary antibody (1:900) for the non-acetylated H4 histone. Both secondary antibodies were coupled with horseradish peroxidase. Primary antibodies were diluted in PBST containing 5% BSA, secondary antibodies in PBST containing 5% fat-free milk. Signals were detected using an enhanced chemiluminescence system (SuperSignal®west Dura Extended Duration Substrate, Thermo Scientific) and then exposing them to clear-blue X-ray film. After film exposure, densitometry was used to quantify protein levels on the western blots by means of Quantity One 1-D software (Bio-Rad).

RNA extraction and cDNA synthesis for gene expression analysis

RNA from 12 sea bass livers and 12 intestines was extracted using a semi-automatic system (Maxwell® 16 Instrument, Promega) and a total RNA purification kit (Maxwell® 16 Tissue LEV). RNA quality and concentration were assessed by a ND-2000 spectrophotometer (NanoDrop product, Thermo Scientific).

One hundred nanograms of the total extracted RNA were reverse transcribed to cDNA using

SuperScript III and random hexamers (Life Technologies, Italy) following the manufacturer's instructions. Two rounds of cDNA synthesis per sample were carried out and then merged.

Quantitative real-time PCR (qRT-PCR)

We applied two strategies for real-time PCR primer design: 1) for the already cloned genes in European sea bass, FASTA sequences were taken from the NCBI repository (http://www.ncbi.nlm.nih.gov/) and primers were designed by using Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi); 2) for the genes not cloned yet, exon sequences from other fish species (stickelback or tilapia) were taken from the Ensembl Genome Browser (http://www.ensembl.org/) and blasted against the European sea bass genome database [42]. Only when the match was annotated in the sea bass genome the exon was considered for primer design (Supporting information Table 1). Primer efficiency was checked by analyzing the slope of a linear regression from six different dilutions. Efficiencies ranged from 1.8 to 2.4. In addition, the correct binding of the primers was checked by adding a melting-curve analysis (95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s) after the amplification phase.

qRT-PCR was performed on an ABI 7900HT (Life Technologies) under a standard cycling program (UDG decontamination cycle: 50ºC for 2 min; initial activation step: 95ºC for 10 min; 40 cycles of 15 s denaturation at 95ºC and 1 min annealing/extension at 60ºC). A final dissociation step was also added (95ºC for 15 s and 60ºC for 15 s).

For qRT-PCR gene analysis, cDNA was diluted 1:10 for all the target genes except for the reference gene, *r18S,* which was diluted 1:500. All samples were run in triplicate in a 384-well plate in a final volume of 10 µl. Each well contained a mix of 5 µl SYBR Green Supermix (Life Technologies), 2 µl distilled water, 2 µl primer mix (forward and reverse at 10 µM concentration), and 1 µl cDNA. Negative controls were added in duplicate. The software SDS

2.3 and RQ Manager (Life Technologies) were used to collect data and calculate gene expression levels (cycle thresholds, Cts), respectively. The expression of housekeeping gene *r18S* (the endogenous control) was used to correct for intra- and inter-assay variations.

Data analysis

qRT-PCR raw data analysis.

Ct values were adjusted, taking into account primer efficiencies per each gene when calculati ng 2^ddCt values. Expression data for each target gene were also normalized to the housekee ping gene (*r18S*) and fold-change calculations were made based on the [43] method.

qRT-PCR statistical analysis.

qRT-PCR analyses were performed using 2^ddCt values in IBM SPSS Statistics 19 software. Data were checked for normality and homoscedasticity of variance; outliers were eliminated when needed.

Treated versus control groups, in liver and intestine, were analyzed in two ways: 1) by analyzing fold-change differences with respect to the controls [43] and 2) by a Student *t*-test analysis. In addition, a two-way analysis of variance (ANOVA) was carried out, taking into consideration both treatment and tissue for analyzing not only the contributions of each variable but also their interactions.

Results

Effect of butyrate on growth performance

The initial weight of 14.91±1.73 g of the control fish group (Fig 1) increased to 20.63±4.17 g after 4 weeks of feeding and to 30.22±5.61 g after 8 weeks of feeding. The

difference with respect to time zero's mean body weight became significant only at the end of the feeding experiment. Fish receiving the butyrate supplemented diet had an initial mean body weight of 15.80±1.60 g, which increased to 20.51±4.74 g after 4 weeks and to 28.97±8.09 g after 8 weeks of feeding. In this group, too, the increase in body weight became significantly different from time zero value only at the end of the feeding trial. However, the differences in fish growth performance between treatments did not reach statistical significance (Fig 1). Survival was high (around 95%) with no significant differences between the fish groups fed different diets. The SGR of fish fed the butyrate-supplemented diet was 1.06±0.02 after 4 weeks of feeding and 1.19±0.03 at the end of the experiment, whereas that of the control group was 1.34±0.04 and 1.33±0.07 after 4 and 8 weeks of feeding, respectively. There were no significant differences in SGR between the fish fed control and butyrate diet.

Effect of butyrate on core histone acetylation

To investigate the effect of dietary supplementation of sodium butyrate on histone acetylation in European sea bass, we performed an immunoblotting analysis on liver core histone extracts. The result of this analysis is presented in Fig 2, whereas the intensity values ($OD*mm^2$) of each band are reported in Table 2. Among the primary antibodies furnished by the Acetyl-Histone Antibody Sampler Kit (Cell Signaling Technology) only anti-H2A (non-acetylated form), anti-H3 (acetylated and non-acetylated forms), and anti-AcH4 (acetylated form) worked correctly in sea bass. In contrast, anti-H2B, anti-AcH2B, anti Ac-H2A, and anti-H4 antibodies did not recognize any epitope in sea bass. The kit manufacturer guaranteed cross-reactivity with fish (zebrafish) only for anti-H3 (acetylated or non-acetylated forms) and anti-H4 (non-acetylated) antibodies, but only the former worked properly in our species.

Table 2. Quantification of core histone protein expression (Vol OD*mm^2).

	Vol OD*mm^2	
	BUTYRATE	**CONTROL**
H2A	1.12 ± 1.10*	5.46 ± 2.62
H3	5.85 ± 2.19	8.03 ± 0.40
AcH3	6.42 ± 1.33	8.36 ± 1.20
AcH4	1.51 ± 0.23*	0.51 ± 0.31

(*) indicates significant differences between the two diets ($P < 0.05$).

Immunoblotting on hepatocyte core histone extracts (Table 2) revealed that dietary butyrate intake decreased the relative protein expression level of the H2A histone ($P<0.05$), which was poorly expressed in butyrate-treated fish but was detected at high amounts (fivefold more) in control fish. Screening of the principal acetylation sites of core histones revealed that butyrate treatment caused hyperacetylation of histone H4. Indeed, the addition of sodium butyrate to the diet significantly increased acetylation of histone H4 at lysine 8 ($P<0.05$), leading to an approximately threefold increase in comparison to the control group (no butyrate) (Table 2). In contrast, the acetylation state of histone H3 at Lysine 9 was not significantly influenced by butyrate dietary intake. Interestingly, two different isoforms of histone H3 were separated on in the immunoblots, which could correspond to the H3.1 and H3.2 isoforms previously found in chicken [7].

Genes related to epigenetic regulatory mechanisms

Regardless of treatment, a 2-way ANOVA showed that the differences between hepatic and intestinal levels of expression of five target genes related to epigenetic regulatory

372 mechanisms were statistically significant (P<0.05) or highly significant (P<0.01; P<0.001)
373 (Table 3), being in general higher in the intestine. However, pairwise individual comparisons
374 between control and treated fish for each tissue and gene analyzed by a Student's t-test showed
375 no differences in any case, despite fold-change ranges of 0.49 to 2.66 in the intestine and of
376 1.67 to 14.74 in the liver. This could be due to the high variability observed between fish.
377 Furthermore, regardless of tissue, *ehmt2* showed significant differences due to butyrate
378 treatment (P=0.002), with significant differences (P=0.010) for the interaction between tissue
379 and treatment, too. Similarly, *dicer1* and *hdac11* showed statistically significant differences
380 due to the interaction between tissue and treatment (P=0.050 and P=0.038, respectively). Fold-
381 change differences in the expression of genes that reached significance due to tissue, treatment,
382 or both are shown in Fig 3 A-C.

Table 3. **Statistical analysis of the expression of genes related to epigenetic regulatory mechanisms.**

Gene	Intestine	Liver	Intestine		Liver		2- way ANOVA	
	FC ± SEM^{+}	FC ± SEM^{+}	Student t-test		Student t-test			
dicer 1	2.40 ± 1.864	5.88 ± 3.393	t	0.548	t	1.068	F (Ts)	14.661 (*P*=0.001)***
			P-value	0.596	P-value	0.313	F (Tr)	0.025 (*P*=0.875)
							F (Ts x Tr)	2.219 (*P*=0.050)*
ehmt2	1.18 ± 0.869	2.52 ± 1.617	t	0.498	t	1.421	F (Ts)	61.878 (*P*=0.000)***
			P-value	0.629	P-value	0.169	F (Tr)	13.426 (*P*=0.002)**
							F (Ts x Tr)	8.093 (*P*=0.010)**
pcgf2	1.29± 0.932	14.75 ± 9.485	t	0.114	t	-0.15	F (Ts)	7.211 (*P*=0.014)*
			P-value	0.911	P-value	0.886	F (Tr)	0.003 (*P*=0.096)
							F (Ts x Tr)	0.024 (*P*=0.878)
jarid2a	2.66 ± 2.186	11.06 ± 4.028	t	0.893	t	0.898	F (Ts)	6.159 (*P*=0.022)*
			P-value	0.395	P-value	0.378	F (Tr)	0.825 (*P*=0.374)
							F (Ts x Tr)	0.385 (*P*=0.542)
hdac11	0.49 ± 0.200	1.67 ± 0.774	t	-1.388	t	1.791	F (Ts)	45.051 (*P*=0.000)***
			P-value	0.19	P-value	0.099	F (Tr)	0.002 (*P*=0.969)
							F (Ts x Tr)	4.843 (*P*=0.038)*

Note: Asterisks mark statistical differences (*P<0.05; ** P<0.01; *** P<0.001). +qRT-PCR gene expression fold change (butyrate-treated versus control fish).

Genes related to mucosal protection and inflammatory response

Statistical analysis by 2-way ANOVA revealed that the expression of four (*il1β*, *il8*, *irf1,* and *tnfa*) out of seven target genes related to inflammatory response and immune system was significantly different ($P<0.05$) between the two analyzed tissues (liver and intestine) but only the *il10* gene showed differences in expression ($P=0.003$) due to the butyrate treatment (Table 4). This effect was also demonstrated with pairwise comparisons using Student's *t*-test (P=0.002). In contrast to what was observed with the epigenetic regulatory mechanism-related genes and with the exception of *il10* in the liver (fold change 25.09±17.18; Fig. 3D), the magnitude of fold change in the other two genes (*il6*, *muc2*) was lower (range 0.01-4.74). Furthermore, in contrast to the epigenetic regulatory mechanism-related genes, the interaction effect between tissue and treatment did not reach statistical significance for any of the seven target genes related to the inflammatory response and mucosal protection.

Table 4. **Statistical analysis of the expression of genes related to inflammatory response, mucosal protection, and immune homeostasis.**

Gene	Intestine	Liver	Intestine		Liver		2- way ANOVA	
	FC ± SEM^{+}	FC ± SEM^{+}	Student t-test		Student t-test			
il1β	1.83 ± 0.693	0.07 ± 0.035	t	1.208	t	-2.155	F (Ts)	11.368 (P=0.003)**
			P-value	0.255	P-value	0.083	F (Tr)	0.000 (P=1.000)
							F (Ts x Tr)	0.000 (P=1.000)
il6	0.47 ± 0.113	0.01 ± 0.006	t	-0.109	t	-1.071	F (Ts)	2.068 (P=0.165)
			P-value	0.915	P-value	0.309	F (Tr)	1.126 (P=0.301)
							F (Ts x Tr)	0.949 (P=0.341)
il8	1.77 ± 0.907	0.69 ± 0.342	t	0.874	t	-0.603	F (Ts)	8.129 (P=0.009)**
			P-value	0.411	P-value	0.560	F (Tr)	0.632 (P=0.435)
							F (Ts x Tr)	0.660 (P=0.425)
il10	1.13 ± 0.242	25.09 ± 17.176	t	1.792	t	3.361	F (Ts)	0.036 (P=0.851)
			P-value	0.098	P-value	0.002**	F (Tr)	10.881 (P=0.003)**
							F (Ts x Tr)	1.007 (P=0.326)
irf1	0.17 ± 0.034	4.74 ± 2.777	t	1.398	t	1.329	F (Ts)	48.930 (P=0.000)***
			P-value	0.19	P-value	0.211	F (Tr)	2.401 (P=0.136)
							F (Ts x Tr)	1.505 (P=0.233)
tnfα	1.54 ± 0.303	0.34 ± 0.323	t	1.761	t	-1.144	F (Ts)	55.649 (P=0.000)***
			P-value	0.109	P-value	0.282	F (Tr)	0.000 (P=1.000)
							F (Ts x Tr)	0.000 (P=1.000)
muc2	0.74 ± 0.226	0.22 ± 0.146	t	-0.553	t	-1.820	F (Ts)	4.241 (P=0.059)
			P-value	0.591	P-value	0.291	F (Tr)	0.148 (P=0.706)
							F (Ts x Tr)	0.070 (P=0.795)

Note: Asterisks mark statistical differences (*P<0.05; ** P<0.01; *** P<0.001). +qRT-PCR gene expression fold change (butyrate versus control fish).

Discussion

Currently, there is strong interest in the use of organic acids and their salts as natural feed additives since such products seem to have growth-promoting effects in livestock. Their positive effects are well documented in terrestrial livestock production [28,44,45], but some questions remain regarding their efficacy in fish farming. Indeed, following the experiments in pig and poultry feeding, a wide variety of organic acids and their salts were tested in aquaculture diets for different fish species. These included carnivore species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Arctic charr (*Salvelinus alpinus*), herbivorous tropical warm-water species such as tilapia (*Oreochromis niloticus*), and omnivore fish such as carp (*Cyprinus carpio*), and catfish (*Ictalurus punctatus*) [46]. However, conflicting reports exist on the subject. Growth was significantly enhanced in some species, such as rainbow trout (*O. mykiss*), when fed an organic acid blend supplement mainly consisting of formate and sorbate [47], but not in trout fed other commercial aquaculture supplements such as lactic acid [48] or citric acid [48,49]. On the other hand, neither hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) fed potassium diformate [50] nor Atlantic salmon (*S. salar*) fed sodium salts of acetic, propionic, and butyric acid (5:5:2 w/w/w) showed any growth enhancement [51, 30]. The results of our study are in line with those of Gislason et al., [51] and Bjerkeng et al., [30], as we did not find differences in the growth of European sea bass fed a diet supplemented with Na-butyrate.

To date, literature related to the use of butyric acid or its salts in fish feed is still scarce and mainly focused on the effects of butyrate on fish growth performance, intestinal morphology, and metabolism [52,31,16,53]. However, only few reports have described butyrate-induced epigenetic and transcriptional changes in intestinal and hepatic genes of farmed fish [15,53]. In view of this scarcity of information, the present work contributes to our

current understanding of the epigenetic regulatory effects of butyrate in European sea bass, which is one of the most important species in Mediterranean aquaculture.

Butyrate belongs to a well-known class of epigenetic factors known as histone deacetylase inhibitors (HDACi) [4]. Histone deacetylases (HDACs) are critical enzymes involved in epigenetic transcriptional regulation, i.e., histone acetylation associated with chromatin structure and function [54]. Acetylation of core histones at specific lysine residues in the NH_2-terminal tails results in a decrease in the overall positive charge of histone tails, which diminishes their strength of binding to the negatively charged DNA. The effect is an opened chromatin structure, which makes DNA more accessible to transcriptional factors [55]. Therefore, as a result, HDACs act as transcriptional repressors, whereas HDACi upregulate gene expression. There are very compelling data showing that sodium butyrate increases the quantities of acetylated H3 and H4 histone proteins in certain cells and tissues. Thus, butyrate exposure caused hyperacetylation of histones H3 and H4 in vertebrate cell lines [56] and the same result was obtained in several *in vitro* studies using cultured mammalian cells [57,58,59]. However, very limited evidence can be found in the literature regarding butyrate-induced histone acetylation *in vivo*. The only data available were obtained in chicken, mice, and pigs [7,60,61,62]; hence, the present study represents the first in fish. Our results on sea bass hepatic histones clearly confirmed the capability of butyrate to induce histone hyperacetylation even *in vivo*. In agreement with what Mátis and colleagues [7] observed in liver of chickens fed a low dose of butyrate (0.25 g/kg body weight, BW), no significant differences were found in the acetylation state of total histone H3 at lysine 9 after the dietary administration of 2 g/kg feed of Na-butyrate in sea bass. Interestingly, a higher dose of butyrate (1.25 g/kg BW) caused, instead, a relevant increase in H3 acetylation ratio in chicken [61]. This indicates that the level of histone H3 acetylation was dose-dependent and therefore the failed hyperacetylation observed in sea bass fed butyrate could be explained by the amount of Na-butyrate in the diet

(2 g/100g feed), which was perhaps not sufficient to induce histone H3 hyperacetylation. Moreover, in sea bass and likewise in chicken, two isoforms of histone H3 were separated on the immunoblots; in mammals, in contrast, three H3 variants have been characterized (H3.1, H3.2, H3.3) [63]. Butyrate treatment undoubtedly induced an increase of histone H4 acetylation in sea bass liver. In chicken, hyperacetylation of histone H4 occurred independently of the dietary intake levels of butyrate [7]. Similarly, acetylation of histone H4 in mammals [61] seemed to be independent of the butyrate dose, since both low and high diet content of Na-butyrate increased acetylated H4 levels in mouse hippocampus; on the other hand and as in chicken, acetylation of H3 histone was improved only at a higher dose [61]. Furthermore, in functional studies such as transcription factor-binding assays or gene expression analysis, acetylation of histone H4 was often found to be inversely correlated with acetylation of H3 [64,65]. In a recent *in vitro* study using microplate-scanning FRET (Fluorescence Resonance Energy Transfer) analysis, Gansen et al. [66] showed that acetylation of histone H3 promoted nucleosome opening and disassembly, whereas acetylation of H4 alone increased unwrapping of the DNA ends, but did not enhance disassembly. The same study also revealed that H4 acetylation significantly counteracted H3 acetylation in nucleosome disassembly. Therefore, it would not be surprising if histone H3 and H4 differ from each other in response to dietary butyrate.

Among all core histones, H2A has the largest number of variants. In mammalian Jurkat cells, at least thirteen H2A variants were identified [67]. According to Brower-Toland et al., [68], and Ishibashi et al., [69] acetylation of H2A is involved in conformational changes of nucleosomes, which influence some strong, specific, and key histone-DNA interactions. In contrast, Gansen et al. [66] suggested that acetylation of H2A and H2B histones did not influence nucleosome stability, but could instead affect the nucleosome entry-exit region. However, multiple studies revealed that butyrate caused hyperacetylation of H2A both *in vivo*

[7] and in cell culture [57,69,70]. Unfortunately, we could not verify in sea bass whether butyrate induced H2A hyperacetylation since the antibody we used did not work in our species. However, we found that dietary butyrate caused a significant decrease in the total amount of H2A histone in European sea bass hepatocytes.

Concerning gene transcript abundance analysis, this study clearly showed tissue-dependent differences in the expression of five target genes involved in epigenetic regulatory mechanisms [71]; the expression was in general, higher in the liver than in the intestine. As previously found in European sea bass reared in different temperatures [35], three of these genes (*dicer1*, *ehmt2*, and *hdac11*) exhibited increased expression in the liver as a consequence of butyrate treatment, suggesting that these genes are involved in physiological processes in charge of coping with external insults.

The Dicer1 family is known to participate in the innate immune response to pathogens, mainly in RNA silencing-based antiviral immunity [72,73]. Indeed, studies in the past twenty years have established a completely new RNA-based immune system against viruses that is mechanistically related to RNA silencing or RNA interference. This viral immunity begins with recognition of viral double-stranded or structured RNA by the Dicer nuclease family of host immune receptors, also known as pattern recognition receptors (PRRs). The double-stranded (dsRNA)-specific Dicer nucleases represent a distinct family of PRRs. Upon viral infection, one domain of PRRs interacts directly with microbial signatures shared by major classes of microbes, whereas the second protein-protein interaction domain activates the downstream signaling events, leading to transcription of immunity effector genes with broad-spectrum anti-microbial activities [72]. Moreover, *dicer1* knockdown experiments showed an increase in the interferon response against pathogens [73]. Although our results showed a slightly increase in the expression of *irf1*, a higher expression of *dicer1* was also observed in the liver in the intestine, suggesting that in butyrate-treated fish dicer 1 was inhibiting an interferon response

against the external insult.

The higher expression of *ehmt2* found in both tissues due to butyrate treatment could probably be related to the histone H3 dimethylation of lysine residue 10, as this is the expected effect of this enzyme. As demonstrated previously, this creates an epigenetic mark on nucleosomes associated to the *il6* promoter that may repress its expression and alter the *il6* signaling pathway [74]. A similar effect is possible in our experiment with butyrate treatment since *il6* expression was downregulated in both the intestine and liver.

Finally, *hdac11* has also been related to the immune system by downregulating the expression of *il10* in antigen-presenting cells [75]. Overexpression of *hdac11* is thought to inhibit *il10* expression and activate T-cell responses. Our results in intestine showed a decrease in *hdac11* expression and a slight increase in *il10* levels. This suggests that, in butyrate-treated fish, antigen-specific T-cell responses could be impaired, which probably activates immune tolerance. This situation is known to prevent self-tissue damage [76] and the scenario fits nicely with the known anti-inflammatory effect of butyrate in the fish that received the supplemented diet.

Conclusions

Results of the 8-week-long feeding trial showed no significant differences in weight gain and SGR (specific growth rate) of sea bass that received 2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate.

Butyrate in the feed significantly increased the acetylation state of histone H4 at lysine 8, leading to a threefold increase in comparison to the control group, but no changes were found in the acetylation of histone H3 at Lys9. Interestingly, for histone H3 two different isoforms were separated on the immunoblots, which could correspond to H3.1 and H3.2 isoforms

previously found in terrestrial animals.

Concerning gene expression, butyrate applied as a nutritional supplement caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1*. Statistical analysis by 2-way ANOVA for these genes showed significant differences due to the butyrate treatment (P=0.002) and to the interaction between tissue and treatment (P=0.010). The expression of four (*il1β*, *il8*, *irf1,* and *tnfa*) out of seven target genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only for the *il10* gene were differences observed in the expression (P=0.003) due to the butyrate treatment.

Authors contribution

Conceived and designed the experiments: GT, MS, FP. Performed the experiments: SR, ND, CC. Analyzed the data: SR, ND. Contributed reagents/materials/analysis tools: FP, GT, MS. Wrote the paper: GT, SR.

References

1. Bergman FN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990; 70:567–590.
2. Cummings JH, Macfarlane GT. The Control and Consequences of Bacterial Fermentation in the Human Colon. Journal of Applied Bacteriology. 1991; 70(6):443–459.
3. Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. From

548 the gut to the peripheral tissues: the multiple effects of butyrate. Nutr Res Rev. 2010;
549 23:366–384.
550 4. Canani RB, Di Costanzo M, Leone L. The epigenetic effects of butyrate: potential
551 therapeutic implications for clinical practice. Clinical Epigenetics. 2012; 4(1):4.
552 doi:10.1186/1868-7083-4-4.
553 5. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing
554 bacteria from the human large intestine. FEMS Microbiol Lett. 2009; 294:1-8.
555 6. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. The
556 Proceedings of the Nutrition Society. 2003; 62(1):67–72.
557 7. Mátis G, Neogrády Z, Csikó G, Kulcsár A, Kenéz Á, Huber K. Effects of orally applied
558 butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver
559 of chicken – a randomized controlled trial. Nutrition & Metabolism. 2013; 10:12.
560 8. Gálfi P, Neogrády S. The pH-dependent inhibitory action of n-butyrate on
561 gastrointestinal epithelial cell division. Food Res Int. 2002; 34:581–586.
562 9. Wong JMWRD, de Souza RRD, Kendall CWC, Emam A, Jenkins DJA. Colonic
563 Health: Fermentation and Short Chain Fatty Acids. Journal of Clinical
564 Gastroenterology. 2006; 40(3):235–243.
565 10. Vinolo MA, Hatanaka E, Lambertucci RH, Curi R. Effects of short chain fatty acids on
566 effector mechanisms of neutrophils. Cell Biochem Funct. 2009; 27(1):48–55.
567 11. Toden S, Bird AR, Topping DL, Conlon MA. Dose-dependent reduction of dietary
568 protein-induced colonocyte DNA damage by resistant starch in rats correlates more
569 highly with caecal butyrate than with other short chain fatty acids. Cancer Biol Ther.
570 2007; 6(2):e1–e6.
571 12. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review
572 article: the role of butyrate on colonic function. Alimentary Pharmacology &

573 Therapeutics (Aliment Pharmacol Ther). 2008; 27:104–119.
574 13. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential
575 beneficial effects of butyrate in intestinal and extraintestinal diseases. World J
576 Gastroenterol. 2011; 17:1519–28.
577 14. Biancotto C, Frigè G, Minucci S. Histone modification therapy of cancer. Adv Genet.
578 2010; 70:341–386.
579 15. Scollo G, Terova G, Rimoldi S, Bernardini G, Antonini M, Saroglia M. Does butyrate
580 have a role in the protection of fish intestine? Results from a preliminary study on
581 European sea bass (*D. labrax*) Aquaculture Europe. Book of abstracts. Prague. 2012.
582 16. Robles R, Lozano AB, Sevilla A, Márquez L, Nuez-Ortín W, Moyano FJ. Effect of
583 partially protected butyrate used as feed additive on growth and intestinal metabolism
584 in sea bream (*Sparus aurata*). Fish Physiol. Biochem. 2013; 39: 1567–1580.
585 17. Björkholm B, Bok CM, Lundin A, Rafter J, Hibberd ML, Pettersson S. Intestinal
586 microbiota regulate xenobiotic metabolism in the liver. PLoS ONE. 2009; 4:e6958.
587 18. Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, Dejong
588 CH. Short chain fatty acids exchange across the gut and liver in humans measured at
589 surgery. Clin Nutr. 2009; 28:657–661.
590 19. Demigné C, Yacoub C, Rémésy C. Effects of absorption of large amounts of volatile
591 fatty acids on rat liver metabolism. J Nutr. 1986; 116:77–86.
592 20. Beauvieux MC, Tissier P, Gin H, Canioni P, Gallis JL. Butyrate impairs energy
593 metabolism in isolated perfused liver of fed rats. J Nutr. 2001; 131:1986–1992.
594 21. Gallis JL, Tissier P, Gin H, Beauvieux MC. Decrease in oxidative phosphorylation yield
595 in presence of butyrate in perfused liver isolated from fed rats. BMC Physiol. 2007; 7:8.
596 22. Gallis JL, Gin H, Roumes H, Beauvieux MC. A metabolic link between mitochondrial
597 ATP synthesis and liver glycogen metabolism: NMR study in rats re-fed with butyrate

598 and/or glucose. Nutr Metab. 2011; 8:38.
599 23. Evans JA. The effects of diet and feeding frequency on peripheral nutrient supply and
600 growth traits of the lamb. PhD Thesis. University of Nottingham. 1998.
601 http://eprints.nottingham.ac.uk/14420/.
602 24. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of
603 resistant starch and non-starch polysaccharides. Physiol Rev. 2001; 81: 1031–64.
604 25. Miller SJ. Cellular and physiological effects of short-chain fatty acids. Mini Rev Med
605 Chem. 2004; 4(8):839–845.
606 26. Gálfi P, Bokori J. Feeding trial in pigs with a diet containing sodium n-butyrate. Acta
607 Vet Hung. 1990; 38(1–2):3–17.
608 27. Kotunia A, Wolinski J, Laubitz D, Jurkowska M, Rome V, Guilloteau P, Zabielski R.
609 Effect of sodium butyrate on the small intestine development in neonatal piglets fed
610 correction of feed by artificial sow. J Physiol Pharmacol. 2004; 55 Suppl 2:59–68.
611 28. Hu Z, Guo Y. Effects of dietary sodium butyrate supplementation on the intestinal
612 morphological structure, absorptive function and gut flora in chickens. Anim Feed Sci
613 Tech. 2007; 132:240–249.
614 29. Zheng, RG. The effect of sodium butyrate on the growth performance and intestinal
615 mucous structure of fresh water fish. PhD thesis (2009). Available:
616 http://www.globethesis.com/?t=2143360272979206.
617 30. Bjerkeng B, Storebakken T, Wathne E. Cholesterol and short-chain fatty acids in diets
618 for Atlantic salmon Salmo salar (L.): effects on growth, organ indices, macronutrient
619 digestibility and fatty acid composition. Aquaculture Nutrition. 1999; 5:181–191.
620 31. Gao Y, Storebakken T, Shearer KD, Penn M, Øverland M. Supplementation of fishmeal
621 and plant protein-based diets for rainbow trout with a mixture of sodium formate and
622 butyrate. Aquaculture. 2011; 311: 233–240.

623 32. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies:
624 integrating mammalian biology. Cell. 2001; 104(4):487–501.
625 33. Brien JD, Daffis S, Lazear HM, Cho H, Suthar MS, Gale M Jr, et al. Interferon
626 Regulatory Factor-1 (IRF-1) Shapes Both Innate and CD8+ T Cell Immune Responses
627 against West Nile Virus Infection. PLoS Pathog. 2011; 7(9): e1002230.
628 34. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin.
629 Int J Biochem Cell Biol. July 1998; 30(7):797–801.
630 35. Díaz N, Piferrer F. Lasting effects of early exposure to temperature on the gonadal
631 transcriptome at the time of sex differentiation in the European sea bass, a fish with
632 mixed genetic and environmental sex determination. BMC Genomics. 2015 Sep
633 4;16(1):679.
634 36. Jaskiewicz L, Filipowicz W: Role of Dicer in posttranscriptional RNA silencing.
635 Current Topics in Microbiology and Immunology. 2008; 320:77–97.
636 37. Nishio H, Walsh MJ. CCAAT displacement protein/cut homolog recruits G9a histone
637 lysine methyltransferase to repress transcription. PNAS. 2004; 101:11257–11262.
638 38. Duan Z, Zarebski A, Montoya-Durango D, Grimes HL, Horwitz M. Gfi1 coordinates
639 epigenetic repression of p21Cip/WAF1 by recruitment of histone lysine
640 methyltransferase G9a and histone deacetylase 1. Molecular and Cellular Biology.
641 2005; 25: 10338–10351.
642 39. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a polycomb group protein, regulates cell
643 proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc
644 oncoproteins. Molecular Biology of the Cell. 2007; 18:536–546.
645 40. Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of
646 HDAC11, a novel member of the human histone deacetylase family. The Journal of
647 Biological Chemistry. 2002; 277:25748–25755.

41. Kim TG, Kraus JC, Chen J, Lee Y. JUMONJI, a critical factor for cardiac development, functions as a transcriptional repressor. The Journal of Biological Chemistry. 2003; 278: 42247–42255.
42. Tine M, Kuhl H, Gagnaire P-A, Louro B, Desmarais E, Martins RS, et al. European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. Nature Commun. 2014; 5:5770. doi:10.1038/ncomms6770.
43. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature Protocols. 2008; 3(6):1101–1108.
44. Øverland M, Granli T, Kjos NP, Fjetland O, Steien SH, Stokstad M. Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing–finishing pigs. Journal of Animal Science. 2000; 78:1875–1884.
45. Øverland M, Kjos NP, Borg M, Skjerve E, Sørum H. Organic acids in diets for entire male pigs: effect on skatole level, microbiota in digesta, and growth performance. Livestock Science. 2008; 115:169–178.
46. Lückstädt C. The use of acidifiers in fish nutrition. CAB Reviews: Perspectives in Agriculture Veterinary Science. Nutr and Nat Resour. 2008; 3(044):1–8.
47. De Wet L. Organic acids as performance enhancers. Aqua Feeds: Formulation and Beyond. 2005; 2:12–14.
48. Pandey A, Satoh S. Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. Fisheries Science. 2008; 74:867–874.
49. Vielma J, Ruohonen K, Lall SP. Supplemental citric acid and particle size of fish bone-meal influence the availability of minerals in rainbow trout *Onchorynchus mykiss* (Walbaum). Aquaculture Nutrition. 1999; 5:65–71.
50. Zhou Z, Liu Y, He S, Shi P, Gao X, Yao B, Ringø E. Effects of dietary potassium

673 diformate (KDF) on growth performance, feed conversion and intestinal bacterial
674 community of hybrid tilapia (*Oreochromis niloticus*×*O. aureus*). Aquaculture. 2009;
675 291:89–94.
676 51. Gislason G, Olsen RE, RingØ E. Lack of growth-stimulating effect of lactate on
677 Atlantic salmon, *Salmo salar* L. Aquaculture and Fisheries Management. 1994; 25:861–
678 862.
679 52. Owen MAG, Waines P, Bradley G, Davies S. The effect of dietary supplementation of
680 sodium butyrate on the growth and microflora of *Clarias gariepinus* (Burchell, 1822).
681 XII international symposium fish nutrition and feeding. 2006 May 28–June 1; Book of
682 abstracts: pp 149.
683 53. Liu W, Yang Y, Zhang J, Gatlin DM, Ringø E, Zhou Z. Effects of dietary
684 microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune
685 response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with
686 or without oxidized oil. Br J Nutr. 2014; 112:15–29.
687 54. Dokmanovic M, Marks PA. Prospects: histone deacetylase inhibitors. J Cell Biochem.
688 2005; 96:293–304.
689 55. Waterborg JH. Dynamics of histone acetylation *in vivo*. A function for acetylation
690 turnover? Biochim Cell Biol. 2002; 80:363–78.
691 56. Candido EPM, Reeves R, Davie JR. Sodium butyrate inhibits histone deacetylation in
692 cultured cells. Cell. 1978; 14:105–113.
693 57. Mohana KB, Song HJ, Cho SK, Balasubramanian S, Choe SY, Rho GJ. Effect of
694 histone acetylation modification with sodium butyrate, a histone deacetylase inhibitor,
695 on cell cycle, apoptosis, ploidy and gene expression in porcine fetal fibroblasts. Journal
696 of Reproduction and Development. 2007; 53:903–913.
697 58. Koprinarova M, Markovska P, Iliev I, Anachkova B, Russev G. Sodium butyrate

enhances the cytotoxic effect of cisplatin by abrogating the cisplatin imposed cell cycle arrest. BMC Molecular Biology. 2010; 11:49.

59. Paskova L, Trtkova KS, Fialova B, Benedikova A, Langova K, Kolar Z. Different effect of sodium butyrate on cancer and normal prostate cells. Toxicology in Vitro. 2013; 27:1489–1495.
60. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD, Newgard CB, Farese RV Jr, de Cabo R, Ulrich S, Akassoglou K, Verdin E. Suppression of oxidative stress by *b*-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science. 2013; 339: 211.
61. Gundersen BB, Blendy JA. Effects of the histone deacetylase inhibitor sodium butyrate in models of depression and anxiety. Neuropharmacology. 2009; 57:67–74.
62. Kien CL, Peltier CP, Mandal S, Davie JR, Blauwiekel R. Effects of the *in vivo* supply of butyrate on histone acetylation of cecum in piglets. JPEN-Parenter Enter Nutr. 2008; 32:366–384.
63. Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J, Bazett-Jones DP, Allis CD, Hunt DF. Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem. 2006; 281:559–568.
64. Kurdistani SK, Tavazoie S, Grunstein M. Mapping global histone acetylation patterns to gene expression. Cell. 2004; 117:721–733.
65. Agricola E, Verdone L, Di Mauro E, Caserta M. H4 acetylation does not replace H3 acetylation in chromatin remodeling and transcription activation of Adr1-dependent genes. Mol Microbiol. 2006; 62:1433–1446.
66. Gansen A, Tóth K, Schwarz N, Langowski J. Opposing roles of H3- and H4-acetylation in the regulation of nucleasome structure-a FRET study. Nucleic Acid Research. 2015; 43(3):1433–1443.

67. Bonenfant D, Coulot M, Towbin H, Schindler P, van Oostrum J. Characterization of histone H2A and H2B variants and their post-translational modifications by mass spectrometry. Mol Cell Proteomics. 2006; 5:541–552.
68. Brower-Toland B, Wacker DA, Fulbright RM, Lis JT, Kraus WL, Wang MD. Specific contributions of histone tails and their acetylation to the mechanical stability of nucleosomes. J Mol Biol. 2005; 346:135–146.
69. Ishibashi T, Dryhurst D, Rose KL, Shabanowitz J, Hunt DF, Ausió J. Acetylation of vertebrate H2A.Z and its effects on the structure of nucleosome. Biochemistry. 2009; 48: 5007–5017.
70. Tobisawa Y, Imai Y, Fukuda M, Kawashima H. Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. J Biol Chem. 2010; 285:6750–6760.
71. Piferrer F. Epigenetics of sex determination and gonadogenesis. Developmental Dynamics. 2013; 242:360–370.
72. Aliyari R, Ding SW. RNA-based viral immunity initiated by the Dicer family of host immune receptors. Immunology Review. 2009; 227(1):176–188.
73. Chiappinelli KB, Haynes BC, Brent MR, Goodfellow PJ. Reduced DICER1 elicits an interferon response in endometrial cancer cells. Molecular Cancer Research. 2012; 10: 316–325.
74. Takahashi H, Katayama K, Sohya K, Miyamoto H, Prasad T, Matsumoto Y, Ota M, Yasuda H, Tsumoto T, Aruga J, Craig AM. Selective control of inhibitory synapse development by Slitrk3-PTPd trans-synaptic interaction. Nature Neurosci. 2012; 15: 389–400.
75. Villagra A, Cheng F, Wang HW, Suarez I, Glozak M, Maurin M, Nguyen D, Wright KL, Atadja PW, Bhalla K, Pinilla-Ibarz J Seto E, Sotomayor EM. The histone deacetylase

HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nature Immunology. 2009; 10(1): 92–100.

76. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson Jr WR, Muller W, Rudensky AY. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008; 28:546–558.

773 **Figure legends**

774

775 **Figure 1. Effects of dietary butyrate on European sea bass growth.** The data were tested by
776 ANOVA followed by Tukey's HSD test to determine whether there were any significant
777 differences between different groups. Different letters indicate significant differences ($P < 0.05$).
778 Data as mean ± SEM.

779

780 **Figure 2. Effects of butyrate on the acetylation state in European sea bass histones from**
781 **isolated hepatocytes.** One–dimensional immune-blotting analysis of histones H2A and H3 as
782 well as H3, H4 acetylated histones are shown. For histone H3, the upper band represents the
783 H3.1 isoform and the lower band the H3.2 isoform. Ponceau staining was used as loading
784 control.

785

786 **Figure 3. Effects of dietary butyrate on gene expression in two tissues of the European**
787 **sea bass: liver and intestine, as determined by qRT-PCR analysis.** Only those genes that
788 showed statistical differences for the interaction between tissue and treatment (A: *dicer1*, B:
789 *ehmt2* and C: *hdac11*), or differences in expression solely due to the treatment (D: *il10*) are
790 depicted.

791

792

793

794

Supporting information

S1 Table. Quantitative real time PCR primer characteristics

Figure 1

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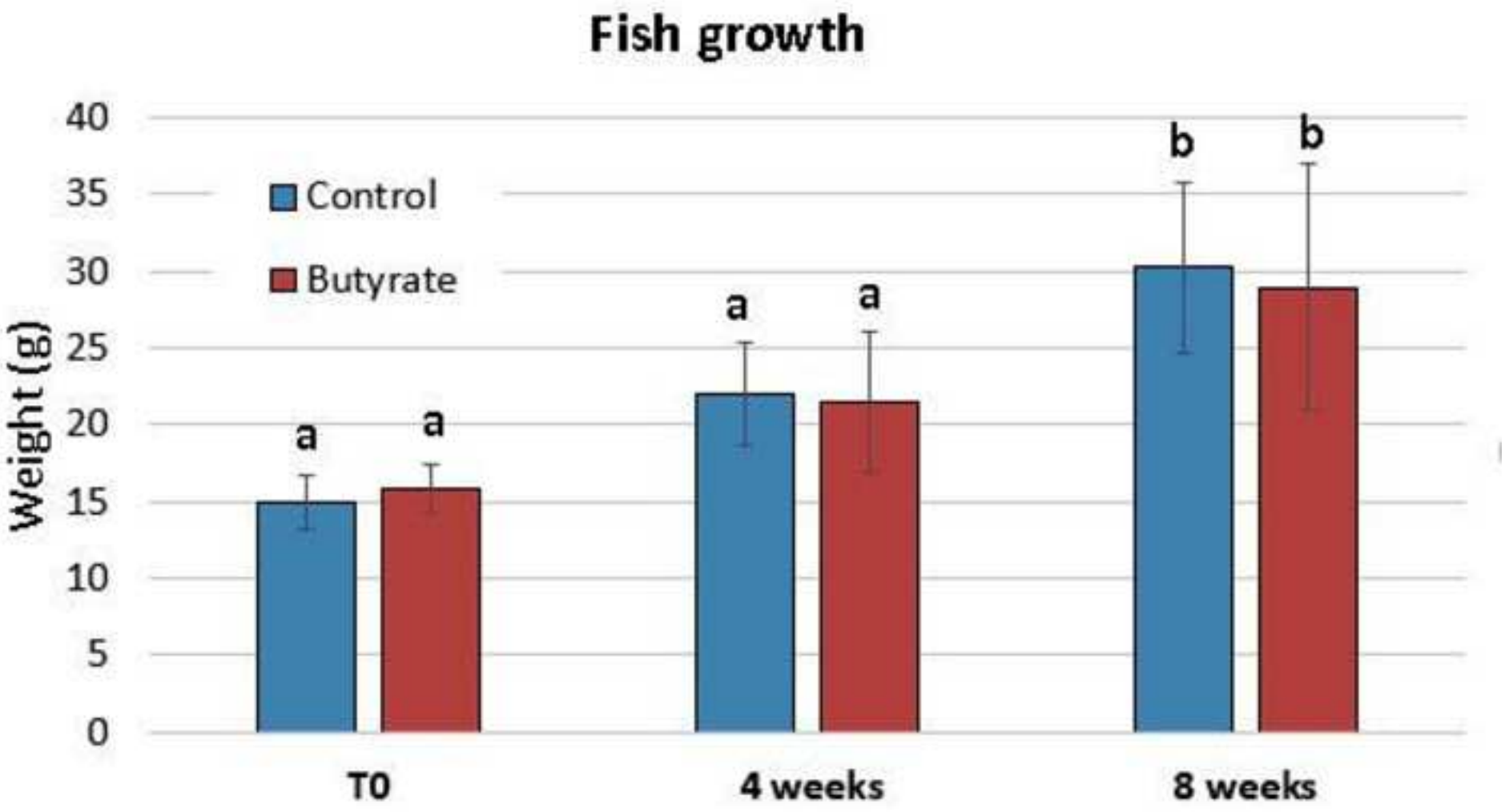

Figure 2

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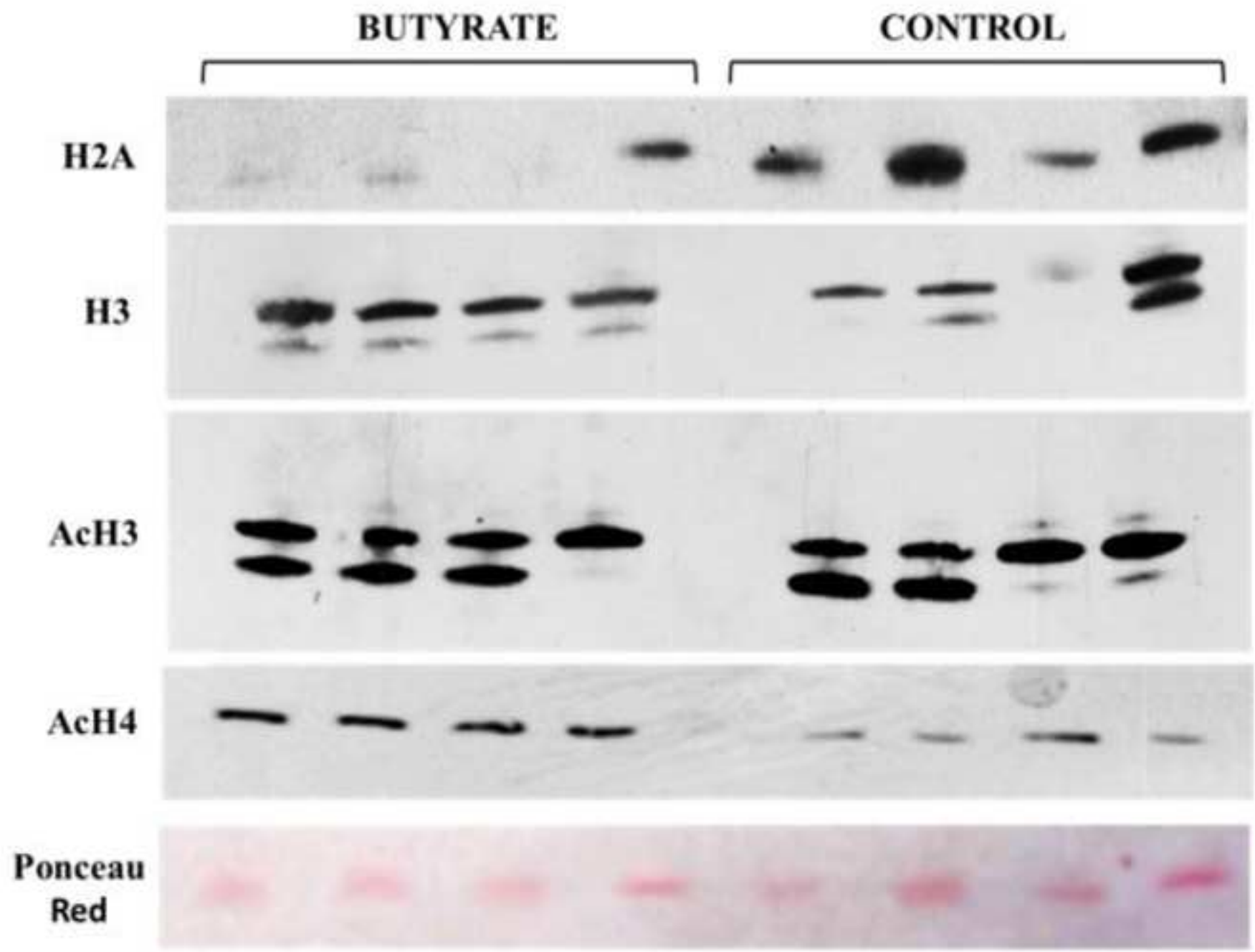

Figure 3.1

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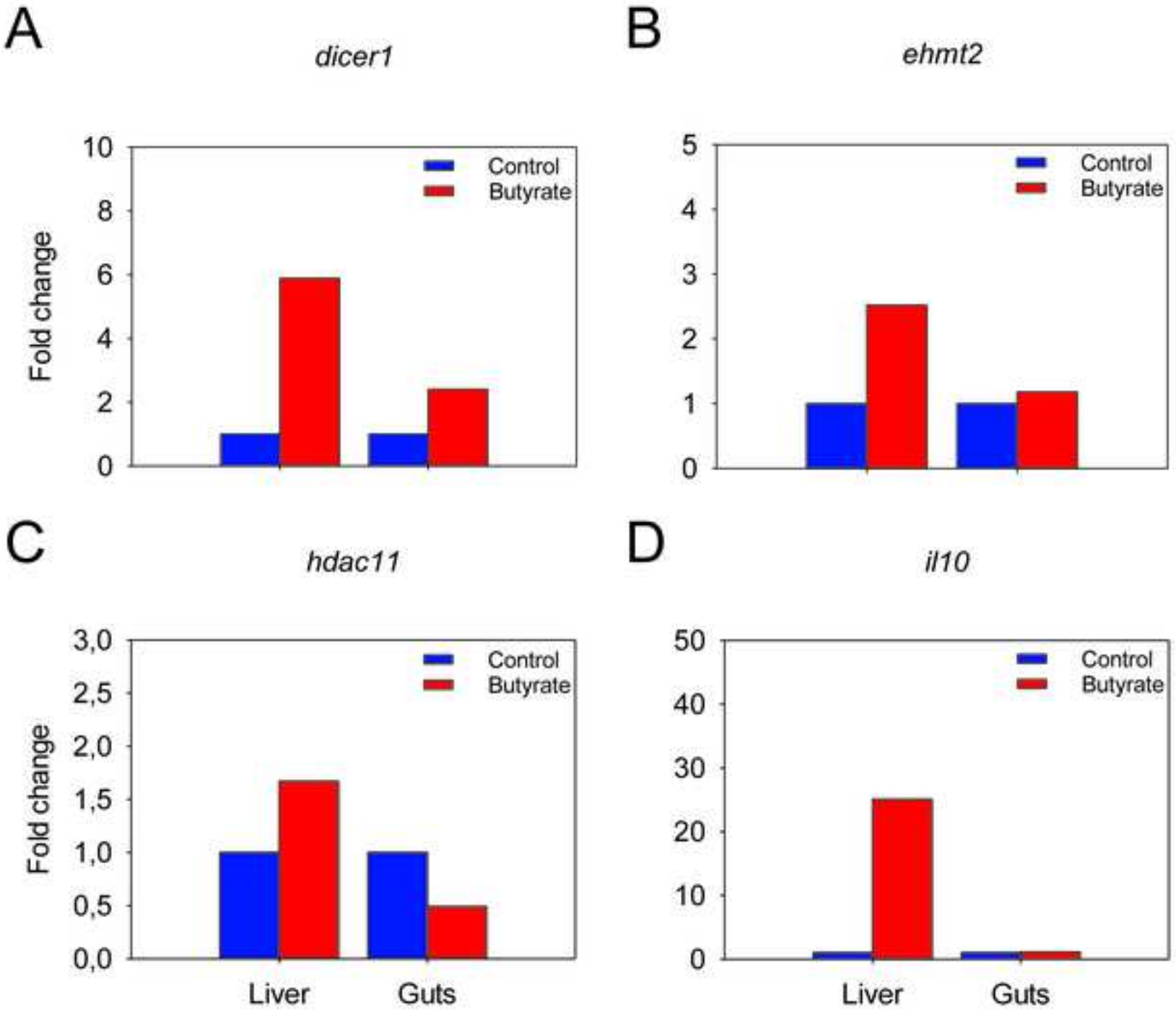

Figure 3.2

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