Background

Early weaning (EW) is a widespread practice in modern settings of pig production. At that time, piglets are exposed to a variety of stressors including abrupt separation from sow and changes in diet and environment, which jointly result in a period of transient anorexia, gut mucosal atrophy, and intestinal dysfunction [1–3]. The weaning-induced deterioration of gut integrity could be partly related to the marked reduction in circulating glucagon-like peptide (GLP)-2 that typically accompanies EW in pigs [4, 5].

GLP-2 is an intestinotrophic peptide released by the enteroendocrine L cells mainly in response to luminal nutrients [6, 7]. Of interest, exogenous GLP-2 restores mucosal growth, transcellular transport, and the expression of tight junction (TJ) proteins that control paracellular permeability in a number of animal models of intestinal atrophy or dysfunction [8–10]. Recently, it has been found that the chronic administration of GLP-2 at supraphysiological levels to neonatal pigs for 42 days increased villus height and crypt depth in the small intestine and colon 21 days after EW [11]. More important, the administration of a long-acting analog of GLP-2 at pharmacological doses to 25-days-old suckling piglets increased intestinal weight and enzyme activity 5 days after weaning [12]. Although available evidence suggests that GLP-2 treatment can contribute to improve intestinal adaptation to weaning, it is reasonable to expect that strategies capable of enhancing secretion and (or) stability of endogenous GLP-2 might be equally effective.
but easier to implement under commercial schemes of pig production.

In recent years bile acids have emerged as potent hormonal regulators capable of stimulating the secretion of GLP-1 (a co-product of proglucagon, released in parallel with GLP-2) from the intestine. This action is mediated by the G-protein-coupled bile acid receptor 1 (GPBAR1, also known as TGR5), which is a bile acid sensor expressed on the luminal surface of intestinal L cells [13, 14]. Interestingly, the continuous enteral administration of chenodeoxycholic acid (CDC), a primary bile acid known to activate TGR5, to newborn piglets fed parenterally increased the plasma concentration of GLP-2 and prevented gut atrophy otherwise resulting from the lack of enteral nutrition [15]. In a later study, we investigated whether CDC could induce a similar response in weaning pigs. In this study, piglets weaned at 21 days of age, fed a cereal-based diet, and infused intragastrically with a single dose of CDC had increased circulating GLP-2 and tended to have a longer and heavier intestine than their control counterparts [16]. As proposed in that report, it is plausible that the dose of CDC and administration procedure used in our study might have limited the impact of increased GLP-2 secretion on intestinal adaptation to EW. It is important to note, however, that bile acids may also control the integrity of the intestinal barrier by regulating the expression or cellular distribution of TJ proteins through mechanisms unrelated to GLP-2 [17, 18].

In summary, available evidence indicates that activating intestinal signaling pathways controlled by bile acids allows stimulating the release of endogenous GLP-2 and thereby improving gut integrity in experimental models of intestinal atrophy and dysfunction. Therefore, the aim of this study was to expand previous work to establish whether the inclusion of CDC in the diet of early-weaned piglets fed according to current standards of pig production can improve gut integrity and animal performance immediately after weaning.

**Methods**

**Animals and housing**

All experimental procedures were approved by the Laboratory Animal Care Advisory Committee of the Faculty of Veterinary Sciences of the Universitat Autònoma de Barcelona, Spain. A total of 36 pigs (Large White x Landrace x Pietrain; 18 of each sex) weaned at 20 ± 0.9 days of age and 6.2 ± 0.34 kg of body weight (BW) were used in a study conducted at the Swine Experimental Unit of Lucta S.A. (Girona, Spain). At arrival, piglets were distributed into 36 individual pens (0.35 m²/pen) thoroughly cleaned and equipped with fully-slatted plastic floor plus a nipple drinker and a feeder. Animals were randomly assigned to receive a standard prestarter diet (CONd; n = 18; 50:50 male to female ratio) or the same diet supplemented with 60 mg of CDC (Sigma-Aldrich) per kg of initial BW (CDCd). Animals were fed the solid diets from weaning until day 14; thereafter, all pigs were fed the same (untreated) starter diet for 21 days until the end of experiment on day 35 (Table 1). During the study, all pigs had *ad libitum* access to feed and water. Starting at weaning BW was measured weekly, whereas feed intake was recorded daily until day 13 and weekly from day 15 to 35.

**Table 1** Composition of the prestarter and the starter diets, % as fed basis, unless otherwise indicated

| Ingredient                  | Prestarter | Starter |
|-----------------------------|------------|---------|
| Corn                        | 34.9       | 35.0    |
| Wheat                       | 11.0       | 22.6    |
| Barley                      | 8.0        | 9.5     |
| Extruded soybeans           | 14.6       | 5.0     |
| Soybean meal (56 % CP)      | 4.2        | -       |
| Soybean meal (47 % CP)      | -          | 13.7    |
| Sweet milk whey powder      | 12.6       | 2.94    |
| Fishmeal                    | 7.0        | 5.0     |
| Soybean oil                 | 4.0        | -       |
| Lard                        | -          | 2.06    |
| Trace elements and vitamin premix | 1.55 | 1.52 |
| Calcium carbonate           | 0.82       | 0.50    |
| Monocalcium phosphate       | 0.64       | 1.40    |
| Salt                        | -          | 0.34    |
| L-Lysine-HCl                | 0.36       | 0.32    |
| DL-Methionine               | 0.16       | 0.05    |
| L-Theonine                  | 0.13       | 0.04    |
| L-Tryptophan                | 0.04       | 0.03    |

**Calculated nutrient composition**

|                        | Prestarter | Starter |
|------------------------|------------|---------|
| Crude protein          | 19.4       | 18.7    |
| Digestible amino acids$^2$ |            |         |
| Lysine                 | 1.24       | 1.14    |
| Methionine             | 0.49       | 0.37    |
| Methionine + cysteine  | 0.72       | 0.62    |
| Threonine              | 0.76       | 0.64    |
| Tryptophan             | 0.23       | 0.22    |
| Digestible energy (MJ/kg) | 14.9  | 14.4    |
| Net energy (MJ/kg)     | 11.2       | 10.3    |

$^1$Containing the following: vitamin A, 10,000 UI; vitamin D$_3$, 2000 UI; vitamin E (alfa-tocopherol), 25 mg; vitamin B$_6$, 1.5 mg; vitamin B$_3$, 3.5 mg; vitamin B$_4$, 2.4 mg; vitamin B$_12$, 20 μg; vitamin K$_1$, 1.5 mg; calcium panthotenate, 14 mg; nicotinic acid, 20 mg; folic acid, 0.5 mg; biotin, 50 μg; iron sulfate, 120 mg; calcium iodate, 0.75 mg; cobalt carbonate, 0.6 mg; copper sulfate, 150 mg; manganese oxide, 60 mg; zinc oxide, 110 mg; sodium selenite, 0.37 mg; amoxicillin trihydrate 10 %, 300 mg; colistin sulphate 4 %, 80 mg; zinc oxide, 2610 mg

$^2$Ileal standardized digestibility
Plasma collection and analysis
Blood samples were obtained from six randomly-chosen pigs per treatment via jugular venipuncture on day 1, 7, and 14 after 12 h of feed deprivation. Samples were collected into tubes containing EDTA and aprotinin (BD Vacutainer®), held in ice-cold water for 30 min, centrifuged at 2000 × g for 10 min, stored at −80 °C, and analyzed later on for bioactive GLP-2 by radioimmunoassay as described previously [19].

Tissue collection
On day 15 after 3 h of feed deprivation, 6 pigs per treatment were euthanized with an intravenous injection of sodium pentobarbital (200 mg per kg of BW; Faro Ibérica, Spain). The abdomen was opened and the intestines were removed and dissected into sections arbitrary designated as jejunum (from the pyloric sphincter to the first Peyer’s patch), ileum (from the first Peyer’s patch to the ileocecal valve) and large intestine (from the ileocecal valve to the rectum). Intestinal sections were measured, flushed with saline, and weighted. A 10-cm segment was removed from the midsection of the jejunum and ileum, divided into 5-cm halves, and opened longitudinally. Half of these samples were fixed in 10 % buffered formalin for subsequent histological examination, whereas mucosal scrapings were taken from the other half and stored in RNA later® (Ambion, USA) at −80 °C until analysis of gene expression.

Morphometric analysis
Samples of jejunum and ileum were dehydrated and embedded in paraffin, sectioned (~4 μm), and stained with hematoxylin and eosin. Villus height, crypt depth, number of intraepithelial lymphocytes in villi, and number of goblet cells in crypts were measured in 10 well-oriented villi and crypts using a light microscope (BHS, Olympus) and a linear ocular micrometer (Olympus, Microplanet). All determinations were done by the same person, who was blinded to treatments, at 400× magnification as described previously [20].

Real-time RT-qPCR analysis
Total RNA from intestinal mucosal scrapings was extracted and first strand cDNA synthesized as previously described [16]. Approximately 2 μg of RNA with an average A260/A280 of 1.9 were retrotranscribed. Primers and optimal PCR conditions for porcine interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), glucagon-like peptide-2 receptor (GLP-2R), proglucagon (GCG), sodium-dependent bile acid transporter (ASBT), tata box-binding protein (TBP), beta actin (ACTB) [16], zonula occludens-1 (ZON-1), occludin (OCLN) [21] epidermal growth factor receptor (EGFR) [22] and interleukin-6 (IL-6) [23] were taken from literature. These genes were previously shown to play a role in mediating the effects of bile acids on gut mucosal inflammation and barrier function [16–18]. Gene expression was determined in the jejunum and ileum, except for EGFR and IL-6 that were examined only in ileal samples. All samples were run in triplicate in an ABI Prism 7300 Sequence Detector System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) and specific primers for each gene, as previously described [16].

Statistical analysis
Analyses were performed using the mixed-model procedure of SAS (release 9.2, SAS Institute Inc.). Performance data (BW, average daily gain, feed intake and feed conversion) for animals that were slaughtered on day 15 (n = 6) and those that completed the study on d 35 (n = 12) were analyzed separately. These results and GLP-2 data were analyzed using a mixed-effect model with repeated measures in which pig within treatment was used as random variable whereas treatment, time (day or week), and the interaction treatment by time were considered fixed. The smallest value for the Akaike’s information criterion was used to identify the most appropriate covariance structure. The same model but without repeated measures was used to analyze intestinal weight, length, and morphology. To achieve normality, data for GLP-2 were transformed prior to analysis. Least squares means were separated into significant effects using the Fisher adjustment option of SAS. Differences in gene expression resulting from the comparison of the CDCd group with the CONd group were determined using a linear mixed-model in which treatment was included as fixed effect and the sample as random [24]. Gene specific residual variance (heterogeneous residual) was fitted to the gene effect [25]. For genes displaying efficiencies different from 2 (E ≠ 2), Ct values were adjusted according to the model described by Stein et al. [24]. The geometric mean of the reference genes TBP and ACTB was used to correct Ct values of target genes [26]. Differences among treatments were considered to be significant when P < 0.05, whereas when P > 0.05 but < 0.10 differences were considered to indicate a trend towards a significant effect.

Results
Animal performance
The onset of feed consumption after weaning and its time course during both the first 13 days of exposure to treatments (Fig. 1) and the 5 weeks of study (Fig. 2) were similar between treatment groups. Likewise, supplementing the CONd prestarter diet with CDC did not alter weight gain of piglets that ended the study either on days 15 or 35 (Table 2). In both treatment groups the incidence of diarrhea was low and did not differ between
them (Table 2). In addition, animals exhibited normal behavior and signs of adverse treatment effects were not observed during the study.

**Plasma GLP-2**
Although mean plasma GLP-2 did not differ between treatments (Fig. 3A), feeding CDCd tended (P < 0.07) to increase the concentration of circulating GLP-2 by 39% on day 14 after 2 weeks of treatment exposure (Fig. 3B).

**Intestinal growth and morphology**
The inclusion of CDC in the prestarter diet tended to enhance the weight of the large intestine (P < 0.10) but did not modify the size (weight and length) of the small intestine (Table 3). In addition, the morphology of the mucosa from the jejunum and ileum was similar between treatments, with the exemption of the ileal crypts that were deeper (P < 0.04) in pigs fed CDC (Table 4).

**Intestinal gene expression**
The feeding of CDCd during the 14 days that followed EW did not modify the relative concentration of mRNA transcripts from genes examined in the jejunal mucosa (data not shown). Although expression of OCLN, GLP-2R, ASBT, EGFR and GCG was similar between groups (data not shown), the expression of ZON-1 (P < 0.02), TNF-a (P < 0.02), IL-10 (P < 0.006), and IL-6 (P < 0.01) increased 1.5, 1.9, 2.2 and 2.4 folds, respectively, in the ileum of CDCd-fed piglets relative to their CONd counterparts (Fig. 4).

**Discussion**
Feeding piglets a cereal-based diet supplemented with CDC for 2 weeks after EW induced genes involved in the barrier function and protection of the intestinal mucosa with marginal effects on the concentration of circulating GLP-2 and mass of the intestine. The enteroprotective action of CDC
was not associated with changes in food intake and BW gain either during the period of CDC supplementation or after withdrawal of CDC from the diet. The low incidence of diarrhea observed in both treatment groups suggests that keeping pigs in individual pens under high sanitary conditions might have hindered improvements in animal performance otherwise mediated by CDC. Taken together, data indicate that the oral administration of bile acids to weaned pigs has the potential to improve the protection of the intestinal mucosa independently of relevant changes in gut growth.

Weaning-induced intestinal atrophy and dysfunction are associated with a transient decrease in circulating GLP-2 [4, 5]. In a previous study, we have showed that the intragastric administration of a single dose of CDC to piglets during the first 6 days after EW remarkably increased the plasma concentration of endogenous GLP-2 but that this response tended to enhance only the length and weight of the ileum [16]. Thus, we speculated that supplementing the postweaning diet with CDC with the aim of distributing its enteral supply throughout the day may augment the nutrient-dependent secretion of GLP-2 and improve its efficacy to preserve gut integrity immediately after EW. We observed that plasma GLP-2 tended to increase at the end of the period of exposure to CDC on day 14, when food intake increased on average by 160 g/d (178 %) relative to the first postweaning week. Coincidentally, in our previous study piglets consumed during the first 6 days after EW about 66 % more feed (+48 g/d) than in the present study [16]. It seems therefore that there is a minimum of enteral nutrition required for CDC to potentiate the release of GLP-2 in animals fed solid diets. Although this effect was paralleled by a deepening of the ileal crypts, which is a distinctive trophic action of GLP-2 [5], only the mass of the

**Table 3** Intestinal weight and length of piglets fed CONd or CDCd prestarter diet

| Organ weight (g/kg BW) | Treatments | SEM | P > F |
|-----------------------|------------|-----|-------|
|                       | CONd | CDCd |       |       |
| Duodenum + jejunum    | 32.8  | 34.7 | 1.32  | 0.33  |
| Ileum                 | 8.0   | 7.9  | 0.30  | 0.80  |
| Small intestine       | 40.9  | 42.7 | 1.45  | 0.41  |
| Large intestine       | 16.1  | 17.9 | 0.71  | 0.10  |
| Whole intestine       | 57.0  | 60.5 | 1.64  | 0.16  |
| Organ length (cm/kg BW) |       |       |       |       |
| Duodenum + jejunum    | 100   | 102  | 4.8   | 0.83  |
| Ileum                 | 19.8  | 18.2 | 0.70  | 0.13  |
| Small intestine       | 12.0  | 12.0 | 4.9   | 0.06  |

*Data are least squares means, n = 6. CONd, prestarter diet; CDCd, prestarter diet supplemented with 60 mg chenodeoxycholic acid per kg of initial body weight

**Table 4** Intestinal morphology of piglets fed CONd or CDCd prestarter diet

| Jejunum | Treatments | SEM | P > F |
|---------|------------|-----|-------|
| Villus height (μm) | CONd | 454 | 419 | 23.8 | 0.31 |
| Crypt depth (μm)   | 186   | 181 | 5.6  | 0.59 |
| Villus:Crypt ratio | 2.5   | 2.3 | 0.14  | 0.48 |
| Intraepithelial lymphocytes (n/villus) | 30.7 | 29.2 | 1.77 | 0.55 |
| Goblet cells (n/villus) | 6.8  | 7.0  | 0.44 | 0.70 |

| Ileum | Treatments | SEM | P > F |
|-------|------------|-----|-------|
| Villus height (μm) | CONd | 403 | 410 | 21.6 | 0.82 |
| Crypt depth (μm)   | 133   | 153 | 6.0  | 0.04 |
| Villus:Crypt ratio | 3.1   | 2.7 | 0.16  | 0.13 |
| Intraepithelial lymphocytes (n/villus) | 27.7 | 30.3 | 1.60 | 0.27 |
| Goblet cells (n/villus) | 6.9  | 7.5  | 0.49 | 0.37 |

*Data are least squares means, n = 6. CONd, prestarter diet; CDCd, prestarter diet supplemented with 60 mg chenodeoxycholic acid per kg of initial body weight
intestine was marginally increased. Paradoxically, enteral administration of CDC prevented gut atrophy in newborn piglets fed via parenteral [15]. In line with our findings, however, recent studies also observed modest enlargements of the epithelium of the intestine of enterally-fed weaning pigs in response to prolonged treatment with exogenous GLP-2 [11, 12]. Collectively, data indicate that the intestine of pigs remains responsive to the trophic effect of bile acids after weaning, an action presumably mediated by GLP-2, but that the magnitude of this effect is rather small and likely irrelevant from a developmental standpoint. However, one cannot rule out that the deepening of crypts induced by CDC might accelerate the recovery of the intestinal mucosa function after weaning and contribute to maintain TJ and adequate enterocyte turnover.

A critical function of the intestinal epithelium is to form a dynamic physical barrier to luminal contents to protect the host from infection and chronic exposure to inflammatory stimuli. Adjacent mucosal cells accomplish this by interacting through TJ proteins that are connected to the actin cytoskeleton and regulate the intestinal paracellular permeability [27]. Importantly, mounting evidence links increased gut permeability with intestinal inflammation, systemic immune activation, and disease progression in humans and animals [28]. Because EW dysregulates intestinal permeability in pigs [3, 29], targeting TJ proteins may illuminate ways to maintain the integrity of the intestinal barrier and thereby improve piglet health, growth, and welfare during the weaning period. Based on this prediction and the notion that bile acids [17, 18] and GLP-2 [30] regulate the expression and or cellular distribution of TJ proteins, we decided to examine the impact of dietary supplementation with CDC on the expression of some genes involved in the control of the barrier function of the intestinal mucosa. We found that feeding CDCd resulted in proinflammatory (i.e., TNF-α and IL-6 expression) and anti-inflammatory (i.e., IL-10 expression) responses that were associated with increased concentration of ZON-1 transcripts in the epithelium of the distal small intestine. Considering that during the development of intestinal inflammation TNF-α disrupts TJ [31] whereas IL-10 antagonizes its action [32], it seems reasonable to suggest that CDC triggered a homeostatic immune response that ultimately appeared to enhance the integrity of TJ of the intestinal epithelium. The question as to whether these effects were mediated directly by CDC via activation of the bile acid sensors TGR5 [18] and farnesoid X-activated receptor (FXR) [17] or indirectly via enhanced released of GLP-2 [30] cannot be addressed with data reported herein. However, the observation that the anti-inflammatory action of GLP-2 involves suppression of both crypt-cell proliferation and inflammatory cytokines through a mechanism unrelated to Th2 cytokines such as IL-10 [33], suggests that the tolerogenic response triggered by CDC was not associated with GLP-2. Yet, it is more important to note that in a recent study with early-weaned pigs weaning disrupted intestinal permeability partly by repressing the expression of TJ proteins, including ZON-1, and that this effect lasted for 14 days postweaning albeit the morphology of the intestinal mucosa was fully recovered by then [34]. Therefore, our findings support the proposal that the oral administration to pigs of bile acids, or compounds that mimic their action, holds potential for enhancing the integrity of the mucosal barrier at weaning and beyond this critical time.

It is widely accepted that disorders caused by EW, including increased susceptibility to diarrhea and growth retardation, mainly result from the transient absence of feed consumption that follows EW [1]. Expectably, supplementing the postweaning diet with CDC comprehended the risk of aggravating EW-induced anorexia because of reduced diet acceptability and or enhanced satiety mediated centrally by GLP-2 [35]. We found, however, that feeding CDCd did not affect the onset of feed intake following EW nor the amount of feed consumed during the 5-weeks study. Likewise, feed intake of weaning pigs was not affected when the plasma concentration of GLP-2 was increased via the intragastric infusion of CDC [16] or the administration of exogenous GLP-2 at a supraphysiological dose [11]. Despite the absence of anorectic effects, the aforementioned entero-protective impact of feeding CDCd did not translate into
improved animal performance (i.e., BW gain and incidence of diarrhea). As suggested before, the high sanitary conditions under which this study was conducted might have accounted for such results. Certainly, the efficacy of endogenous GLP-2 for improving gut integrity during EW was most evident when pigs developed severe diarrhea [12]. Furthermore, bile acid-mimicking compounds administered orally to mice suppressed intestinal inflammation and signs of diarrhea in models of chemically-induced colitis [18, 36]. Thus, available data provide a rationale for exploring the value of bile acids and compounds that mimic their action as dietary supplements to improve performance of pigs under situations of increased incidence of enteric disorders.

Conclusions

Supplementing the diet of early-weaned pigs with CDC enhanced the expression of genes involved in the protection and barrier function of the mucosa of the distal small intestine. These effects, however, were only associated with a trend towards increased concentration of endogenous GLP-2 and intestinal growth. Even though dietary supplementation with CDC did not affect feed intake, the high sanitary conditions that prevailed in this study might have negated improvements in piglet performance resulting from the enteroprotective action of CDC. Results from this study warrant further research to examine the use of bile acids and compounds that mimic their action as dietary interventions to improve gut health and performance of pigs under situations of increased susceptibility to enteric inflammation and infection (e.g., poor environmental hygiene, increased microbial exposure, physical stress, etc.).

Abbreviations

EW: Early weaning; GLP: Glucagon-like peptide; GPBAR1: TGR5, G-protein-coupled bile acid receptor 1; CDC: Chenodeoxycholic acid; BW: Body weight; CONd: Control standard prestarter diet; CDCd: Standard prestarter diet supplemented with 60 mg CDC per kg of body weight; IL-10: Interleukin-10; TNF-a: Tumor necrosis factor alpha; GLP-2R: Glucagon-like peptide-2 receptor; GCG: Proglucagon; ASBT: Sodium-dependent bile acid transporter; TBP: Tata box-binding protein; ACTB: Beta actin; ZON-1: Zonula occludens-1; OCLN: Occludin; EGFR: Epidermal growth factor receptor; IL-6: Interleukin 6; TJ: Tight junction; FXR: Farnesoid X-activated receptor.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

IRI and AM designed the research; IRI, NdDC, AM, DM, and JIH conducted the research; IRI, AM, NdDC, and DM analyzed the data; IRI, NdDC, DM, JIH, and AM wrote the manuscript; and IRI had primary responsibility for the final content. All authors read and approved the final manuscript.

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