Strain-dependent induction of primary bile acid 7-dehydroxylation by cholic acid.

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Abstract (250 words)

Bile acids (BAs) are steroid-derived molecules with important roles in digestion, the maintenance of host metabolism and immunomodulation. Primary BAs are synthesised by the host and their concentrations tightly regulated, while secondary BAs are produced by the gut microbiome through transformation of the former. Regulation of microbial production of secondary BAs is not well understood, particularly the production of 7-dehydroxylated BAs, which are the most potent agonists for host BA receptors. The 7-dehydroxylation of cholic acid (CA) is well established and is linked to the expression of a bile acid-inducible (*bai*) operon. However, little to no 7-dehydroxylation has been reported for other host-derived BAs (e.g., chenodeoxycholic acid, CDCA or the mouse ursodeoxycholic acid, UDCA) and it is unknown whether they induce *bai* gene expression. Here, we demonstrate that the 7-dehydroxylation of CDCA and UDCA by *Clostridium scindens* is induced by CA suggesting that CA-dependent transcriptional regulation of 7-dehydroxylation is generalisable to CDCA and UDCA. Nevertheless, this effect is strain-specific and the murine isolate *Extibacter muris* did not respond to CA exposure, suggesting that *bai* genes are regulated differently in this strain. Moreover, the accessory gene *baiJ* was only upregulated in the *Clostridium scindens* ATCC 35704 strain, implying mechanistic differences amongst isolates. Interestingly, the *C. scindens* strains were also capable of 7-DH-ing muricholic acids (MCAs) to a limited extent. This study shows novel 7-dehydroxylation activity *in vitro* as a result of CA-driven induction and suggests that it is likely that a different BA plays the same role in the murine gut.

Keywords

7-dehydroxylation, *Clostridium scindens*, *Extibacter muris*, gut microbe, deoxycholic acid (DCA), lithocholic acid (LCA), muricholic acid (MCA), CA co-induction, *bai* gene expression, conjugated bile acids

Introduction

Primary bile acids (BAs) are metabolites synthesised from cholesterol by hepatocytes while secondary BAs are produced by the gut microbiome through the transformation of primary BAs (Figure 1). In the liver, the BA are conjugated to glycine or taurine. The three main microbial BA transformations are deconjugation (loss of the amino acid group), oxidation (of one or several of the hydroxyl groups), and 7 α -dehydroxylation (7-DH-ion), the loss of a hydroxyl group at the C7 position¹. These microbial transformations increase the diversity of the BA pool (Figure 1) and determine BA affinity to host receptors. In particular, 7-DH-ion turns primary BAs such as cholic acid (CA) and chenodeoxycholic acid (CDCA) into the 7-dehydroxylated (7-DH-ed) BAs deoxycholic acid (DCA) and lithocholic acid (LCA), respectively².

BAs act as detergents to solubilise dietary fats, but also have important metabolic and immunomodulatory roles through activation of their target receptors³. The two best-studied BA receptors are the Farnesoid X Receptor (FXR), a nuclear receptor, and the G Protein-Coupled Bile Acid Receptor, GPBAR1, also known as Takeda G-Protein Receptor 5 (TGR5), which is a membrane receptor. FXR is activated through the binding of BA agonists, particularly the 7-DH-ed LCA but also DCA⁴. FXR activation results in the inhibition of primary BA synthesis through repression of the cholesterol 7 α -hydroxylase CYP7A1. Regulation of BA production limits BA concentration and therefore, toxicity. On the other hand, BA dysregulation can cause health issues such as cholestasis, Irritable Bowel Syndrome (IBS), gallstone disease, or even the induction of colon cancer⁵⁻⁷. Besides BA homeostasis, FXR also has focal roles in glucose and lipid homeostasis⁸. Similarly, TGR5 is a multifunctional regulator involved in glucose homeostasis, energy expenditure, and the modulation of the inflammatory response^{9,10}. LCA, DCA and their tauro-conjugated forms TLCA and TDCA, are among the strongest agonists of TGR5^{11,12}, highlighting the importance of microbial transformation, particularly 7-DH-ion, in TGR5 activation. Moreover, DCA and LCA have protective properties against *Clostridium difficile* infection^{13,14}.

The study of BAs has traditionally been based on mouse models¹⁵. Besides CA and CDCA, mice (and other rodents) also generate muricholic acids (MCAs) such as α -MCA and β -MCA (Figure 1) and re-hydroxylate DCA and LCA in the liver¹⁶. Additionally, the mouse liver is capable of producing primary ursodeoxycholic acid (UDCA)¹⁶ although there is evidence that the gut microbiome is responsible for a significant fraction of UDCA in the gut¹⁴ (Figure 3 in manuscript provided for reviewers). On the other hand, UDCA is exclusively a secondary BA in humans⁷. Because 7-DH-ion plays a major role in host homeostasis, significant effort has been expended to study 7-dehydroxylating (7-DH-ing) bacteria.

Nonetheless, experimental evidence of 7-DH-ion is limited to a few species of the *Clostridiales* order. One of the best characterised is the human isolate *Clostridium scindens* ATCC 35704, the type strain of *C. scindens*¹⁷. The *Extibacter muris* DSM 28560 (JM40) strain was recently isolated from mice and identified as a 7-DH-ing organism^{18,19}. The ability of *C. scindens* ATCC 35704 to 7-dehydroxylate both *in vivo* and *in vitro* is well established²⁰; *E. muris* DSM 28560 has also been shown to 7-dehydroxylate *in vivo*, transforming primary BAs CA, CDCA, α MCA, β MCA and UDCA into their respective secondary BAs DCA, LCA and MDCA (Figures 3 and 5 in manuscript provided for reviewers).

The biochemical machinery for 7-DH-ion is encoded in the *bai* (bile acid inducible) eight-gene operon (*baiBCDEA2FGHI*)²¹. In addition, the *C. scindens* ATCC 35704 strain harbours the accessory gene *baiJ* (HDCHBGLK_03451)²² whereas the *E. muris* DSM 28650 genome includes a *baiJKL* pseudogene cluster²³. Most of the published work on the 7-DH-ion pathway has been performed with another *C. scindens* strain, VPI 12708^{24,25}. A recent publication by Funabashi *et al.* cloned the *bai* operon of *C. scindens* VPI 12708 into *Clostridium sporogenes* which then showed *in vivo* 7-DH-ing activity²⁵. Different strains exhibit varying efficiency in 7-DH-ing CA *in vitro*. The *C. scindens* ATCC 35704 and VPI 12708 strains show almost complete transformation to DCA while *E. muris* DSM 28560 has more limited activity^{19,20,26}. Other known 7-DH-ing strains such as *Clostridium hylemonae* and *Peptacetobacter hiranonis* have been reported as harbouring weak and strong activity, respectively²⁷ and a new strain of *P. hiranonis* recently isolated from dog faeces displayed *in vitro* 7-DH-ion at around 30% conversion of CA to DCA²⁸. Notably, *in vitro* 7-DH-ion of other primary BAs has been reported to be minor (CDCA) or non-existent (MCAs and UDCA)^{20,23,29}.

The limited *in vitro* 7-DH-ion of primary BAs other than CA (i.e., CDCA and MCAs) is striking considering that secondary 7-DH-ed forms of these BAs are routinely detected at significant concentrations in the host^{30,31}. Most studies tackling *in vitro* primary bile acid 7-DH-ion consider each BA in isolation. In addition, significant overexpression of the *bai* operon in response to CA has been reported^{32,33} but

there is no information about the potential induction of this operon by other primary BAs (i.e., CDCA and MCAs) and whether induction by CA also results in the transformation of the latter. We hypothesise that CA-dependent induction of the *bai* operon promotes 7-DH-ing activity of other BAs when they occur together with CA. Moreover, we posit that primary BAs other than CA cannot induce their own transformation.

Here, the expression of *bai* genes was measured *in vitro* in the presence of CA, CDCA, α MCA, β MCA and UDCA, with and without co-induction with CA to test whether the overexpression of *bai* genes was exclusive to CA and whether CA-induced overexpression was sufficient to promote the 7-DH-ion of other BAs. The experiments were performed with three strains, the human isolates *C. scindens* ATCC 35704 and VPI 12708 and the murine isolate *E. muris* DSM 28561 (SJ24). The results show that the response to CA co-induction was strain-variable. It was highly effective for *C. scindens* strains and sufficient to promote the transformation of other primary BAs.

This work highlights the importance of the presence CA for the 7-DH-ion of other BAs. Moreover, the lack of activity from *E. muris* SJ24 points at potential host-related differences whereby CA may not be the key inducer for BA 7-DH-ion in the murine gut.

Results

In vitro bile acid transformation and impact of ^{13}C -CA co-induction

Two human isolates *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 and one murine isolate *E. muris* DSM 28561 (SJ24) were tested for their ability to 7-dehydroxylate human and mouse primary BAs *in vitro*. Moreover, parallel experiments were performed by amending the cultures with ^{13}C -CA in order to test whether the 7-DH-ion of CDCA, α MCA, β MCA and UDCA could be induced by CA (Supplementary Table 1). As expected, all three strains 7-DH-ed CA but to varying extents (Figure 2). *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 showed strong 7-DH-ing activity with 97% and 80% CA conversion to 7-DH-ed BAs after 48 hours, respectively. *E. muris* SJ24, on the other hand, only converted 9% of the CA provided into 7-DH-ed forms (Figure 2). Similar results were obtained when this experiment was repeated as part of the uninduced batch of conditions (Supplementary Figure 1). *C. scindens* ATCC 35704 produced up to 52.26 μM DCA after 32 hours. Some of the DCA was subsequently oxidised to 12-oxolithocholic acid (12-oxoLCA) (Figure 2A). In contrast, *C. scindens* VPI 12708 produced the highest amount of DCA after 48 hours (71.42 μM), with little to no oxidised DCA forms (0.19 μM of 3-oxoDCA at 48 hours) (Figure 2B). The lack of 12-oxo forms from the *C. scindens* VPI 12708 strain was expected since the 12 α -hydroxysteroid dehydrogenase (12 α -HSDH) required for this process was not detected by PCR in this strain (the full genome is currently unavailable) (data not shown). Finally, *E. muris* SJ24 only produced 8.1 μM of DCA after 48 hours with very low amounts of oxidised forms of DCA (0.21 μM of 12-oxoLCA) (Figure 2C). It is important to highlight that *E. muris* does not possess a 3 α -HSDH encoded by *baiA2* which was recently identified as an important component of the CA 7-DH-ion pathway²⁵. This strain may perform hydroxyl group oxidations at the C3 position using *BaiA1/3* which has lower affinity to CA than *BaiA2*³⁴ and is present outside the *bai* operon³⁵. All three strains also showed a modicum of 7-oxidation activity (Figure 2), resulting in the formation of 7-oxoDCA, which cannot be 7-DH-ed.

CDCA 7 α -dehydroxylation is limited for all three strains. Indeed, *C. scindens* ATCC 35704 only produced 1.59 μM LCA and *C. scindens* VPI 12708 only 1.55 μM LCA (Figure 3AB), whereas for *E. muris*, no LCA was detected. The latter is in line with previous reports²³. The amendment of ^{13}C -CA significantly increased the transformation of CDCA for both *C. scindens* strains (p -value < 0.001 two-way ANOVA) but had no impact on *E. muris* SJ24 (Figure 3C). Indeed, the LCA yield increased to 9.77 μM for strain

ATCC 35704 and to 40.4 μM for strain VPI 12708 (Figure 3AB). No change was observed for *E. muris* SJ24.

As for UDCA, 7 α -dehydroxylation to LCA was observed only upon amendment with ^{13}C -CA (Figure 4). None of the strains exhibited any detectable level of activity from cultures that included only UDCA. 14.92 μM of LCA as well as extremely low amounts of 3-oxoLCA (with a maximum of 0.12 μM at 32 hours) were detected when *C. scindens* ATCC 35704 was co-induced. An unknown oxidised form labelled X-oxoUDCA was detected with a maximum concentration of 2.41 μM after 32 hours (Figure 4A). It is likely that this BA corresponds to 3-oxoUDCA (3-oxo-7 β -Hydroxy-5 β -cholan-24-oic acid) as we can exclude 7-oxoLCA (the other product of oxidation of UDCA) (Figure 1). Another BA with the same ionised mass as UDCA was detected at a maximum concentration of 4.24 μM after 24 hours. We propose that this could be an isoform of UDCA with the hydroxyl group of the C3 carbon in β conformation (3 β ,7 β -Dihydroxy-5 β -cholan-24-oic acid). However, the identity of these compounds remains unconfirmed due to the lack of standards. Upon co-induction, the 7-DH-ing activity of *C. scindens* VPI 12708 was comparable to that of the co-induced ATCC strain, with 13.61 μM of LCA and 0.68 μM of 3-oxoLCA after 48 hours. X-oxoUDCA was also detected at very small concentrations around 0.5 μM from 12 hours until the end of the experiment. The potential isoform of UDCA was detected at up to 9.92 μM at the 24-hour time point. Following the same trend observed with the other primary BAs, *E. muris* SJ24 did not show any detectable activity with UDCA even after co-induction. The chromatograms for the unknown bile acids can be found in Supplementary Figure 2.

As expected, neither *C. scindens* strain nor *E. muris* were capable of α MCA 7 α -dehydroxylation in the absence of ^{13}C -CA (Figure 5). *C. scindens* ATCC 35704 only produced minute amounts of an unknown oxo form of α MCA (labelled Y-oxo α MCA) (0.85 μM at 32 hours). Once co-induced with ^{13}C -CA, 6-oxoMDCA was detected at 2.7 μM after 48 hours in the *C. scindens* ATCC 35704 culture (Figure 5A). This secondary bile acid has been 7 α -DH-ed but also the hydroxyl at C6 oxidised. Moreover, several intermediates for which standards are unavailable were also detected after 48 hours. These were unknown oxidised forms of α MCA (labelled X- and Y- oxo α MCA) at concentrations not exceeding 5 μM each. A third unknown BA was detected (albeit at very low concentrations, 0.41 μM at 32 hours) with the same mass as 6-oxoMDCA, suggesting that it is an MCA species with one oxidation and one dehydroxylation. This would indicate the production of another 7 α -dehydroxylated form of α MCA *in vitro* (Figure 1, Figure 5A). As for the ATCC 35704 strain, *C. scindens* VPI 12708 exhibited an increase in the quantity of products from α MCA transformation in the presence of ^{13}C -CA relative to its absence (Figure 5B). This includes the 7-DH-ed BA 6-oxoMDCA that reached a concentration of 8.18 μM after 48 hours and the X- and Y- α MCA forms that were detected at maximum concentrations of 4.22 μM (4 hours) and 1.69 μM (32 hours), respectively. The aforementioned α MCA-derived bile acid with one ketone group and one dehydroxylation was also detected at a maximum concentration of 3.49 μM after 48 hours (Figure 5B). Surprisingly, *E. muris* SJ24 did not exhibit any observable 7-DH-ing activity with or without ^{13}C -CA. Nevertheless, a small amount of X-oxo α MCA was detected at all time points, with a stable concentration at around 2.4 μM without and 1.9 μM with ^{13}C -CA (Figure 5C). The results for β MCA were very similar to those for α MCA and are discussed in further detail in the supplementary information.

The concentration of ^{13}C -CA was also measured over time to ascertain that CA was being metabolised. It was observed to decrease until disappearing after 48 hours in the *C. scindens* strains except in the presence of CDCA, for which the concentration decreased slowly over time. We attribute this observation to the toxicity of CDCA in that concentration²⁰. On the other hand, the concentration of ^{13}C -CA in *E. muris* remained stable over time and in all conditions (Supplementary Figure 3).

Induction of *bai* genes expression in the presence of primary bile acids

In order to assess whether the amendment of ^{13}C -CA to the culture induced the *bai* operon as hypothesised, the relative expression of *baiCD* and of *baiE* were measured. In addition, the expression of *baiJ* (an accessory gene) was also monitored. Gene expression was normalised using at least three reference genes and was calculated relative to the expression levels in a control group without BAs. Both *E. muris* strains, that is JM40 (DSM 28560) and SJ 24 (DSM 28561), have a truncated *baiJ* gene²³. However, while the *baiJ* pseudogene is interrupted by stop codons in strain JM40, it is not in strain SJ24, making it worthwhile to investigate *baiJ* in the latter strain (as was done in this study). Additionally, *baiO* was also analysed for *E. muris* SJ24 as an alternative accessory *bai* gene³⁶.

Results show that the expression of *bai* operon genes in both *C. scindens* strains was highly upregulated as a response to exposure to CA or to CDCA but not to the other BAs (Figure 6). CA and CDCA are also the only two primary BAs for which *in vitro* 7-DH-ion data are already available²⁰. Moreover, it is worth highlighting that CDCA was tested using a concentration of 200 μM vs. 100 μM for CA.

For *C. scindens* ATCC 35704, the three genes tested were highly upregulated when ^{13}C -CA was present along with another BA (UDCA, αMCA , or βMCA) (Figure 6A). In the CDCA dataset, statistically significant differences relative to the uninduced conditions were observed and all genes were slightly more upregulated in the presence of ^{13}C -CA (p -value < 0.001 linear model), but *baiCD* was more so than the other genes (Figure 6A). Most interestingly, none of the other primary BAs activated the expression of *bai* genes on their own, consistent with the lack of 7-DH-ing activity with these BA substrates alone. However, the expression was brought up to levels higher than those observed for CA once co-induced (Figure 6A).

A similar pattern was observed for *C. scindens* VPI 12708 but with the significant difference being that *baiJ* was not overexpressed under any conditions (Figure 6B) (p -value < 0.001 linear model). In the CDCA dataset, the co-induction with ^{13}C -CA had an upregulatory effect if assessed with a paired Wilcoxon test (Supplementary Figure 4) but this effect was not found to be significant when using the linear statistical model displayed in Figure 6. It is worth noting that the expression data were obtained from the mid- to late-log exponential phase (around 18 hours) when differences in activity between uninduced and co-induced conditions were not very large (Figure 3B). As with *C. scindens* ATCC 35704, co-induction had a dramatic effect on the expression levels of *baiCD* and *baiE* in the presence of UDCA, αMCA , or βMCA , with upregulation reaching the expression levels observed with CA or CDCA alone (Figure 6B).

E. muris SJ24 showed a slight upregulation of *baiCD* and *baiE* in the CA dataset but it was not significant and did not translate to any of the other conditions (Figure 6C), mimicking its very poor 7-DH-ing activity (Figure 2). As a matter of fact, the increased *baiCD* and *baiE* gene expression ratio observed in the CA group was probably caused by one of the biological replicates which had a higher expression level than the others.

Thus, CA had a large effect on *bai* expression which was strain specific. Genes of the *bai* operon in the two *C. scindens* strains (ATCC 35704 and VPI 12708) exhibited a similar response to CA induction but the accessory *baiJ* differed in its response. It was upregulated in strain ATCC 35704 but not in strain VPI 12708. In contrast, CA had no significant effect on the expression of all the *bai* genes considered in *E. muris* SJ24.

The *rhaS_1* gene (HDCHBGLK_01429) is immediately upstream of the *bai* operon promoter on the opposite strand and has also been proposed as *b*ile *a*cid-regulatory (*bar*) due to its potential

implication in *bai* regulation⁷. The expression of *rhaS1* and *rhaS2* (a copy of *rhaS1* elsewhere in the genome) was shown to have background levels across BAs (Supplementary Figure 5). This was tested in *C. scindens* ATCC 35704 without the amendment of ¹³C-CA. Results indicate that *rhaS* is not upregulated by any of the tested BAs.

Discussion

Bile acid chemistry is a relevant field in human and veterinary medicine not only because of BA detergent function during digestion but also for the wide range of roles related to host physiology and homeostasis^{37,38}.

The *bai* operon was originally described in *C. scindens* VPI 12708 in 1990 and this strain has become the reference for biochemical studies of the 7-DH-ion pathway^{21,35,39}. However, genetic differences occur in the *bai* operon amongst 7-DH-ing strains^{35,40}. Moreover, the presence of accessory *bai* genes with as-of-yet undefined roles varies across strains^{35,39}.

Out of all primary BAs, only CA has been consistently reported to be 7-DH-ed *in vitro*, with low levels of 7-DH-ion for CDCA and none for other murine primary BAs (α MCA, β MCA or UDCA)^{20,23,41–44}. We confirm that the two *C. scindens* human isolates upregulated the expression of *bai* operon genes in response to CA (Figure 6). However, we observed differences between the two strains in the response of *baiJ*. This accessory gene is annotated as an urocanate reductase and a member of the oxidoreductase family. Current pathway data indicate that the gene does not play a significant role in 7-DH-ion of CA in strain VPI 12708²⁵, which would align with the lack of expression observed from our data. In contrast, the high levels of upregulation in the ATCC 35704 strain match previously published data³² and suggest that *baiJ* may play a role in the 7-DH-ion of CA or other BAs in that strain. On the other hand, *E. muris* SJ24, the murine isolate, barely registered any response to CA, nor activity in general, highlighting the potential differences in the 7-DH-ion pathway between human and mouse isolates.

UDCA is the 7 β isomer of CDCA and is reported to have significant therapeutic properties^{2,7,45}. In humans, UDCA is synthesised from CDCA by gut microbes containing 7 α - and 7 β -HSDHs^{7,26,46,47} and is thus, a secondary BA; in mice, it is produced by the liver, thus, it is a primary BA, and is used as a precursor for β MCA^{15,16}. Nevertheless, colonisation of mice lacking any 7-dehydroxylators with 7-DH-ing bacteria increases UDCA levels, implying that the gut microbiome also plays a significant role in the UDCA production of mice¹⁴ (Studer *et al.*, and appended manuscript Figures 3 and 5). Despite its importance, little is known about the capacity of bacteria to 7 β -dehydroxylate UDCA *in vitro* and previous data have shown no activity by *C. scindens* strain ATCC 35704²⁰. In accordance with our hypothesis, co-induction with ¹³C-CA not only greatly upregulated *bai* gene expression (Figure 6B) but also provided evidence of UDCA 7 β -dehydroxylation as LCA was detected in the culture (Figure 4). The absence of CDCA in the culture suggests that UDCA was not 7-epimerised to CDCA prior to its 7 α -dehydroxylation. Moreover, two unidentified compounds were detected, providing further evidence of the 7 β -dehydroxylation of UDCA and its associated unique set of intermediates. The oxidised intermediate is very likely to be 3-oxoUDCA (3-oxo-7 β -Hydroxy-5 β -cholan-24-oic acid), as based on the mass, the only alternative would have been 7-oxoLCA, which was quantified (Figure 4). The other unknown compound had the same mass as UDCA albeit a different retention time, suggesting this could be an iso- form of UDCA. It follows that this would be 3 β -UDCA, tentatively named isoUDCA (3 β ,7 β -Dihydroxy-5 β -cholan-24-oic acid). Isoforms are well known in the BA pool and particularly in CDCA-related intermediates^{20,25,33,48} so it is likely that UDCA follows a similar pattern. Despite the production of unknown intermediates, the activity response to co-induction suggests that UDCA 7 β -dehydroxylation uses the same *bai* machinery as the 7-DH-ion of CDCA.

The BA pool of mice is significantly more diverse than that of humans due to the primary production of muricholic acids (with a hydroxyl group at the C-6 position) and of UDCA. Their secondary bile acid pool includes DCA and LCA but also MDCA and its 6 α counterpart, hyodeoxycholic acid (HDCA). Furthermore, mice can rehydroxylate TDCA back into TCA in the liver¹⁵. In general, the secondary BAs derived from muricholic acids seem to be in low abundance in the gut, hinting at the difficulty in 7-DH-ing these BAs. This is perhaps the reason why primary BAs such as β MCA are dominant in the bile acid pool⁴⁹. While we initially hypothesised that the lack of activity was due to the lack of expression, our co-induced data show that *bai* gene expression in the human isolates is not sufficient for the production of MDCA.²³ On the other hand, *E. muris* showed no significant expression.

The human isolates showed marginal activity for α MCA and β MCA upon co-induction. 7-DH-ed forms such as 6-oxoMDCA were detected (Figure 5 & Supplementary Figure 6) but the full 7-dehydroxylation to MDCA was not observed. These results imply that other elements besides the *bai* operon (and *bail*) might be needed to completely 7-dehydroxylate MCAs. A potential reason could be a missing gene such as a 6 β -HSDH required for complete 7-DH-ion.

The mouse isolate *E. muris* was a weak CA 7-dehydroxylator and had no activity with the other BAs. This was coupled with a complete lack of gene upregulation in response to any BA. Nonetheless, recently published *in vivo* data from Studer *et al.*, 2022 (appended manuscript Figure 3) quantified MDCA as a direct result of *E. muris* colonisation, demonstrating that this strain is capable of 7-DH-ion of MCA *in vivo*. To explain these differences, it is possible that an untested BA could be relevant for inducing 7-DH-ion of MCAs. An option could be T β MCA, but conjugated forms were not tested here. Both of these BAs are highly abundant in the BA pool of mice¹⁶. Finally, experimental conditions might have not been optimal to stimulate the 7-DH-ing activity⁵⁰.

The novel activity for α MCA and β MCA yielded several intermediate BAs that we were not able to fully characterise due to the lack of appropriate standards. All human and mouse BAs share a backbone of four rings (Figure 1), this makes mass fractionation in the mass spectrometer unsuitable for identification. Therefore, we currently rely on comparison of ionised mass and retention time to standards. However, several assumptions can be made to speculate what these compounds could be. MCAs oxidised at the C-3, C-6 or C-7 position (Figure 1). A C-7 oxidation would yield 7-oxoMDCA regardless of the primary MCA. Meanwhile, other oxidations would be differentiated by the α or β conformation of the C-7 hydroxyl. It is possible that one of the intermediates that we detected was 7-oxoMDCA but none shared retention times across MCAs, meaning that different intermediates were produced for each of the two MCA substrates (Supplementary Figure 2). Considering that two oxidised intermediates are detected from both MCAs, we hypothesise that those are the 3-oxo and 6-oxo forms of α MCA and β MCA (3-oxo α MCA: 3-oxo-6 β ,7 α -Dihydroxy-5 β -cholan-24-oic acid; 6-oxo α MCA: 6-oxo-3 α ,7 α -Dihydroxy-5 β -cholan-24-oic acid; 3-oxo β MCA: 3-oxo-6 β ,7 β -Dihydroxy-5 β -cholan-24-oic acid; and 6-oxo β MCA: 6-oxo-3 α ,7 β -Dihydroxy-5 β -cholan-24-oic acid). A third intermediate was also detected from both MCAs, with the ionised mass corresponding to secondary BAs with one dehydroxylation and a ketone group (e.g., 7-oxoLCA) (Figure 5 & Supplementary Figure 6). Three options are plausible: 1) A dehydroxylation at the C-3 position. This would yield a novel family of BAs with a 6 β - and 7 α / β - hydroxyls which is highly unlikely as it would have been identified previously by the multiple studies investigating the murine BA pool^{15,16,51–54}. 2) An oxidation paired with a 6-dehydroxylation would yield 3-oxoCDCA, 7-oxoLCA, or 3-oxoUDCA. 3-oxoCDCA and 7-oxoLCA were included in our analysis (Supplementary Table 3) and would have been detected if present. Meanwhile, the retention time of this compound is distinct from that of the compound proposed to be 3-oxoUDCA from the transformation of UDCA (see above and Supplementary Figure 2). Thus, this is not likely to be 3-oxoUDCA. 3) A 7-dehydroxylation could allow for ketone groups at the C-3 and C-

6 positions. 6-oxoMDCA was available as a standard but the other option, 3-oxoMDCA (3-oxo-6 β -Hydroxy-5 β -cholan-24-oic acid), was not. It is therefore possible that this compound corresponds to 3-oxoMDCA, but this remains unconfirmed.

Conclusion

The findings presented here are threefold. First and foremost, we demonstrated that the previously reported^{32,33} strong upregulation of *bai* genes by CA increases the extent of 7-DH-ion of other primary BAs. This was particularly true for UDCA which has been reported to be converted to LCA *in vitro* for the first time. Secondly, the upregulation of *bai* genes exhibited strain-specific differences. *C. scindens* ATCC 35704 upregulated the *bai* operon genes *baiCD* and *baiE* as well as the *bai* accessory gene *baiI*. While for *C. scindens* VPI 12708, it upregulated the *bai* operon genes, but *baiI* expression was found to be at a background level. This is consistent with the lack of involvement of *baiI* in 7-DH-ion in strain VPI 12708²⁵. *E. muris* SJ24 was the third strain tested, a murine isolate with *in vivo* 7-DH-ing capabilities (appended manuscript Figure 3 and 5). Our findings with the SJ24 strain showed weak 7-DH-ion of CA and no upregulation of any of the *bai* genes tested. The lack of activity of this murine strain even in the presence of murine primary BAs suggests that there is a missing factor, maybe an untested BA or other co-factors, required for an efficient *bai* expression and 7-DH-ing activity. It is also possible that 7-DH-ion of MCAs occurs through a distinct pathway with different enzymatic requirements. Finally, *C. scindens* human isolates can partially 7-dehydroxylate MCAs. Nevertheless, there might be missing a key component such as a 6- β HSDH that would complete the reductive arm of 7-DH-ion to MDCA.

In conclusion, these data provide novel insights into the intricacies of 7-DH-ion and the significant differences amongst 7-DH-ing bacteria. The CA-dependent co-induction can be attributed to the abundance of this compound in the BA pool of humans and mice. However, the inducing factor for *E. muris* activity remains elusive. Moreover, multiple novel BAs were observed and their identity surmised based on mass spectrometry information. Further work is required to investigate the 7-DH-ion pathway of CDCA, α MCA, β MCA and UDCA as well as exploring the strain-specific differences regarding the 7-DH-ion pathway of CA.

Materials and Methods

Bacterial strains and growth conditions

The strains used were *Clostridium scindens* ATCC 35704, *Clostridium scindens* VPI 12708 and *Extibacter muris* DSM 28561 (SJ24). Bacteria were grown in Brain Heart Infusion Supplement – Salts (BHI-S) medium, consisting of 37g BHI, 1g L-cysteine, 5g yeast extract, 2.5g fructose, 50mL salts solution (0.2g CaCl₂, 0.2 MgSO₄, 1g K₂HPO₄, 1g KH₂PO₄, 10g NaHCO₃ and 2g NaCl per L of ddH₂O) per L ddH₂O. The salts solution and media were sterilised by autoclaving. Static growth was carried out at 37°C in an anaerobic chamber (Coy Laboratory Products, 95% N₂, 5% H₂). A pre-inoculum was prepared from glycerol stocks (using BHI-S) before inoculating 25 mL of BHI-S in Falcon tubes at a starting OD₆₀₀ of 0.05. Results of the growth curves for the experiments can be found in the Supplementary Information.

In vitro 7-dehydroxylation assays

Bacteria were grown in presence of BAs: CA (100 μ M), CDCA (200 μ M), α MCA (100 μ M), β MCA (100 μ M) and UDCA (100 μ M) or the same volume of ethanol (solvent control). A sterile control (media with ethanol solvent) was also included. Co-induction was performed by adding an additional 100 μ M of ¹³C-CA to CDCA, UDCA, α MCA, or β MCA). ¹³C-CA was chosen so its transformation products could be separated from those of the other primary BAs during the quantification process. All uninduced

experiments were conducted at the same time while all the co-induced ones were run simultaneously at a different time. Both experiments included a condition consisting of the amendment of only 100 μ M CA, which allows for comparison of the results from co-induced and uninduced experiments. Growth was monitored by periodically measuring the OD₆₀₀. During the main time points (0, 4, 8, 12, 24, 32 and 48 hours), 1mL samples were collected for BA extraction in a 2 mL bead-beating resistant tube. All conditions were performed in triplicates.

Bile acid extraction

Samples were vacuum dried overnight (ON). Approximately 450 mg of 0.5 mm zirconium beads were added to the dried samples as well as 500 μ L ice-cold alkaline acetonitrile (acetonitrile – 25% ammonia 4:1 v/v) and 100 μ L of ISTD solution (CA-d₄, CDCA-d₄, TCA-d₄, TUDCA-d₄, DCA-d₄ and LCA-d₄, each at 100 μ M in methanol). Samples were homogenised in a Precellys 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 6500 rpm 3x 30" beat 30" rest. Samples were vortexed for 1 hour and centrifugated for 15 minutes at 16000 rcf at room temperature. Approximately 500 μ L of suspension was carefully collected over the beads level and transferred into a new 1.5 mL epi tube which was then vacuum dried overnight. Finally, the samples were reconstituted in 1 mL of ammonium acetate [5mM] – methanol (50:50 v/v) and a 1:20 dilution with the same solvent was prepared in LC-MS glass vials, ready for injection.

RNA extraction and reverse transcription

1 mL of sample was collected in a 15 mL falcon tube during the mid-log to late-log phase for the RNA extraction. The sample was stored with RNa protect following the manufacturer protocol (Protocol 5 from RNa protect Bacteria Reagent Handbook 01/2020, Qiagen) at -80°C until processed. All conditions were performed in triplicates. Lysis and RNA purifications were done using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Bacterial lysis was performed following Protocol 5: Enzymatic Lysis, Proteinase K Digestion and Mechanical Disruption of Bacteria (RNa protect Bacteria Reagent Handbook 01/2020, Qiagen), with 20 μ L of proteinase K for each sample and the required volumes for a number of bacteria $<7.5 \times 10^8$. The cell lysis was performed using a Precellys 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 6500 rpm 3x 10 seconds beat 10 seconds rest. RNA purification was performed following Protocol 7: Purification of Total RNA from Bacterial Lysate using the RNeasy Mini Kit. Centrifugations were carried out at 15000 rcf except for the 2 min centrifugation which was done at 18000 rcf.

Purified RNA was further subject to a DNase treatment using the RQ DNase I (Promega, Madison, WI, USA) following the manufacturer protocol with small modifications: The final volume was adjusted to a 100 μ L and incubation was extended to 1 hour at 37°C. The treated RNA was cleaned-up using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the RNA Clean-up protocol from the manufacturer (RNeasy Mini Handbook 10/2019) with the 2 min centrifugation done at 18000 rcf. Concentration and purity of RNA was measured with a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA).

100 ng of RNA was reverse transcribed into cDNA using the GoScript™ Reverse Transcription Mix, Random Primers (Promega, Madison, WI, USA) following the manufacturer protocol. The process was done in duplicates with one group using water instead of the reaction buffer as a non-reverse transcription control (NRT).

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCRs were prepared using the Myra liquid handling system (Bio Molecular Systems, software version 1.6.26) and performed using the Magnetic induction cyler (Mic) platform (Bio Molecular Systems, Upper Coomera, QLD, Australia) with the micPCR software (v2.10.5).

The list of primers used can be found in Supplementary table 2. Samples were prepared with the SensiFAST SYBR No-ROX Kit (Meridian Bioscience, Cincinnati, OH, USA) at a final volume of 10 μ L. All runs were performed with the following program, with small modifications: Initial hold at 95°C for 5 minutes with a cycle of 95°C for 5 seconds, 54.5°C for 20 seconds (54.1°C for *E. muris* SJ24) and 72°C for 9 seconds. 40 cycles were done for *C. scindens* ATCC 35704 and 50 for *C. scindens* VPI 12708 and *E. muris* SJ24. The melting curve, temperature control and acquisition settings were left as default. The quantification was done using three or more reference genes (Supplementary Table 2) based on their expression stability across conditions. NRTs as well as no template controls (NTCs) were included to check for residual DNA or contaminations. Four technical replicates were done for each biological triplicate, totalling at 12 data points per condition. Note that expression data presented in Figure 6 for the CA-only condition (labelled CA) correspond to the pooled expression results (for the condition in which only 100 μ M Ca was added) from the two sets of experiments presented above (referred to as the co-induced and the uninduced experiments, respectively).

Liquid chromatography – mass spectrometry (LC-MS)

The quantitative method was performed on an Agilent ultrahigh-performance liquid chromatography 1290 series coupled in tandem to an Agilent 6530 Accurate-Mass Q-TOF mass spectrometer. The separation was done on a Zorbax Eclipse Plus C18 column (2.1 x 100mm, 1.8 μ m) and a guard column Zorbax Eclipse Plus C18 (2.1 x 5mm, 1.8 μ m) both provided by Agilent technologies (Santa Clara, CA, USA). The column compartment was kept heated at 50°C. Two different solutions were used as eluents: ammonium acetate [5mM] in water as mobile phase A and pure acetonitrile as mobile phase B. A constant flow of 0.4 mL/min was maintained over 26 minutes of run time with the following gradient (expressed in eluent B percentage): 0-5.5 min, constant 21.5% B; 5.5-6 min, 21.5-24.5% B; 6-10 min, 24.5-25% B; 10-10.5 min, 25-29% B; 10.5-14.5 min, isocratic 29% B; 14.5-15 min, 29-40% B; 15-18 min, 40-45% B; 18-20.5 min, 45-95% B; 20.5-23 min, constant 95% B; 23-23.1 min, 95-21.5% B; 23.10-26 min, isocratic 21.50% B. The system equilibration was implemented at the end of the gradient for 3 minutes in initial conditions. The autosampler temperature was maintained at 10°C and the injection volume was 5 μ L. The ionisation mode was operated in negative mode for the detection using the Dual AJS Jet stream ESI Assembly. The QTOF acquisition settings were configured in 4GHz high-resolution mode (resolution 17000 FWHM at m/z 1000), data storage in profile mode and the high-resolution full MS chromatograms were acquired over the range of m/z 100-1700 at a rate of 3 spectra/s. The mass spectrometer was calibrated in negative mode using ESI-L solution from Agilent technologies every 6 hours to maintain the best possible mass accuracy. Source parameters were setup as follows: drying gas flow, 8 L/min; gas temperature, 300°C; nebulizer pressure, 35psi; capillary voltage, 3500V; nozzle voltage, 1000V. Data were processed afterwards using the MassHunter Quantitative software and MassHunter Qualitative software to control the mass accuracy for each run. In the quantitative method, 42 bile acids were quantified by calibration curves (Supplementary Table 3). The quantification was corrected by addition of internal standards in all samples and calibration levels. Extracted ion chromatograms were generated using a retention time window of $\pm$ 1.5 min and a mass extraction window of $\pm$ 30ppm around the theoretical mass of the targeted bile acid. Unknown BAs were identified when found within the retention time window of a standard with the same ionised mass. Approximate quantification of these unknown BAs was done by using the nearest standard (by retention time) with the same ionised mass.

Statistical analysis and data visualisation

Graphpad Prism 9.2.0 (GraphPad) was used to generate the figures shown in this paper and perform pairwise comparisons, the gene expression data was analysed with a linear model (LM) in R language v4.1.2⁵⁵ using RStudio⁵⁶. The statistical significance boundary was established at a *p*-value < 0.05.

Conflicts of interest

The authors declare no conflicts of interest.

Data Availability Statement

The data used for this manuscript are publicly available in the following link:

<https://doi.org/10.5281/zenodo.6034320>

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Figure Legends

Figure 1. List of deconjugated human and rodent bile acids (BAs) discussed here. The characteristic that distinguishes BAs is the presence of a hydroxyl group at the C-3, C-6, C-7 and/or C-12 position. The hydroxyl groups can be in α - or β -conformation, oxidised into a ketone group, or fully removed (dehydroxylated BA). CA and CDCA are primary BAs of both humans and rodents whereas MCAs are exclusively produced by rodents. (*) UDCA is a primary BA in rodents while it is a secondary BA in humans where the gut microbes epimerise it from CDCA. (†) Bile acids that might have been detected in this study but for which no standards were available. (‡) Also known as 6-oxolithocholic acid (6-oxoLCA). See Supplementary Table 3 for full chemical names of the BAs.

Figure 2. *In vitro* 7-dehydroxylation of cholic acid. The 7-dehydroxylation of CA was tested in (A) *Clostridium scindens* ATCC 35704, (B) *C. scindens* VPI 12708 and (C) *Extibacter muris* DSM 28561 (SJ24) over time. All strains were grown anaerobically in BHIS-S containing 100 μ M CA. Bile acids were extracted from the suspended biomass. Error bars represent the standard deviation of the mean of biological triplicates.

Figure 3. *In vitro* 7 α -dehydroxylation of CDCA. The transformation of CDCA into secondary bile acids was tested with and without co-induction with 100 μ M of 13 C-CA. 200 μ M of CDCA were used based on previous experiments by Marion *et al.* (A) *Clostridium scindens* ATCC 35704, (B) *C. scindens* VPI 12708 and (C) *E. muris* DSM 28561 (SJ24). Bile acids were extracted from the suspended biomass. Error bars represent the standard deviation of the mean of biological triplicates.

Figure 4. *In vitro* 7-dehydroxylation of UDCA. The transformation of 100 μ M of UDCA into secondary bile acids was tested with and without co-induction with 100 μ M of 13 C-CA. (A) *Clostridium scindens* ATCC 35704, (B) *C. scindens* VPI 12708 and (C) *Extibacter muris* DSM 28561 (SJ24) were grown anaerobically in BHIS-S. Bile acids were extracted from suspended biomass. Two compounds were detected that could not be identified due to missing standards but their oxidative state can be estimated based on their ionised mass. X-oxoUDCA had the same mass as other bile acids with one ketone group and one hydroxyl group. The other unidentified compound had the same mass as UDCA and therefore it is likely to be an isoform with a β conformation. The retention times for these compounds was unique and therefore could not be identified further. Concentration values of the unknown BAs could only be estimated for the same reason. Error bars represent the standard deviation of the mean of biological triplicates.

Figure 5. *In vitro* 7-dehydroxylation of α MCA. The transformation of 100 μ M of α MCA into secondary bile acids was tested with and without co-induction with 100 μ M of 13 C-CA. (A) *Clostridium scindens* ATCC 35704, (B) *C. scindens* VPI 12708 and (C) *Extibacter muris* DSM 28561 (SJ24) were grown anaerobically in BHIS-S. Bile acids were extracted from suspended biomass. Several compounds were detected that could not be identified due to missing standards but their oxidative state can be estimated based on their ionised mass. X- or Y- oxo α MCA had the same mass as other bile acids with one ketone group and two hydroxyl groups. The other unidentified compound had the same mass as secondary bile acids that have been dehydroxylated (-1 -OH), have one ketone group and one hydroxyl group. The retention times for these compounds did not correspond to that of any known standard. Concentration values of the unknown BAs could only be semi-quantitative for the same reason. Error bars represent the standard deviation of the mean of biological triplicates.

Figure 6. *bai* gene expression in the presence of CA (first panel) or in that of various primary BAs either uninduced or and co-induced with 13 C-CA. Data for the CA-only condition correspond to the pooled expression results from the two sets of experiments (referred to as uninduced and co-induced) that both include this condition, as described in the text. The expression (normalised to at least three reference genes) is relative to the no BA condition including an equivalent volume of solvent (ethanol). (A) *Clostridium scindens* ATCC 35704, (B) *C. scindens* VPI 12708 and (C) *Extibacter muris* DSM 28561 (SJ24) gene expression of *baiCD*, *baiE* (part of the *bai* operon), and accessory genes *baiI* and *baiO* (only in *E. muris* DSM SJ24) was measured. CA, UDCA, α MCA, β MCA and the co-inducing 13 C-CA were used at 100 μ M, CDCA was used at 200 μ M. A detailed view of the *C. scindens* VPI 12708 uninduced vs 13 C-CA co-induced in the CDCA group is found in

667 **Supplementary Figure 4. Coloured dots represent the average and error bars represent the standard deviation of 12**
668 **replicates. Some error bars may look elongated due to the logarithmic scale of the Y axis. (***) indicates a p -value < 0.001**
669 **in a linear model analysis comparing the uninduced vs ^{13}C -CA co-induced factor for each *bai* gene.**

	Host	Bile Acid name	Abbreviations	C-3	C-6	C-7	C-12
Human and rodent	Primary	Cholic acid	CA	α -OH		α -OH	α -OH
		Chenodeoxycholic acid	CDCA	α -OH		α -OH	
		Ursodeoxycholic acid*	UDCA	α -OH		β -OH	
	secondary	Deoxycholic acid	DCA	α -OH			α -OH
		Lithocholic acid	LCA	α -OH			
		3-oxocholic acid	3-oxoCA	=O		α -OH	α -OH
		3-oxodeoxycholic acid	3-oxoDCA	=O			α -OH
		3-oxochenodeoxycholic acid	3-oxoCDCA	=O		α -OH	
		3-oxolithocholic acid	3-oxoLCA	=O			
		3-oxoursodeoxycholic acid†	3-oxoUDCA	=O		β -OH	
		7-oxodeoxycholic acid	7-oxoDCA	α -OH		=O	α -OH
		7-oxolithocholic acid	7-oxoLCA	α -OH		=O	
		12-oxochenodeoxycholic acid	12-oxoCDCA	α -OH			=O
		12-oxolithocholic acid	12-oxoLCA	α -OH			=O
		Isoursodeoxycholic acid†	isoUDCA	β -OH		β -OH	
Rodent	primary	α -Muricholic acid	α MCA	α -OH	β -OH	α -OH	
		B-Muricholic acid	β MCA	α -OH	β -OH	β -OH	
	secondary	Murideoxycholic acid	MDCA	α -OH	β -OH		
		3-oxo- α -muricholic acid†	3-oxo α MCA	=O	β -OH	α -OH	
		3-oxo- β -muricholic acid†	3-oxo β MCA	=O	β -OH	β -OH	
		3-oxomurideoxycholic acid†	3-oxoMDCA	=O	β -OH		

		6-oxo- α -muricholic acid†	6-oxo α MCA	α -OH	=O	α -OH	
		6-oxo- β -muricholic acid†	6-oxo β MCA	α -OH	=O	β -OH	
		6-oxomurideoxycholic acid‡	6-oxoMDCA	α -OH	=O		
		7-oxomurideoxycholic acid†	7-oxoMDCA	α -OH	β -OH	=O	
		ω -Muricholic acid†	ω MCA	α -OH	α -OH	β -OH

A)

C. scindens ATCC 35704 uninduced *C. scindens* ATCC 35704 ¹³C-CA co-induced

B)

C. scindens VPI 12708 uninduced *C. scindens* VPI 12708 ¹³C-CA co-induced

C)

E. muris SJ24 uninduced *E. muris* SJ24 ¹³C-CA co-induced

A)

C. scindens* ATCC 35704 uninduced** C. scindens* ATCC 35704 ¹³C-CA co-induced**

B)

C. scindens* VPI 12708 uninduced** C. scindens* VPI 12708 ¹³C-CA co-induced**

C)

E. muris* SJ24 uninduced** E. muris* SJ24 ¹³C-CA co-induced**

A)

***C. scindens* ATCC 35704 uninduced**

***C. scindens* ATCC 35704 ¹³C-CA co-induced**

B)

***C. scindens* VPI 12708 uninduced**

***C. scindens* VPI 12708 ¹³C-CA co-induced**

C)

***E. muris* SJ24 uninduced**

***E. muris* SJ24 ¹³C-CA co-induced**